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Understanding the Molecular Mechanism through which Aspirated Bile Triggers Chronic *Pseudomonas aeruginosa* Infections in Respiratory Disease

A Thesis Presented to the

National University of Ireland

for the degree of

Doctor of Philosophy

By

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Cork

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Head of Department: Professor Gerald Fitzgerald

March 2019

Dedicated to my fianceé, Fergal and my parents Ann & Jimmy.

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Declaration

Declaration

I, the undersigned Stephanie Flynn, declare that I have not obtained a degree from University College Cork, National University of Ireland, Cork or elsewhere on the basis of this PhD thesis and that the results presented in this thesis were derived from experiments undertaken by myself at University College Cork, National University of Ireland, Cork with the exception of the transcriptome preparation which was completed by David Woods. The research presented in Chapter 1 & 2 has been published in part with the research presented in Chapter 3 submitted for publication.

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Stephanie Flynn

April 2019

General Abstract

General Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* is the leading cause of morbidity and mortality in Cystic Fibrosis (CF) patients. Extensive genomic adaptation of this organism facilitates its emergence as a dominant organism within the lung microbial community and to its ability to chronically persist within the CF airways. The environmental and host factors contributing to the success of this species *in vivo* have been the subject of intensive research efforts. Gastro-oesophageal reflux (GOR) has recently emerged as a major co-morbidity in CF and a range of other respiratory conditions and is associated with the presence of bile acids in the lungs of CF patients, a consequence of micro-aspiration of refluxed gastric contents. This thesis aimed to investigate the impact that bile exerts on the global lung microbiota and the key CF associated pathogen *P. aeruginosa.*

The detection of bile acids in paediatric CF patients using liquid chromatography mass spectrometry (LC-MS) analysis correlated with a reduction in lung microbial biodiversity and the emergence of dominant respiratory pathogens including *P. aeruginosa*. Bile acids may contribute to the progressive restructuring of the lung microbiota towards a pathogen dominated state associated with worse clinical outcomes. Bile and the active component bile acids were found to be capable of triggering *P. aeruginosa* to transition to a chronic, antibiotic tolerant lifestyle through a combination of transcriptional and phenotypic responses. Functional screens based on biofilm formation and growth on bile identified key two component systems mediating the biofilm response to bile with a connection to central metabolism becoming apparent. The latter screen identified the glyoxylate shunt as a key breakpoint in the suppression of redox potential as part of the bile response. Bile was also found to be capable of selecting for genetic variants in an *in vitro* system known to mimic conditions found within the CF lung environment. Pigmented

derivatives emerged exclusively in the presence of bile with genome sequencing identifying single nucleotide polymorphisms (SNPs) in quorum sensing (*lasR*) and both the pyocyanin (*phzS*) and pyomelanin (*hmgA*) biosynthetic pathways. These mutations have been previously described in various clinical isolates of *P. aeruginosa.* Loss of Pseudomonas Quinolone Signal (PQS) production in the pigmented variants underpinned the loss of redox suppression in response to bile, perhaps a consequence of the anti-oxidant/pro-oxidant activities attributed to the PQS signalling molecule. Bile is therefore capable of influencing the evolutionary trajectory of this respiratory pathogen, a key finding in understanding the emergence of genotypic and phenotypic heterogeneity within the lungs of patients with respiratory disease.

Collectively, this research supports the role for bile in the progression of chronic infection in CF through its impact on *P. aeruginosa* and other respiratory pathogens. Therefore, the early detection and profiling of bile acids utilising rapid point of care devices could lead to the identification of high risk paediatric patients and to the development of increasingly effective intervention strategies to prevent the establishment of chronic respiratory microbiota.

Abbreviations

Abbreviations

CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
H. influenzae	*Haemophilus influenzae*
EU	European Union
WHO	World Health Organisation
ERS	European Respiratory Society
FDA	Food and Drug Administration
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
BALF	Bronchoalveolar Lavage Fluid
FEV1	Forced Expiratory Volume in one second as measured during a Forced Volume Capacity test
NGS	Next Generation Sequencing
DNA	Deoxyribonucleic Acid
T3SS	Type Three Secretion System
T6SS	Type Six Secretion System
QS	Quorum Sensing
AHL	Acyl-Homoserine Lactone
AQ	Alkyl Quinolone
PQS	Pseudomonas Quinolone Signal
HHQ	4-Hydroxy-2-Heptylquinoline
PYO	Pyocyanin
HSI	Hcp-Secretion Island
eDNA	Extracellular DNA
EPS	Extracellular polymeric Substances
IL	Interleukin
SCFA	Short Chain Fatty Acid
GOR	Gastro-oesophageal Reflux
GORD	Gastro-oesophageal Reflux Disease
LOS	Lower Oesophageal Sphincter
PPI	Proton Pump Inhibitor
BOS	Bronchiolitis Obliterans Syndrome
NAD	Nicotinamide Adenine Dinucleotide
LCMS	Liquid Chromatography Mass Spectrometry
TNF	Tumour Necrosis Factor
FXR	Farnesoid X Receptor
HIF-1	Hypoxia Inducible Factor
AREST CF	Australian Respiratory Early Surveillance Team for Cystic Fibrosis
CT	Computed Topography
SI	Shannon Index

HB	High Bile
MB	Moderate Bile
LB	Low Bile
BA+	Bile Acid Positive
T	Transitioning
BA-	Bile Acid Negative
PCA	Principal Component Analysis
NE	Neutrophil Elastase
BRC	BIOMERIT Research Center
TCS	Two Component System
LB	Luria–Bertani
TSB	Tryptic Soy Broth
MH	Mueller Hinton
ASM	Artificial Sputum Media
HCl	Hydrochloric Acid
TCA	Tricarboxylic Acid Cycle
CUH	Cork University Hospital
OD	Optical Density
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
TLC	Thin Layer Chromatography
BT	Bile Treated ASM isolates
UT	Untreated ASM isolates

General Introduction

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1. The Antibiotic Crisis

The discovery of penicillin in 1928 by Sir Alexander Fleming reshaped the landscape of modern medicine, revolutionizing the treatment of bacterial infections and saving the lives of countless millions in the process (1). Since then, a wide array of antibiotics have been discovered (2). However, this has coincided with the rapid emergence of antimicrobial resistance with resistance strategies described for almost all developed antibiotics **(Fig. 1)** (3). This challenge represents perhaps the greatest threat to modern medicine with urgent action required. The problem of resistance could be combatted, at least in part, by the development of novel antibiotics and the implementation of appropriate stewardship practices. However, there has been a significant reduction in pharmaceutical investment for the research and development of novel antimicrobials, with 15 of the 18 largest pharmaceutical companies abandoning this field of research which has contributed to the drying up of the antibiotic pipeline (4-8). Furthermore, the global over-reliance and over prescription of antibiotics has exacerbated the problem, increasing the rate of emergence of antibacterial resistant isolates. All of which was predicted and forewarned by Fleming following his pioneering penicillin discovery (9, 10).

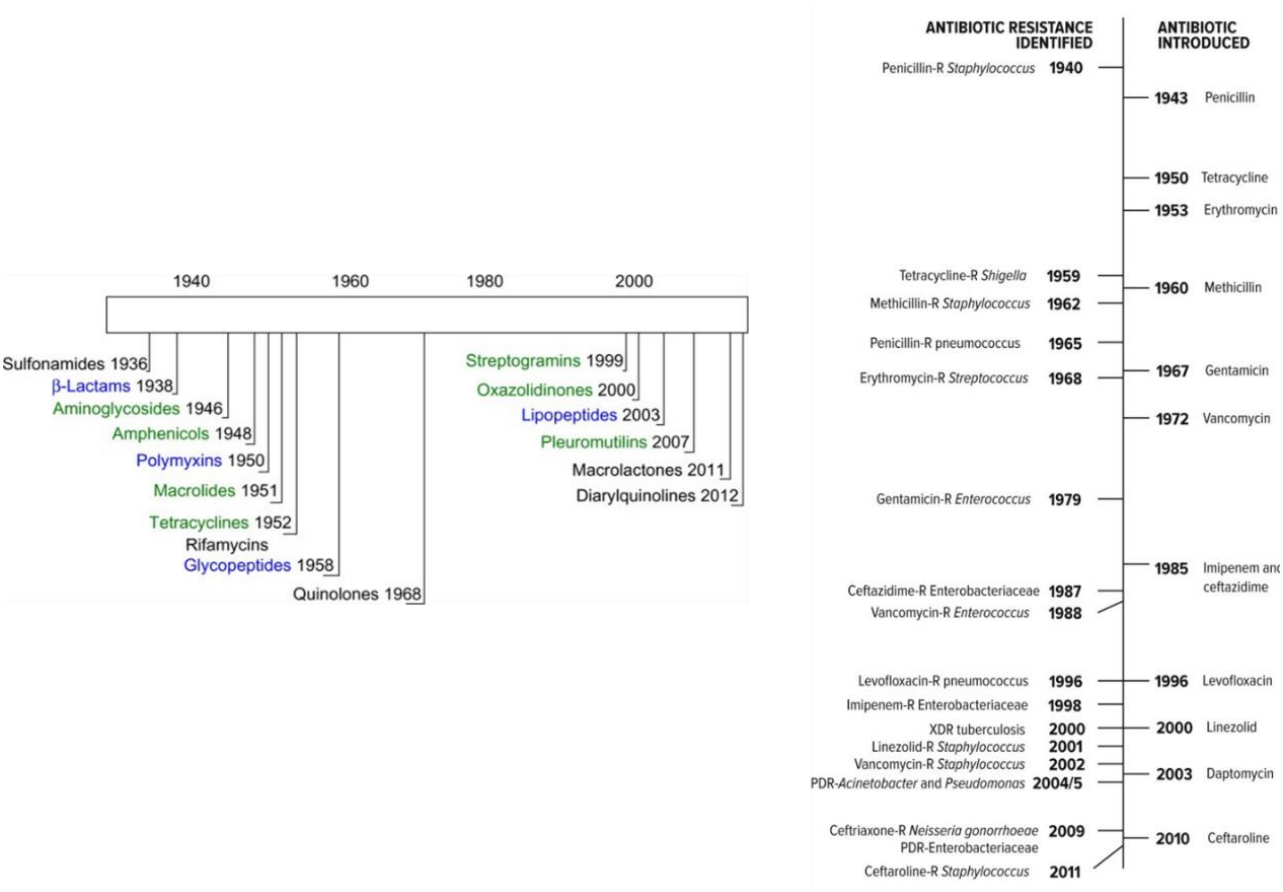


Figure 1. Timeline of the explosion in novel antibiotic discovery accompanied by the years in which there was reports of bacterial antimicrobial resistance. This highlights that for every new antibiotic introduced, the target bacteria evolved resistance strategies ensuring their survival. Adapted from Fair and Tor, 2014 and Ventola, 2015 (2,3).

1.1 Challenges in the Clinical Control of Infection caused by ESKAPE Pathogens

Epidemiologists have become particularly concerned about combatting nosocomial infections caused by the "ESKAPE" pathogens (11). This group encompasses both gram positive and gram negative bacteria and comprises *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species. More recently, inclusion of *Escherichia coli* has been proposed under the acronym ESKAPEE (12). Members of the ESKAPE club are notorious for the numerous strategies of antimicrobial resistance they employ. Significant emphasis has been placed on the elucidation of these

mechanisms which could subsequently be exploited for the development of new therapeutic strategies (11, 13).

In 2017, the World Health Organisation (WHO) outlined a list of 12 priority pathogens, which included ESKAPE pathogens, designating them as either a critical, high or medium threat **(Table 1)**(14). The threat posed by these pathogens is immediate, representing a significant clinical and economic challenge, as uncontrolled infections by these organisms can often be fatal. Though clinical control of infection is still manageable, there is now strong evidence to suggest we are rapidly approaching the onset of a post antibiotic era which may severely limit our capacity to treat currently controllable infections. Of particular concern is the increased incidence of chronic disease globally (15) and with it, the refractiveness of persistent chronic infections to conventional antibiotic treatments. The eradication of pathogens which have transitioned from an acute to chronic lifestyle and become established in the patient can be clinically challenging if not entirely impossible. Understanding the basis of this transition is a key focus in overcoming the ineffectiveness of current antimicrobial regimens.

Table 1; WHO list of priority pathogens for the research and development of novel antimicrobials.

Priority 1: Critical	**Resistance**	**Reference**
Acinetobacter baumanii	Carbapenem	(16)
Pseudomonas aeruginosa	Carbapenem	(17)
Enterobacteriaceae,, ESBL-producing	Carbapenem	(18)
Priority 2: High		
Enterococcus faecium	Vancomycin	(19)
Staphylococcus aureus	Methicillin & Vancomycin	(20)
Helicobacter pylori	Clarithromycin	(21)
Campylobacter spp.	Fluoroquinolone	(22)
Salmonellae	Fluoroquinolone	(23)
Neisseria gonorrhoeae	Cephalosporin & Fluoroquinolone	(24)
Priority 3: Medium		
Streptococcus pneumonia	Penicillin-non susceptible	(25)
Haemophilus influenzae	Ampicillin	(26)
Shigella spp.	Fluoroquinolone	(27)

2. Chronic Respiratory Disease

In recent decades, there has been an increase in the morbidity and mortality arising from chronic disease. Current estimates have placed chronic disease as the third leading cause of death worldwide (28), with the incidence projected to rise with the age profile of the global human population. Chronic respiratory diseases, including Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), asthma, idiopathic pulmonary fibrosis and non CF-Bronchiectasis, are rapidly emerging as a major global health problem (29). Economic losses linked to chronic respiratory disease can be primarily attributed to expenses associated with long term disease management and loss of productivity due to disability (30). In the European Union (EU) alone the annual cost of respiratory disease is predicted to be greater than €380 billion with an

estimated €48.4 billion due to COPD alone and €33.9 billion due to asthma (30). The European Respiratory Society (ERS) reports that the economic costs associated with CF are in the region of 600 million per annum with mutation type shown to influence the cost of CF associated care (30, 31). It is expected with the advent of new and expensive therapeutics that this figure will increase substantially. Whilst the underlying pathophysiology of these respiratory diseases is unique, disease progression is often mediated by chronic infection or chronic inflammation which is accompanied by a gradual loss of lung function (32, 33). In fact, for COPD, CF and non CF-bronchiectasis, the "vicious cycle" hypothesis has been proposed whereby infection or inhalation of toxic substances, such as tobacco smoke, impairs the innate immune system within the lung. This induces an overt immune response resulting in chronic inflammation which further perpetuates chronic airway infection (32-34).

2.1 Overview of the Pathophysiology of CF

CF is the most common autosomal recessive genetic disease within the Caucasian population, affecting more than 70,000 people worldwide (35). This genetic condition results from a mutation in the *Cystic Fibrosis Conductance Regulator (CFTR)* gene encoding an Adenosine Triphosphate (ATP) driven chloride pump (36, 37). This protein is embedded in the cell membrane of all epithelial cells including the cells lining the lungs and gastrointestinal tract (GI tract). The mutation results in a defective CFTR protein which impairs normal airway clearance and results in the accumulation of a viscous mucus on the lining of epithelial cells, hence CF is considered to be a multisystem disorder **(Fig. 2)**. The disease is found to affect the pancreas, resulting in pancreatic insufficiency, and the digestive tract causing gastrointestinal malabsorption and malnutrition. Hepatic defects are also common with a third of patients exhibiting cholelithiasis and biliary cirrhosis (38, 39).

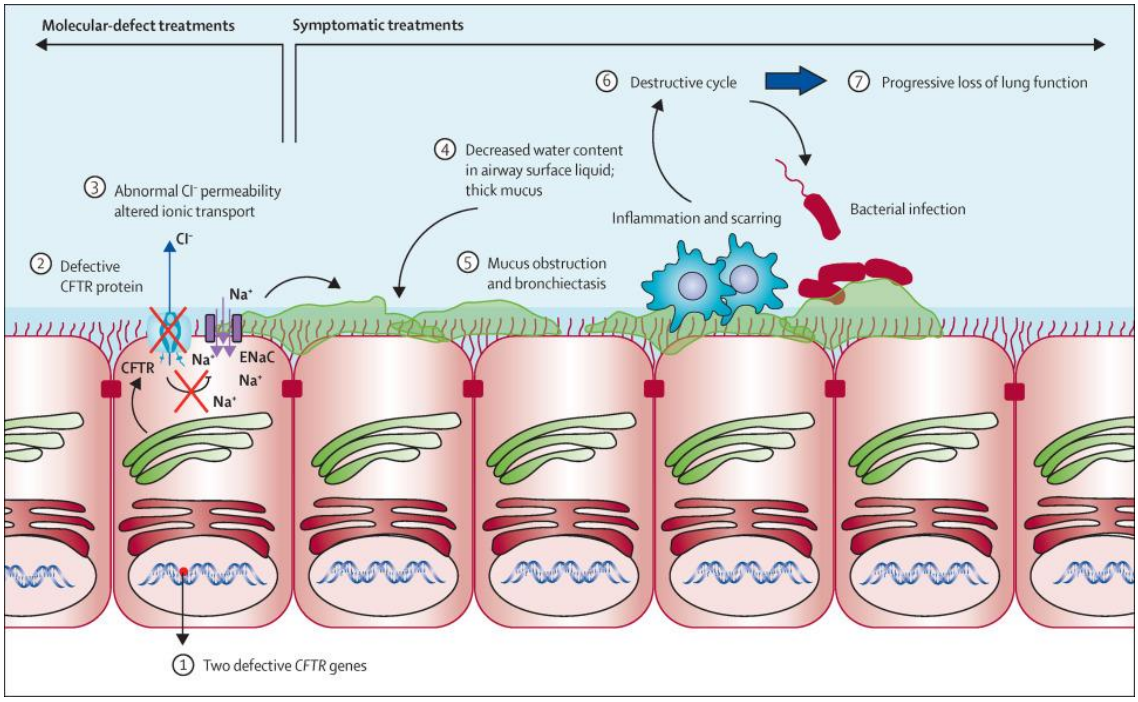


Figure 2. An overview of the pathophysiology of CF. CF results from a genetic defect in the *CFTR* gene resulting in abnormal chloride and water transport in and out of epithelial cells. The subsequent accumulation of a viscous mucus on the lining of the cell leads to a perpetual cycle of infection and inflammation culminating in a progressive loss of lung function. Taken from Boeck and Amaral, 2016 (58).

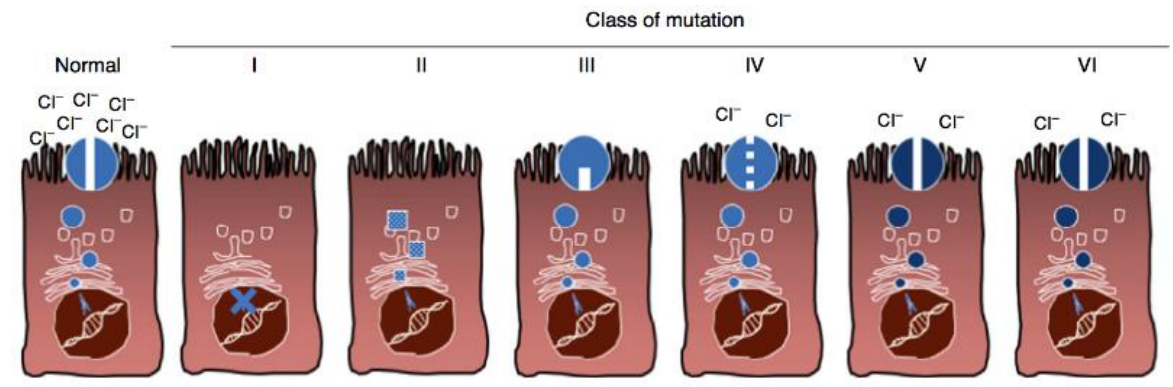
2.2 CF and the Respiratory System

The pathophysiology of CF is primarily considered to be respiratory in nature. The reduced airway surface hydration and build-up of viscous mucus on the lining of lung epithelial cells impairs mucociliary clearance in the airways. This defective mucociliary clearance has been proposed to be central to the progression of lung disease due to the ineffective clearance of pathogenic microorganisms from the lungs which are capable of causing airway infections (40, 41). This results in the generation of an overt immune response culminating in prolonged inflammation in the lungs (42, 43). The heightened pro-inflammatory response to pathogens further exacerbates the problem **(Fig. 2)** (44). It has recently been shown, however, that inflammation can occur independently of infection in paediatric patients less than 3 months old.

Inflammation was detected prior to the onset of bacterial infection, which could be correlated with bronchiectasis at 1 years old (45, 46). Furthermore, mutation of the *CFTR* gene itself has been shown to lead to a dysregulation of host inflammation leading to bronchiectasis further promoting persistent airway infection. CFTR defects have been shown to generate a more acidic pH in the lungs which has been proposed to reduce the action of the pH sensitive innate immune system (47). What is clear is that infection drives inflammation and that inflammation can predispose to infection with these perpetual cycles of infection and inflammation underpinning bronchiectasis and progressive lung decline, the leading cause of morbidity and mortality in the CF cohort (35, 48, 49).

2.3 Genetic Basis of CF

The genetic basis of CF was first elucidated in 1989 (37). Since then over 2,000 mutations have been described in the *CFTR* gene with only 150 of these known to be definitive causative disease associated mutations (50, 51). These mutations can be classified in to six different categories depending on the functional consequence of the mutation in the gene on the protein **(Fig. 3)** (52). Consequently, a spectrum of disease severity ranging from mild to severe is evident in CF depending on the type of mutation present, a major determinant of clinical status. The presence of one partially functioning *CFTR* gene has been shown to dramatically reduce disease severity (53, 54).



Molecular defect	No synthesis	Block in processing	Block in regulation	Reduced conductance	Reduced synthesis	Reduced half-life
Functional abnormality	Protein is not synthesized	Folding defect	Channel opening defect	Ion transport defect	Decreased protein synthesis	Decreased half-life of the protein

Figure 3. Genetic mutations in the *CFTR* gene can be categorized into 6 broad categories on the basis of the molecular defect in the CFTR protein arising from the mutation. The class of mutation present significantly affects disease severity and therapeutics available for treatment. Taken from Quintana-Gallego et al, 2014 (52).

2.3.1 Classes of CFTR Gene Mutation

The classes of *CFTR* gene mutation are outlined in the review by Veit et al. (55). Class I mutations result in the introduction of a stop codon into the messenger RNA leading to the production of a non-functional protein by the ribosome during translation. Class II mutations result in the production of a misfolded protein that is not transported to the cell surface and is therefore targeted by the cell for proteasomal degradation. This class encompasses the most common *CFTR* gene mutation, a deletion mutation of phenylalanine at position 508 (ΔF508), yielding a misfolded protein which is targeted for degradation in the cell. Approximately 90% of CF patients present with at least one copy of this mutation (56). Class III mutations cause the permanent closing of the CFTR channel, preventing any ion transport across the cell membrane. Class IV mutations decrease the conductance of chloride ions through the CFTR channel. Class V mutations reduce the number of CFTR proteins present on the cell surface meaning that even though there is partial functionality, it is significantly reduced relative to normal. Class VI

mutations affect the stability of the CFTR protein so that its half-life is reduced. A new classification system was recently proposed which takes into account the possible corrective therapies available, in addition to severity and mode of action of the mutation (57, 58).

2.4 Prevalence of CF

The availability of prenatal diagnosis and new-born screening has affected the global incidence and prevalence of CF and has resulted in earlier patient diagnosis and improved patient prognosis. As stated previously, CF is the most common genetic disease in Caucasian populations with Canada, UK and Ireland all having particularly high rates of CF births (**Fig. 4)** (59, 60). Ireland has the highest incidence of CF worldwide with approximately 1 in 19 Irish people carrying a mutation in the *CFTR* gene and approximately 1 in 1,400 live births presenting with CF (60-62). Though the life expectancy of affected patients has significantly increased with medical advancements, the estimated life expectancy of 40 years still falls far below average (63, 64). Diagnosis at birth represents a unique opportunity to monitor disease progression which may aid in the design of future treatment plans.

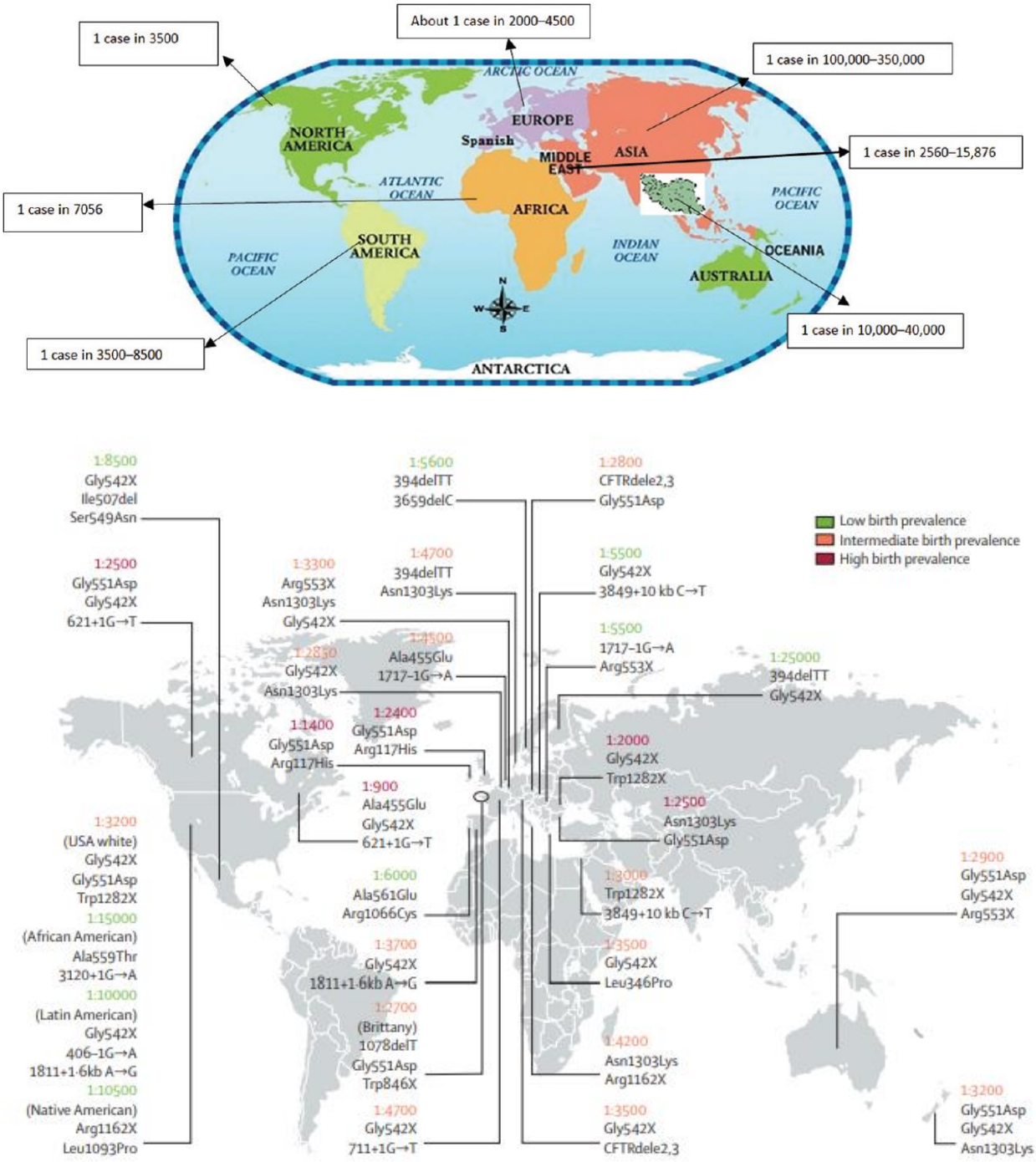


Figure 4. Global prevalence of CF with green denoting low birth prevalence, orange denoting intermediate birth prevalence and red denoting high birth prevalence. The most common mutations identified in each country are also outlined. Ireland has the highest worldwide prevalence of CF. Adapted from Seyed Bashir et al, 2017 and O'Sullivan and Freedman, 2009 (59).

2.5 Clinical Treatment for the Symptomatic Control of CF

A variety of treatments exists for the daily management of CF in an attempt to improve the quality of life of affected individuals. These therapies are primarily targeted to the symptomatic consequences arising from *CFTR* mutation. Clinical strategies consist of a combination of chest physiotherapy to clear excess mucus from the lungs, bronchodilators and/or corticosteroids to relieve symptoms of breathlessness and intensive antibiotic therapy to control frequent airway infections with the primary goal of preventing the onset of chronic bacterial infection (65-67). Inhalation of antibiotics is indicated for patients with chronic *P. aeruginosa* infection; either the nebulized aminoglycoside tobramycin or the inhaled β- lactamase aztreonam (68, 69). The macrolide antibiotic azithromycin is regularly administered to CF patients. However, it has been proposed to function as an anti-inflammatory rather than as an anti-infective (70-72). Anti-inflammatories, including oral corticosteroids are important in the reduction of airway dilation (73). As the build-up of mucus within the lungs significantly impacts lung function it represents a significant interventional target in the clinical control of CF. Routine sessions of chest physiotherapy are directed to the reduction of airway obstruction with the goal of improving mucus clearance from the lungs (74, 75). The use of handheld vibratory devices such as the FLUTTER device can further aid airway clearance (76). Mucolytic therapies such as dornase alfa reduce the viscosity of mucus which has been shown to reduce the number of airway exacerbations (77-79). Additionally, hydrator therapies utilizing hypertonic saline solutions improves mucociliary clearance in an attempt to reduce the frequency of airway infections (80, 81). Unfortunately, the lack of compliance and adherence to recommended treatment plans has serious consequences on patient welfare and prognosis. Generally, as the patient transitions into adult there is a decrease in therapy adherence resulting in worse clinical outcomes (82).

Whilst patient life expectancy and quality of life has undoubtedly improved, these conventional therapies are largely ineffective in modulating disease progression (83-85). In cases of severe lung damage a patient may be required to undergo lung transplantation (86). However, several contraindications exist in the selection criteria for lung transplant such as infection with the pathogen *Burkholderia cenocepacia* (87). As such many CF patients do not qualify as candidates and are excluded from this last resort treatment (88). Additionally, recolonization of the lower airways following lung transplant by *P. aeruginosa* residing in the host is common and is associated with poor clinical outcome (89).

The rapid spread of antibiotic resistance through bacterial populations resulting in the emergence of multi drug resistant pathogens means many antibiotics currently utilised are becoming ineffective in the control of infectious disease particularly pulmonary disease (90). This is further confounded by the fact that antibiotics become largely redundant once respiratory pathogens adopt a chronic biofilm lifestyle (91, 92). These developments signify the urgent need for alternatives to antibiotics. As we enter a new era of medicine it is hoped that treatment will shift from management of symptoms to early treatment interventions in order to prevent deterioration.

2.6 Molecular Therapeutics for the Treatment of CF

As our understanding of the genetic basis of CF has progressed, so too has our application of molecular therapeutic treatments to alleviate symptoms. This is particularly evident in the targeted therapies that aim to correct the effects of *CFTR* gene mutation allowing for the restoration of protein functionality. There are three main classes of drugs currently utilized to achieve this. Premature stop codon suppressors, CFTR correctors and CFTR potentiators.

2.6.1 Premature Stop Codon Suppressors

Premature stop codon suppressors are used in the treatment of class I mutations, which represent approximately 10% of all presenting CF cases, where a stop codon is introduced into the sequence which interferes with translation (93). The aminoglycoside category of antibiotics such as gentamicin and tobramycin were found to be effective in the induction of translational read through so that the ribosome ignored the introduced premature stop codon and continued on to produce a complete functional protein (94-96). However, long term administration of such antibiotics is not advised due to toxicity complications (94, 96). This led to the production of a functionally similar synthetic alternative ataluren which also allows the ribosome to skip the premature stop codon (97, 98).

2.6.2 CFTR Correctors

CFTR correctors are used in the treatment of class II mutations in which the CFTR protein is misfolded and not transported to the cell surface. As this class encompasses the most common *CFTR* mutation it has been the subject and focus of much clinical research. Correctors have been shown to improve the trafficking of the protein to the cell surface (99). The first corrector compound to be introduced onto the market was lumacaftor however clinical trials revealed little improvement in patient symptoms (100, 101).

2.6.3 CFTR Potentiators

The third category of molecular therapeutics; CFTR potentiators are used in the treatment of all other classes of *CFTR* mutations. Potentiators improve the functionality of the CFTR protein at the cell surface (102). Ivacaftor is one such potentiator introduced onto the market in 2012 for the G551D mutation (103, 104). Interestingly, the administration of either lumacaftor (CFTR corrector) or Ivacaftor (CFTR potentiator) alone in the treatment of a homozygous ΔF508

mutation revealed no significant improvement in patient clinical status (105, 106). However, the administration of a combination of lumacaftor and Ivacaftor, referred to as Orkambi on the market, resulted in a 30% increase in cellular chloride transport in patients (101). This is proposed to be due to the combined action of the drugs resulting in an increase in both the delivery of the CFTR protein to the cell surface and enhanced channel opening. The efficacy of Orkambi *in vivo* may also be related to the ability of lumacaftor to stimulate phagocytosis and killing of *P. aeruginosa* by macrophages (107). A significant limitation of these therapies, however, is the age at which treatment can commence. Up until recently Orkambi treatment has only been approved for use in patients greater than 6 years of age (108). In January 2018 the FDA and the EU expanded the approval for the use of Orkambi in children as young as 2 (108). Unfortunately, by this time irreversible lung damage has been shown to have already occurred (109, 110). Additionally, bacterial infections can have also become established, a negative prognostic marker for the effectiveness of Orkambi (111, 112). Furthermore, longer term studies are required to establish the efficacy of these CFTR repair treatments in the prevention of symptomatic decline as patient's age.

2.6.4 Gene Therapy

Gene therapy/editing is emerging as a new approach in the management of genetic disease. However, despite early progress, many significant obstacles remain which continue to delay the development of this strategy as a viable treatment for CF. Evidence from pre-clinical trials have demonstrated modest improvements in lung function as measured by Forced Expiratory Volume (FEV1), however there was no coinciding increase in the quality of life of affected patients (113, 114). Additionally, the numerous risks that accompany the application of gene therapy must be overcome before this approach can ever be considered for widespread use (115).

As medical research has advanced at such a fast pace, many have suggested that we are approaching the age of personalised medicine in the treatment of genetic diseases such as CF. However, response to treatment plans has been shown to drastically vary between patients, even for those individuals with the same mutation (116). Therefore, several challenges must be addressed if personalised medicine is to become a reality. Though, the aforementioned combined corrective and potentiating therapies have considerable potential, it is clear that focus should shift to early intervention strategies. The objective of such strategies should ultimately be the prevention of lung function decline in paediatric CF patients. The identification of factors promoting chronic inflammation and the emergence and persistence of chronic pathogens that contribute to this decline is fundamental in the design and eventual implementation of novel treatment regimens.

3. Lung Microbiome Composition in Patients with Chronic Respiratory Disease

Whilst the development of chronic respiratory disease is multifactorial, the interactions between the host and colonising microbes is central to the pathophysiology of disease progression. Therefore, it follows that a potential alternative strategy could emerge from the study of the underlying microbiology of chronic respiratory disease, particularly how respiratory pathogens residing in the lung are triggered to adopt a chronic biofilm lifestyle.

Several studies have investigated the microbiological basis of chronic pulmonary disease and have revealed that the lungs of both healthy and diseased individuals harbour diverse communities of bacteria and are not sterile environments as was previously believed (117-122). A comprehensive knowledge of the differences between microbial communities (known as the microbiota) residing in the lungs of healthy and diseased patients could further enhance our understanding of their role in the progression of respiratory disease. The composition of the lung

microbiota has been shown to be determined by three primary factors; (1) the degree of microbial immigration, (2) the rate of microbial elimination and (3) the growth rate of the constituent members. The contribution and balance of each of these factors to the respiratory microbiota should be considered when studying differences that may exist between health and disease (123). The lower airways of healthy individuals have been shown to harbour diverse, though low abundance communities of bacteria (124-126). In contrast, the lower airways in respiratory disease consists of highly diverse and highly abundant bacterial communities **(Fig. 5)** (127-133). These differences in abundances between health and disease can potentially be attributed to a higher bacterial burden and ineffective bacterial clearance from the lungs due to impaired mucociliary clearance in pulmonary disease (134). The source of these lower airway communities are thought to be the upper airways and the gastrointestinal tract (135-139). These reservoirs of bacteria can be transmitted via inhalation, micro-aspiration and direct mucosal extension (123, 125). Reports of perturbations of these communities in samples from patients with chronic respiratory disease are frequently described in the literature. Additionally, insights into the influence of host physiological factors that shape the dynamics of these populations could further aid the design of novel therapeutic plans.

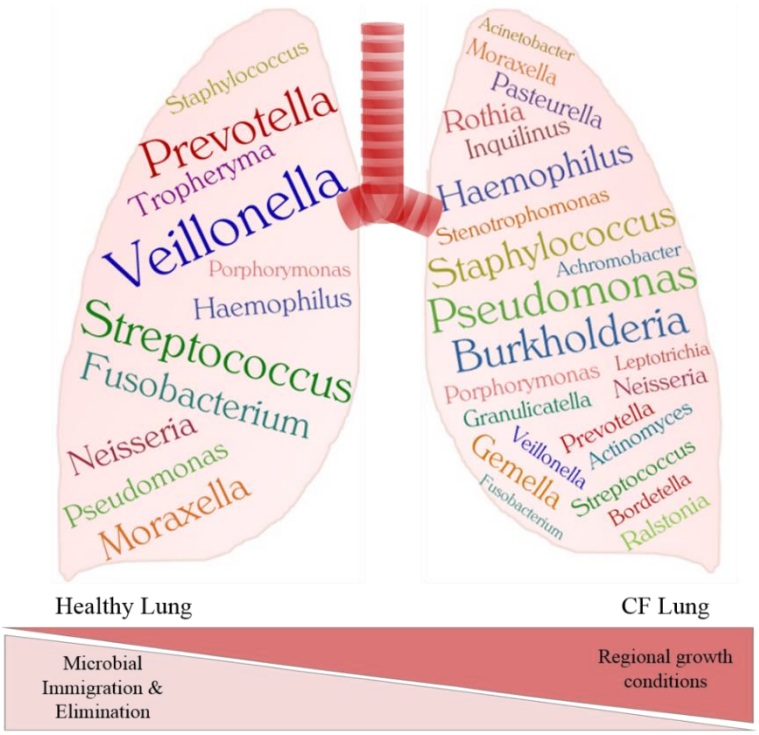


Figure 5. Summary of common microbiota associated with healthy vs CF lungs. Commonly identified taxa are illustrated and the factors governing the dynamics of these communities are presented. Microbes such as the anaerobes *Veillonella* and *Prevotella* are routinely isolated from healthy lungs. While *Staphylococcus* and *Haemophilus* are known to be early colonisers of the lungs of paediatric patients with CF, *Pseudomonas* ultimately achieves a dominant position within the lung microbiota. The balance between microbial immigration and elimination has been described as a primary influence on the community structure of a healthy lung. In contrast, regional growth conditions within the lung microenvironment have been shown to drive population diversification.

3.1 Sampling Techniques for the Identification and Characterisation of the Lung Microbiome

In order to examine the microbial communities residing in the lung, the lung must first be sampled with specific consideration given to the location of sampling and the level of invasiveness (140). There are four types of sample that can be used; 1) bronchoalveolar lavage fluid (BALF), 2) induced or expectorated sputum sampling, 3) deep throat swab and 4) analysis of explanted lungs (120).

3.1.1 BALF Sampling

The gold standard for sampling the lung microbiota is BALF sampling. BALF is obtained during a surgical lung bronchoscopy during which various regions of the lung can be sampled (124, 133, 141). In adults, this procedure is considered to be minimally invasive requiring the administration of local anaesthetic and sedative (142, 143). In contrast, this procedure is considerably more invasive in paediatric patients as a general anaesthetic is required with an increased risk of unintentional trauma to the airway epithelium (144, 145). As many microbiota studies encompass paediatric patients, the level of invasiveness should be a primary concern when choosing a sample type. Furthermore, this option can often become prohibitively expensive for use on a wide scale basis in large clinical cohorts (146).

3.1.2 Sputum Sampling

The preferred alternative to BALF sampling is the use of expectorated or induced sputum samples. Sputum comprises the thick mucus that is coughed up by patients from the lower airways; with expectorated sputum generated solely by coughing and induced sputum obtained by administration and inhalation of a hypertonic saline solution (147-149). The use of sputum, both expectorated and induced, has numerous advantages; it is safe, non-invasive, can be routinely carried out and is relatively inexpensive (150). Therefore, it is the most widely practiced technique of sampling the lung microbiota. Initially, the primary disadvantage associated with the use of sputum was the potential risk of cross-contamination by microbial communities present in the upper respiratory tract (151). However, several studies have identified distinct microbial communities in the upper airways/oropharyngeal tract when compared to that of the lower respiratory microbiota with minimal cross contamination (128, 135, 152, 153).

3.1.3 Explanted Lung Sampling

Lastly, in patients who present with end stage lung disease, a lung transplantation may be required. In these cases microbial sampling of the explanted lungs can be undertaken. Analysis of these microbial communities has provided further evidence confirming that BAL and sputum are effective means of sampling the lung environment (127). Interestingly, these studies of explanted lungs highlighted that significant bacterial community heterogeneity exists throughout the lung. The spatial heterogeneity present within the lung, a product of environmental gradients within the lung, may be the basis for the formation of distinct communities of bacteria in different regions of the lungs (123, 154-156).

3.2 Culture Dependent versus Culture Independent Analysis of the Lung Microbiome

What has become increasingly apparent from microbiota analysis of various regions throughout the body is that the use of culture dependent techniques alone is insufficient in providing a fully representative overview of the complexity of microbial communities present. Furthermore, the practice of species-specific cultivation techniques for the identification of pathogenic species has further biased the view of the diversity present within the lung. The primary limitation associated with culturing is that only a small percentage (1%) of bacteria were believed to be culturable. This phenomenon coined the “plate count anomaly” by Staley and Konopka in 1985 described the apparent inability to culture many microbes visible under the microscope (157). To culture this 1% is both challenging and time consuming (158). However, a study by Sibley *et al.* demonstrated that the majority of bacteria present in the CF airways are readily cultured through an enrichment of conventional microbiology techniques. A combination of culture dependent and culture independent techniques increased the sensitivity of bacterial detection within the CF lung (159). The significant advances in culture independent techniques in recent years has provided a

better understanding of the complexity of the human microbiota (160, 161). Next Generation Sequencing (NGS) including Illumina HiSeq/MiSeq and PacBio, has become more affordable allowing increased species level identification offering a more representative view of the lung microbiome (131, 162). The emergence of third generation sequencing technologies such as Oxford nanopore technologies and ion torrent require just a single molecule of DNA to sequence which could potentially translate into faster and cheaper sequencing of lung microbiomes (163).

The availability of such a wide array of technology means studies conducted on the lung environment generates a vast amount of information. However, inter-study comparisons should be interpreted cautiously as standardised practices have yet to be established. This includes choice of sample type, method of DNA extraction and sequencing technology; all of which can introduce biases into microbiome studies. Hence, what is critical for this rapidly emerging field is the design of standardised practices, an issue that must be addressed for future research. Nevertheless, microbiota studies have been conducted for many respiratory conditions with a pervasive microbiome reported for CF which has been correlated with patient disease status (128). Signature microbiota profiles are currently being investigated for other respiratory diseases including COPD, asthma and bronchiectasis (164, 165).

4. A Pervasive Lung Microbiome Exists in CF Patients

The lungs of CF patients harbour diverse microbial communities with independent studies detecting up to 100 genera of bacteria **(Fig. 5)** (166-168). This is significantly more diverse than previously estimated and is unsurprising considering the CF lung is a warm, humid environment, organically rich with defective mucociliary clearance favouring the exponential proliferation of microbial communities without removal. This favourable niche is exploited by a plethora of microbial communities (168-170). At the phylum level, the majority of the genera present belong

to Proteobacteria, Bacteroidetes, Fusobacteria and Firmicutes (166, 168). The signature CF microbiome is reported to be heavily dominated by the classical CF-associated pathogens; *P. aeruginosa*, *S. aureus*, *Haemophilus influenzae* and *B. cepacia* complex (36, 42, 128, 171). It is thought that there is a general pattern of colonization of the CF lung; first by *H. influenzae* and *S. aureus* succeeded by *P. aeruginosa*, *Stenotrophomonas* and *Burkholderia* (**Fig. 6)** (35, 171).

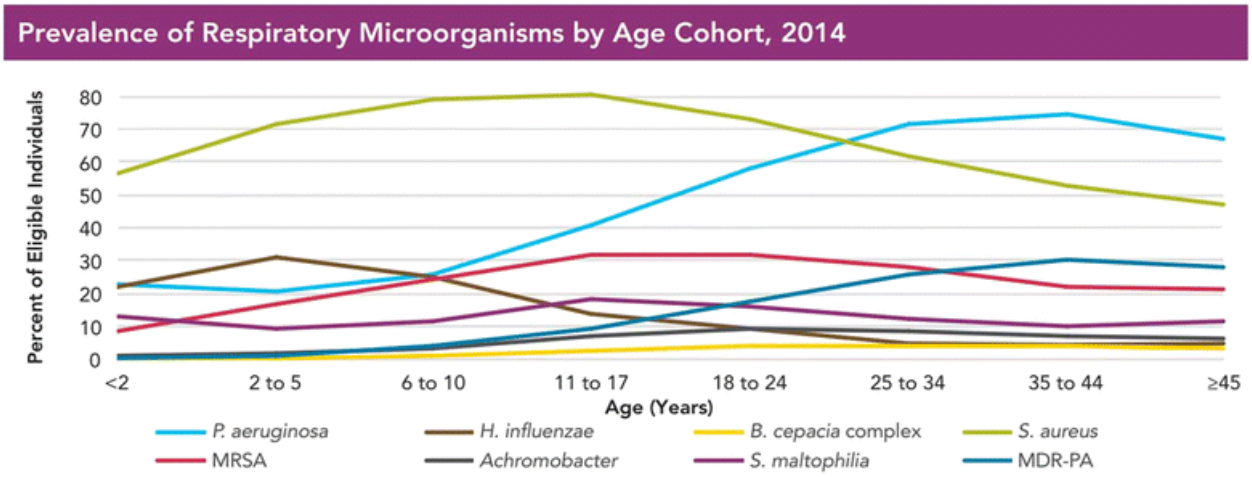


Figure 6. General pattern of microbial colonisation within the CF airways with *S. aureus* a primary coloniser of the paediatric CF lung and *P. aeruginosa* a primary coloniser of the adult CF lung. Taken from CFF Annual data report, 2014 (1).

It has been shown that these pathogenic bacteria can represent a tiny portion of the total microbiota in early stage lung disease with evidence that *Streptococcus*, *Porphorymonas*, *Prevotella*, *Veillonella*, *Rothia*, *Actinomyces*, *Gemella*, *Granulicatella*, *Fusobacterium*, and *Neisseria* comprise the core microbiota with a relatively high proportion of both facultative and obligate anaerobic bacteria present (168, 172). The presence of anaerobes may be explained by the thick mucus present on lung epithelial cells which is anoxic in nature due to the slow diffusion of oxygen and rapid utilisation of available oxygen by aerobic bacteria. This results in the generation of both anaerobic and microaerophilic pockets in the lungs which are occupied by

strict and facultative anaerobes (169, 170, 173). The discovery that strict anaerobes are commonly present in the CF lungs further highlights the limitations of conventional microbiology as these bacteria went largely undetected due to difficulties culturing within clinical settings (168, 172).

4.1 Polymicrobial Communities and Age Associated Decline in Diversity are Observed in CF Microbiomes

The previously held view that respiratory infections in CF are of a mono-microbial nature has been challenged by the growing understanding of the microbiological basis of the disease. This has highlighted that a limited number of pathogenic species are not solely responsible for infection. Rather, pulmonary exacerbations, described as a temporary decline in lung function, are driven by multiple microbial species with community composition also impacting antibiotic efficacy (174, 175). These observations have shifted our attention to the role of polymicrobial communities and inter-kingdom interactions in infection and in the factors contributing to the restructuring of these communities throughout disease progression (130, 176-182). These studies have also highlighted the limitations associated with treatment strategies based solely on the identification of pathogenic bacteria through routine culturing. Therefore, analysis of the microbiota as a whole is becoming increasingly important (121, 130, 159, 177). Ecological networking strategies have played an important role in deciphering microbial community dynamics throughout the infection process (178).

Findings from numerous cross-sectional studies have revealed progressive increases in the diversity of bacterial communities as a patient ages (129, 166, 183). However, as patients transition to adulthood, there is a gradual decrease in diversity where communities become dominated by a single pathogenic species. Recent studies have shown that this process is largely completed by the

age of 25 (129, 132, 166, 183). Whilst the microbiome is relatively constant in clinically stable patients, patients experiencing an exacerbation display dynamic changes in community structure referred to as microbial dysbiosis though there is no coinciding significant change in bacterial load. This dysbiosis is characterised by a shift towards a low diversity, pathogen dominated microbiota with the infection process beginning as early as infancy, further highlighting the importance of community diversity in the maintenance of lung health (184-188). The "Keystone pathogen hypothesis" could explain this observation. This attempts to describe the role of polymicrobial interactions within the lung whereby low abundance members of the community such as the *Gemella* genera, play a major role in the remodelling of the microbiota towards that of a dysbiotic pathogenic community (185, 189, 190). There is evidence of early homeostatic disruption to the CF lung microbiota when compared to healthy controls, indicating the occurrence of a fundamental disturbance at an early age (191). The similarity in community composition between paediatric and adult microbiota further supports the finding that microbial dysbiosis becomes established early on in life and is maintained throughout disease progression (135).

The trend towards reduced microbial diversity is characterised by a reduction in evenness and richness of microbial populations and is negatively correlated with clinical status and pulmonary function. This observation is becoming a predominant feature of many chronic inflammatory conditions (192, 193). Furthermore, microbial communities in older patients are composed of more phylogenetically related populations which are potentially resistant to antimicrobial treatment strategies (166, 194). Whilst data generated from these studies are informative, they are significantly limited by their cross-sectional study design and small sample size (186, 195). A concerted shift to longitudinal analyses and clinical follow-up would prove more useful in the long

term as these studies would provide insights into microbiome evolution with respect to patient aging.

The association of microbial community diversity and disease status has been previously described for several human conditions. A reduction in biodiversity and consequential alteration of the functional capabilities of the microbial community are evident in the gut microbiota for inflammatory diseases and diabetes and in the oral cavity microbiota with periodontitis (196, 197). The recurrent administration of antibiotics during exacerbations has been proposed to contribute to the age-related decline in diversity in CF. Whilst some studies have proposed significant determining effects (132, 198), others have demonstrated only transient effects (128, 199). However, the microbiota has been shown to recover to baseline level after exacerbations and antibiotic treatment (186, 200). In addition to antibiotics, the environment and inflammation have been proposed to play a role in the shaping of the lung microbiota. However, the relative contribution of each of these factors is unknown, in particular that of the influence of antibiotics (132, 198-202). It is clear, however, that the frequent disruptions to these communities are associated with increased morbidity and mortality. Even with the emergence of new treatments such as Orkambi (203-205) which have revolutionised the clinical management of CF, understanding the CF microbiota in terms of both low and high abundance genera, as well as the relative importance of fungal species and viruses, remains a key research question. Particularly, as those receiving treatment will have a degree of pre-existing airway destruction and an increased risk of infection (171, 206).

5. *P. aeruginosa* Pathogenesis & CF

Pathogenic domination of the CF lung microbiota is largely due to the emergence of *P. aeruginosa,* which chronically infects up to 70% of CF patients (207, 208). Environmental conditions within

the lung such as reduced oxygen tension promotes the outgrowth of *P. aeruginosa*. Chronic infection by *P. aeruginosa* is a primary indicator of poor clinical prognosis and is correlated with a lower FEV1, a clinical measurement of lung function (207, 209). A recent study has highlighted that chronic infection with the key CF pathogen *P. aeruginosa* drives pulmonary dysbiosis rather than the existence of prior dysbiosis facilitating the emergence of this opportunistic pathogen (210). The ability of *P. aeruginosa* to adopt a biofilm mode of growth which is refractive to antibiotic therapy and to chronically infect the CF lung is widely considered to be the primary driver of progressive lung disease in CF (91).

P. aeruginosa is a gram negative rod belonging to the family Pseudomonadaceae. It is capable of occupying a wide range of niches with its high degree of genome adaptability facilitating rapid adaptation to new environments (211). It is considered an opportunistic human pathogen with the WHO classifying it as a priority one critical pathogen for the development of novel antibiotics (14). *P. aeruginosa* can cause life threatening infections in immunocompromised patients and patients suffering from respiratory conditions such as CF with colonisation found to occur within the first three years of life (212). Significant advances have been made in CF clinical care, in particular early eradication strategies and the prevention of cross patient transmission which has reduced the frequency of chronic colonisation by *P. aeruginosa* and has resulted in an improvement in patient outcome and increased life expectancies (69, 213-219).

5.1 Acute versus Chronic Lifestyle of *P. aeruginosa*

The large proportion of transcriptional regulators (representing approximately 10% of all genes present) within the 6.3 Mb *P. aeruginosa* genome facilitates the survival of this microbe under the range of stressful conditions it encounters such as oxygen stress and nutrient limitation (220). Furthermore, the ability of this organism to grow under both aerobic and anaerobic conditions

and optimal growth at 37˚C underpins its role as a human pathogen (221). The process of airway infection involves an initial acute phase characterised by the production of a suite of virulence factors facilitating invasion and survival in the host (222). During this acute stage of pathogenesis, the pathogen is highly motile, secreting a variety of toxins and proteases with the type three secretion system of particular importance. This lifestyle stage is characterised by severe and significant damage to target host cells. Acute infections are evident in patients with ventilator associated pneumonia, urinary tract infections, infections in immunosuppressed patients and wound infections in burn patients (223). As the organism transitions towards chronicity and long term persistence, there is a reduction in acute virulence factor production which contributes to the successful evasion of this pathogen from the host immune system (222, 224, 225). The emergence of hyper-mutator strains of *P. aeruginosa* increases the ability of the pathogen to adapt to these new host conditions and is central to the success of *P. aeruginosa* as a human pathogen (226, 227). The occurrence of alginate overproducing strains is associated with the development of mucoid strains arising from a loss of function mutation in the *mucA* transcriptional regulator (228, 229). This event is thought to signify the switch to chronic infection and is important in the long-term survival of this organism within the host. Although there is currently no universally accepted clinical definition of chronic *P. aeruginosa* infection (208, 230, 231), the phenotypic changes that occur during the transition have been well characterized in this organism. The acute to chronic switch as outlined by Moradali *et al*. **(Fig. 7)** involves the suppression of key acute virulence determinants such as the Type Three Secretion System (T3SS), phenazine production, and swarming motility, while the chronic persistent lifestyle is adopted through increased biofilm formation and the production of chronic virulence systems such as the Type Six Secretion System (T6SS). Coordinating this switch are several

layers of regulation, including the quorum sensing (QS) signalling pathway and classical two component signalling systems (220).

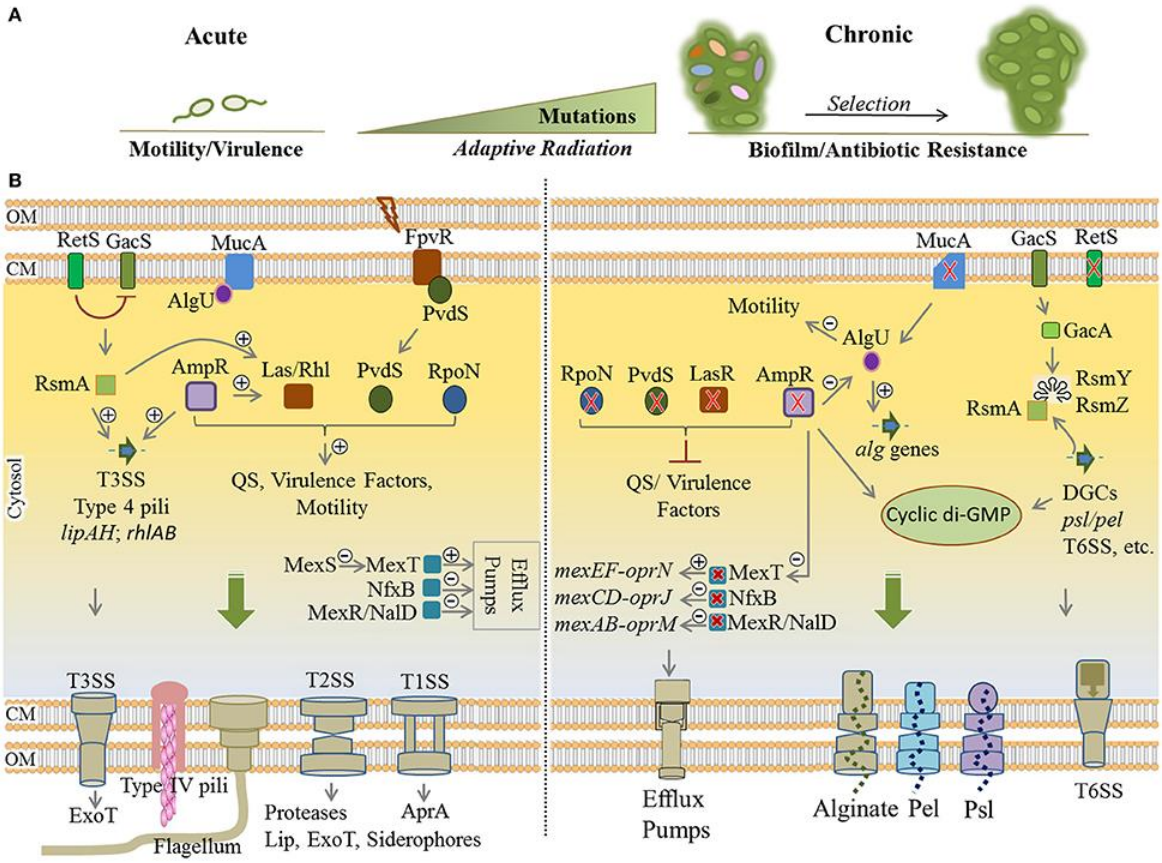


Figure 7. The acute to chronic behavioural lifestyle switch encompasses a wide array of phenotypic and signal transduction changes facilitating persistence within the CF lung enivronment. This includes a combination of clsssical two component systems, transcriptional regulators and virulence factors. Taken from Moradali et al, 2017 (220).

5.1.1 Quorum Sensing

P. aeruginosa employs quorum sensing signalling systems in order to mediate the collective behaviour of communities. These regulatory systems enable coordination of cooperative gene expression to environmental signals. *P. aeruginosa* encodes two classical Acyl-Homoserine Lactone (AHL) QS systems (LasIR and RhlIR), as well as an Alkyl Quinolone (AQ) system controlled by the Pseudomonas Quinolone Signal (PQS) and its precursor HHQ with all 3

systems involving an autoinducer synthesis gene and a cognate regulatory gene. There is also an emerging role for the IQS system, although its interconnection with the classical systems remains to be established (232). These are complex, interconnected pathways mediating the response to a variety of input signals. All three of these systems are involved in cell to cell communication with the AQ system in particular shown to have a role in biofilm formation and persistence (233). Quorum sensing is linked to the production of a wide range of virulence factors, including the redox active phenazine pyocyanin, an important virulence factor in the establishment and persistence of infection in vivo. Phenazines are respiratory pigments that play a role in extracellular electron transport which facilitate survival by mitigating the stress of electron acceptor limitation (234). Pyocyanin has also been shown to contribute to the success of *P. aeruginosa* as the dominant organism within the lung microbial community through its inhibition of respiration of competing microorganisms such as *S. aureus* (235). Quorum sensing has also been implicated in the pathogen's interaction with the host, with PQS shown to induce oxidative stress and repress expression of the anti-oxidant enzyme Heme oxygenase-1 in lung epithelial cells (236). Hence, these signalling molecules are central to *P. aeruginosa* pathogenicity.

5.1.2 Secretion Systems

There are a range of secretion systems facilitating the interaction between *P. aeruginosa*, the host and co-colonising resident micro-organisms. Two of the primary secretion systems are the Type Three Secretion System (T3SS) and the Type Six Secretion System (T6SS) which are capable of injecting effector proteins into host cells and neighbouring bacteria, respectively. The T3SS is employed by *P. aeruginosa* to manipulate the cells of its eukaryotic host. This system is encoded by 36 genes located on 5 operons and consists of a phage needle like structure with the ExsB protein required for correct assembly of the T3SS system and for full virulence *in vivo* (237).

Upon assembly of the needle like structure effector proteins encoded by approximately 6 additional genes can be injected into the host cell. These effectors include ExoS, ExoT, ExoU and ExoY which can induce cell death, inhibit DNA synthesis and disrupt the actin cytoskeleton (238, 239). It is thought that these toxins prevent phagocytosis of the organism with a functional T3SS increasing disease severity. Hence, as the organism transitions to chronicity there is a dampening down of the activity of this system. A study by Subedi and colleagues further confirmed this with CF isolates shown to not possess the *exoU* gene while acute isolates recovered from anterior eye infections and microbial keratitis contained *exoU* which correlated with increased antibiotic resistance (240).

The T6SS also uses a phage-like needle machinery and is utilised by *P. aeruginosa* to manipulate competing bacterial cells through a 'tit for tat' interaction, ultimately inhibiting their growth (241). The T6SS is encoded by three distinct gene clusters; Hcp1 secretion island (HSI)-I, HSI-II and HSI-III. The activity of this system potentially facilitates the dominance of *P. aeruginosa* further enhancing its fitness within the polymicrobial CF lung microbiome.

5.1.3 Antibiotic Resistance

P. aeruginosa displays a high level of resistance to a range of antibiotics **(Fig. 8).** The antibiotic resistance of this organism can be attributed to a combination of natural resistance afforded by (i) the presence of the outer membrane for which a range of antibiotics cannot permeabilise; (ii) intrinsic resistance systems, including an array of efflux systems and regulated porins; and (iii) the ability to reside within a protective biofilm matrix providing an additional barrier to antibiotics.

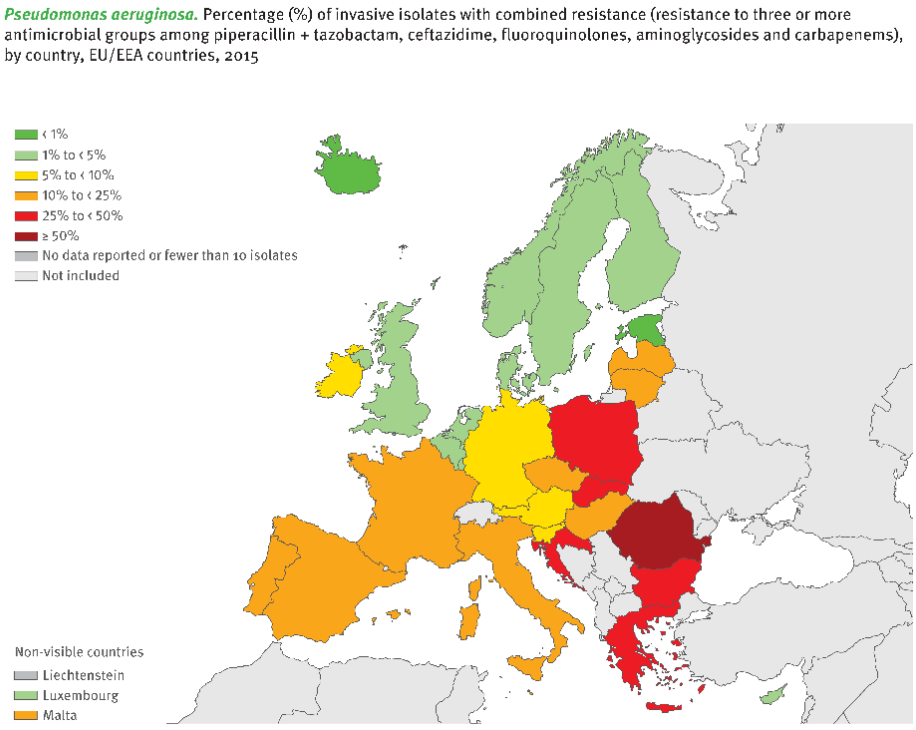


Figure 8. High percentage of *P. aeruginosa* isolates display combined antibiotic resistance to clinically relevant antibiotics throughout Europe. This highlights the effectiveness of this organism at combatting the stress represented by antimicrobials and its numerous strategies to increase its resistance (1).

P. aeruginosa employs a range of mechanisms for efficient antibiotic resistance **(Fig. 9)**, including the production of antibiotic degrading enzymes such as β-lactamases, synthesis of antibiotic modifying enzymes, reduced outer membrane permeability and increased activity of efflux systems (242, 243). MexAB-OprM is one such efflux system contributing to *P. aeruginosa* resistance to β-lactam antibiotics. In clinical isolates, mutations in the regulatory genes of efflux systems have been demonstrated to result in the hyperexpression of the efflux pumps contributing to increased resistance to antibiotics in the clinic (244, 245). Though increased resistance to antibiotics is well described, a recent finding of the acquisition of resistance to the polymyxin class of antibiotics, often considered a last resort antibiotic, is

worrying (246). The implications of enhanced *P. aeruginosa* antimicrobial resistance are far reaching not only in terms of the detrimental impact on patient's quality of life but also the financial burden of clinical management strategies.

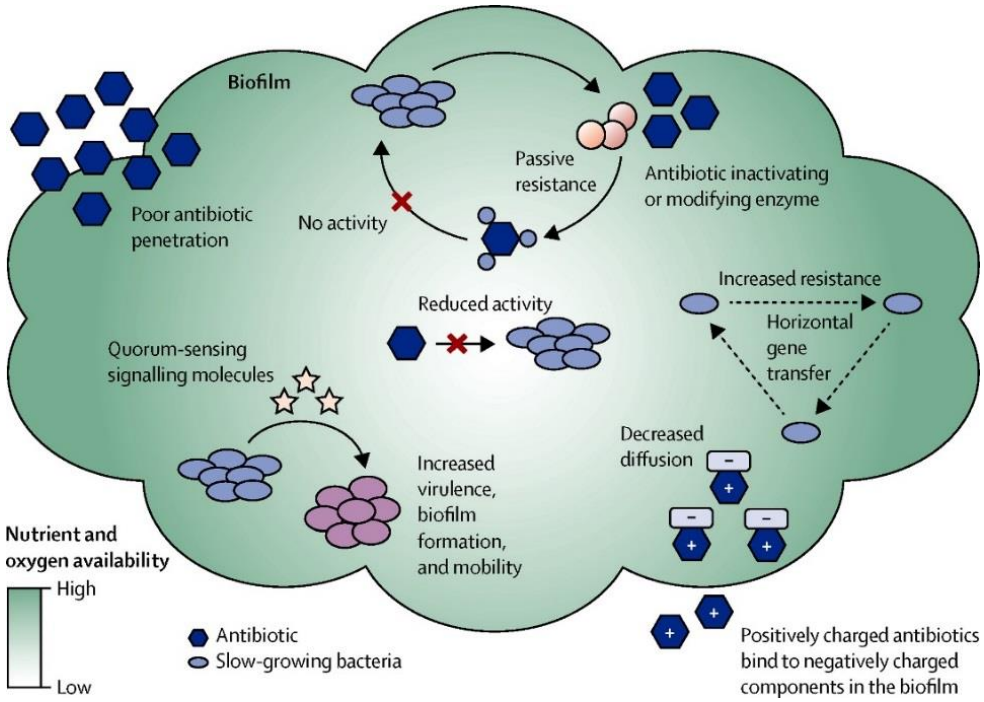


Figure 9. *P. aeruginosa* employs a range of antibiotic resistance strategies to increase its tolerance and resistance to antimicrobials ensuring its persistence within the CF lung. This includes production of a biofilm hindering the penetration of antibiotics, antibiotic modification to prevent its activity and active efflux of antibiotics. Taken from Sherrard et al, 2014 (243).

5.1.4 Biofilm formation

The ability of *P. aeruginosa* to produce a biofilm, a community of microbes residing in a protective matrix of Extracellular Polymeric Substances (EPS), is one of the primary mechanisms underpinning its tolerance to antibiotics within the CF lung (247). The EPS matrix is predominantly composed of exopolysaccharides, extracellular DNA and polypeptides (248,

249). The architecture of the biofilm changes throughout biofilm maturation as described in Figure 10 below (250).

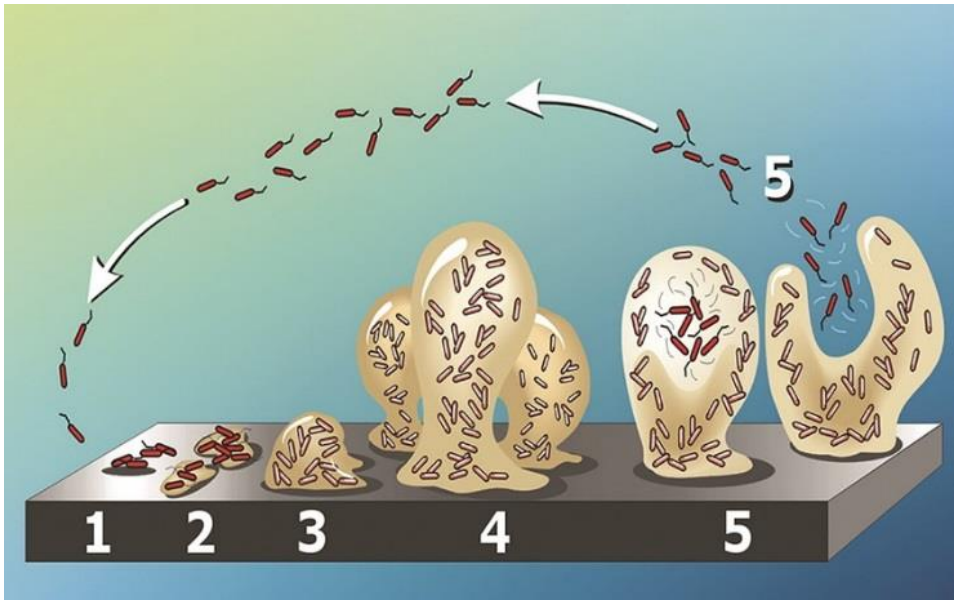


Figure 10. The stages of biofilm development in *P. aeruginosa* involving initial then irreversible attachment, microcolony formation, macrocolony formation and eventually dispersal of the biofilm. The architecture of the resulting biofilm can vary with the 3D mushroom like biofilm typical. Taken from Whitters and Stockley 2011 (250).

P. aeruginosa is known to produce three main polysaccharides; alginate, Pel and Psl which determine biofilm stability, with Pel and Psl required for early stage biofilm formation (251, 252). The modulation of polysaccharide production during chronic infection promotes the strength of biofilms during infection (253). Extracellular DNA (eDNA) is essential for biofilm formation, with degradation of eDNA shown to block the production of a biofilm (254). Additionally, eDNA contributes to biofilm maturation through its impact on motility and its role as nutrient source to bacteria within the biofilm (255, 256). The production of a biofilm involves the coordinated action of several regulatory systems including but not limited to quorum sensing, two component systems (GacS/GacA, RetS/LadS*)* and the bacterial second messenger cyclic-di-GMP (257).

Cyclic-di-GMP has been widely recognised for its importance in biofilm formation and swarming motility, behaviours which are proposed to be inversely regulated. The production of c-di-GMP involves synthesis by GGDEF domain containing diguanylate cyclase proteins and breakdown by EAL and HD-GYP domain containing phosphodiesterase proteins, for which *P. aeruginosa* is known to encode approximately 41 (258). The pools of c-di-GMP in the cell have been shown to modulate the production of polysaccharides, with high levels of c-di-GMP inducing polysaccharide biosynthesis and low levels of c-di-GMP promoting motility (259-262). Interestingly, a recent study has proposed a role for c-di-GMP in the ability of *P. aeruginosa* to evade the host immune system through its interactions with the methyltransferase, WarA (261).

The regulatory control of biofilm development has been linked to quorum sensing (263). The Las system has been implicated in the structural development of a biofilm with LasR involved in the regulation of Psl and PQS linked to the release of eDNA (264-266). Furthermore, the connection between quorum sensing and cellular motility indirectly impacts upon biofilm architecture (267). The GacS/GacA two component system consisting of a transmembrane sensor kinase and response regulator has been demonstrated to be a global regulator of QS biofilm formation (268-270).

The complexity of systems governing *P. aeruginosa* virulence and pathogenesis serves to highlight the challenges faced in elucidating the factors that signal the transition from an acute to chronic lifestyle. Many of the regulatory systems that are involved in controlling multi-cellular behaviour, biofilm formation, motility and secretion are well characterised. Molecular mechanisms have been proposed for a number of these systems and yet the factors responsible for triggering or suppressing their activity remain for the most part unknown. The challenge

therefore is to elucidate the niche factors that cause *P. aeruginosa* and indeed other pathogens to adopt a chronic antibiotic tolerant biofilm in patients with respiratory disease.

5.1.5 Adaptive Evolution

The persistence of *P. aeruginosa* has been proposed to be due in part to its ability to rapidly adapt to novel conditions with several studies reporting phenotypic and genotypic diversification both *in vitro* and *in vivo* (271-281). This adaptive evolution, proposed to be underpinned by recombination, is evidenced in the recovery of a range of distinct clinical isolates from the sputum of CF patients, a product of spatial heterogeneity within the lung (154, 155, 275, 282, 283). From the genomic perspective, these studies have identified several loci that are frequently mutated, which has led to them being classified as pathoadaptive mutations. The evolutionary pathways through which these mutations emerge are distinct between studies, notwithstanding the fact that convergent evolution is proposed to occur (284, 285). Pathoadaptive mutations frequently occur in genes including the global regulators *lasR, rpoN, mucA, mexT, retS, exsD,* and *ampR* as reviewed by Winstanley *et al*. (286). The consequence of these mutations is the emergence of phenotypic variants which display reduced virulence factor production, enhanced antibiotic resistance, enhanced siderophore production and auxotrophy. The non-uniform nature of *P. aeruginosa* populations and the significant genotypic and phenotypic heterogeneity present means that there should be careful interpretation of data generated from clinical isolates. Though there is still not a full understanding of the drivers of pathoadaptation, several factors may contribute to the positive selection on these genes including, iron acquisition (287, 288), antibiotic administration (289-293), sputum conditions (277, 290, 294), biofilm conditions (295, 296) and presence of phage (277, 297). Further investigation is required to unravel the complexities of *P. aeruginosa* evolution *in vivo*. The metabolic evolution of this pathogen should

also be taken into consideration, as it can provide valuable insights into the connections between metabolic specialisation and adaptive phenotypic traits (298). Collectively, this research has the potential to provide new knowledge that may be useful in hindering the establishment of chronic *Pseudomonas* infections.

6. Factors Modulating the Progression of Chronic Respiratory Infection

The microbiota is profoundly shaped by clinical intervention though the importance of host and environmental factors in disease progression has yet to be fully established. Knowledge of the role of host factors is vital to understanding (i) how remodelling of the CF microbiota occurs and (ii) how pathogenic bacteria emerge to chronically dominate the local microbial community. This would underpin the design of novel strategies with the purpose of breaking the vicious cycle of chronic infection and inflammation to prevent progressive respiratory decline. As a result there have been several investigations attempting to identify environmental or host factors correlating with disease progression and clinical outcome (32, 299). Several factors, including, antibiotics, anaerobic conditions, mucin and short chain fatty acids have been demonstrated to influence both the microbiota and the immune response.

6.1 Antibiotic Administration

The repeated administration of antibiotics to CF patients from a very young age has been proposed to significantly contribute to the remodelling of microbial communities within the CF airways (185, 198-200, 300). The utilisation of broad-spectrum antibiotics which alter the global polymicrobial community may underpin these effects. However, no consensus has been reached regarding the long term impact of antibiotics on the airway microbiota with some studies suggesting significant effects (198), while others observed that the microbiota is only transiently altered and highly resilient to antibiotic treatment (185, 199, 200, 300). Therefore, while there is

some evidence that supports the contribution of antibiotic usage to the restructuring of the microbiota, it does not appear to have a determining effect hence other factors must also be at play.

6.2 Hypoxic Environment

The accumulation of viscous mucus, a consequence of airway hypersecretion and airway surface liquid dehydration, results in the generation of steep oxygen gradients throughout the lung (169, 301). These hypoxic conditions function as an environmental signal to potential colonising anaerobic microbes particularly *P. aeruginosa* (169, 302). *P. aeruginosa* has been shown to respond to hypoxic conditions by increasing production of the polysaccharide alginate, a significant biofilm component, further increasing anoxic regions within the lung (169, 303). *S. aureus* has also been shown to transition from a non-mucoid to mucoid phenotype in response to anaerobic conditions (304). In addition to the impact on the microbiota, hypoxic conditions have been demonstrated to trigger an inflammatory response with elevated levels of macrophages, neutrophils and cytokines detected in BALF exposed to hypoxic conditions (305). Neutrophilic inflammation is a key factor in the pathophysiology of CF lung disease (306) with hypoxic conditions shown to contribute to this (307).

6.3 Mucin

The primary component of the viscous mucus present on the lining of airway epithelial cells are mucins; high molecular weight glycoproteins which represent a favourable nutrient source to colonising microbes as a reservoir of carbon and nitrogen (301, 308). Mucin degradation has been shown to occur within the CF lung microbiota with co-colonisation of residential anaerobes essential to this process. This facilitates the outgrowth of opportunistic pathogens potentially facilitating pathogen establishment (301, 309-311). The fermentation of mucin results in the

production of Short Chain Fatty Acids (SCFA) and amino acids, products which are detected at high concentrations in CF sputum (312, 313). There is evidence to suggest that mucin degradation can support the high carbon demands of *P. aeruginosa*, though this organism is capable of utilising multiple carbon sources *in vivo* (314, 315). Hence, the catabolism of host mucin by residential microbes may contribute to the initial progression of lung disease, with its relative importance possibly diminishing as the microbiota becomes dominated by a single organism such as *P. aeruginosa* which cannot efficiently utilise mucin as a sole carbon source in a monoculture (309, 316).

6.4 Short Chain Fatty Acids

SCFAs as stated above are by-products of microbial fermentation by facultative anaerobes within the hypoxic lung environment and are regularly detected in CF sputum (317). SCFA's contribute to the host inflammatory response through its impact on cytokines GM-CSF, IL-6 and IL-8 (318, 319). Hence, SCFA's may enhance the recruitment of neutrophils to the CF airways contributing to the heightened pro-inflammatory response which results in the development of bronchiectasis. Though high concentrations of SCFA's impaired *P. aeruginosa* growth (320) lower concentrations were found to transiently enhance growth in mid-log phase (318). Therefore, this key microbial metabolite has a dual impact on both pathogen growth and airway inflammation.

6.5 Co-morbidities in CF

As the life expectancy of CF patients continues to increase, this brings with it new challenges for the clinical management of the disease. Factors arising from co-occurring morbidities now need to be considered when assessing the progression of CF. It has recently been proposed that there will be a 75% increase in the number of adults with CF by 2025 (321). This figure does not account for improvements in life expectancy arising from the implementation of corrective and

potentiator therapies described above. A detailed and comprehensive review of the co-morbidities associated with CF has been recently published by Ronan and colleagues (322). As might be expected from a multisystem disease, these include pulmonary, pancreatic, hepatobiliary, renal, genitourinary, coronary, bone, malignancies, and gastrointestinal disorders. The latter has received considerable attention in recent years arising from the growing appreciation of the connectivity of the gut-lung axis.

6.5.1 Gastro-oesophageal Reflux and Lung Disease

A significant correlation has emerged between gastro-oesophageal reflux disease (GORD/GERD in the USA) and chronic respiratory disease (323-328). In patients diagnosed with GORD, a variety of pulmonary manifestations have been observed, including chronic cough, bronchitis, bronchial asthma, bronchitis, pneumonia and interstitial fibrosis (329). This has led to suggestions that GORD is an underlying host factor modulating chronic respiratory disease though the exact mechanism through which it elicits this effect has not been thoroughly investigated.

GORD is a condition resulting in the transition of the contents of the stomach in to the oesophagus. It is ultimately a clinical manifestation resulting from the disruption of a normal physiological process where the barriers that control reflux are impaired and no longer function effectively (327, 330, 331). GORD is a spectrum disease comprising three broad categories, where patients exhibit mild to severe disease symptoms; i) non-erosive reflux disease, where there is no evidence of mucosal damage, ii) erosive esophagitis and iii) Barrett's oesophagus, where there is evidence of damage to the mucosa (332, 333). Many physiological risk factors contribute to the development of GORD, such as a defective lower oesophageal sphincter (LOS), increased lower abdominal pressure and delayed gastric emptying (331). These symptoms are

commonly present in patients with underlying respiratory conditions such as CF and COPD and are found to be exacerbated by daily chest physiotherapy treatment regimens (334-336). Typically, diagnosis depends on patient presentation with clinical symptoms such as heartburn, acid regurgitation and stomach pain however, up to 50% of patients do not present with these characteristic symptoms making clinical diagnosis a challenge (327, 337-339). Furthermore, GOR diagnosis is hindered by asymptomatic or silent GOR, therefore diagnosis based solely on symptomatic presentation is inadequate with recommendations that patients be monitored and tested for reflux.

The co-morbidity of GORD with chronic respiratory disease is now widely accepted, however, the mechanisms through which this lung damage is incurred has yet to be elucidated. It was first proposed in 1975 that GORD contributes to progressive lung decline with GORD positive patients exhibiting more severe respiratory disease (340). The correlation between the presence of GORD and increased severity of lung disease led to suggestions that GORD contributes to progressive lung decline. A strong correlation between GORD-derived reflux, pulmonary aspiration, and increased lung damage extends to a wide range of respiratory diseases (341). This includes advanced lung damage following lung transplantation (342, 343), ventilator associated pneumonia (344), Barrett's oesophagus and oesophageal adenocarcinoma (345) and Bile Acid Pneumonia in neonates (346). Based on previous associations between GORD, poor pulmonary function and early acquisition of key lung pathogens in CF patients, GORD is potentially a key factor in the acceleration of CF disease progression (347). It has been reported that up to 80% of CF patients exhibit symptoms of GORD, though this figure may be an under estimation of the prevalence within these populations due to limitations regarding diagnosis and the prevalence of silent GORD (348).

Table 2; Co-morbidity of GORD with a range of respiratory conditions.

Respiratory Condition	Clinical Presentation	Reference
Cystic Fibrosis	Reflux	(327, 328, 349-356)
	Reflux/aspiration	(326, 338, 357-361)
COPD	Reflux	(362-373)
	Reflux/aspiration	(374, 375)
Asthma	Reflux	(376-385)
	Reflux/aspiration	(386)
Chronic cough	Reflux	(387-394)
	Reflux/aspiration	(395-397)
Idiopathic pulmonary fibrosis	Reflux	(398-403)
	Reflux/aspiration	(404, 405)

6.5.1.1 Clinical Diagnosis of GOR

Clinically, there are several methods available enabling GOR diagnosis including endoscopy, manometry, proton pump inhibitor testing (PPI), and multichannel intraluminal impedance pH monitoring. Endoscopic diagnosis is routinely used to visualise mucosal damage, however, its low sensitivity of detection has limited its diagnostic value (406). Alternatively, manometry is effective in investigating oesophageal and LOS functioning. This technique is conventionally performed in the evaluation of a patient's suitability for anti-reflux surgery (407). The prescription of PPI drugs and the evaluation of patient responsiveness to treatment is another diagnostic strategy routinely used (408). However, PPI testing is an ineffective strategy for the diagnosis of GORD due to subjectivity in the assessment of patient responsiveness and symptomatic resolution. The gold standard for the accurate diagnosis of GORD is intraluminal oesophageal impedance pH monitoring which detects both acid and non-acid reflux. The procedure involves placing a pH catheter into the oesophagus and monitoring oesophageal pH over a 24 hour period (409). Alternatively, the use of exhaled breath condensate for the detection

of biomarkers such as pepsin is currently under investigation as a diagnostic tool for GORD (374).

6.5.1.2 Clinical Management of GORD

Acid Suppression Therapy

The current treatment plans of choice for GORD focus on acid suppression therapy through the use of PPIs and Histamine 2 receptor antagonists also known as H2 blockers. Both function through interference with the gastric acid secretion pathway (410). H2 blockers are effective at decreasing gastric secretion after meals and are useful in the treatment of mild forms of GORD. PPI's are much more potent and function through the irreversible binding of the H^+K^+ATPase. Though daily therapy is the recommended treatment strategy which controls the majority of patients symptoms, up to 30% of patients have been reported to not respond to therapy and continue to experience symptoms (411-413). As the rate of refractory GORD is so high with those receiving PPI treatment still experiencing respiratory decline, alternative therapeutic management strategies must be sought for the successful control of GORD. In severe cases of GORD, where symptoms cannot be controlled by medicinal intervention surgical treatment may be required.

Surgical Intervention

The most common surgery for the resolution of GORD is known as a Nissen fundoplication which involves wrapping the upper curve of the stomach around the oesophagus in an attempt to strengthen the lower oesophageal sphincter muscle which, if successful, prevents recurring reflux. This can be a complete 360 degree wrap or a partial wrap with varying degrees of wrapping (414, 415). This surgery has evolved from an open surgical procedure to a laparoscopic procedure (414, 416, 417). Additionally, this surgery can now be performed through the mouth

avoiding the need for any incisions and is known as a transoral incisionless fundoplication (418-420). Surgery has the primary advantage of addressing the underlying physiological complication of GORD and although it is invasive, it is quite effective in the control of GORD. Patients who undergo this surgery have been shown to display significant improvements in lung function which further reinforces that GORD and potentially aspiration of gastric contents into the lungs is contributing to pulmonary decline (421-424).

Endoscopic Interventions for the Control of GOR

Endoscopic techniques have been developed and are currently being tested for their effectiveness in the management of GORD. The goal of such endoscopic techniques is the reduction in the long term reliance on medication or surgical interventions through the creation of a barrier to reflux with very promising results to date. These techniques include radio frequency energy ablation; the most widely utilised technique, endoluminal gastroplasty and numerous implant techniques (417, 425). Radiofrequency ablation also known as the Stretta procedure is one major alternative to surgical intervention. This procedure involves the delivery of consecutive rounds of thermal energy to the LOS through the use of a 4 channel radio frequency generator and catheter system. This is an attractive alternative to medication and surgery as the procedure can be carried out as an outpatient under mild sedation in roughly 35 minutes. Though the mechanism of action is still not fully understood it has been shown to be a safe and effective technique for the control of chronic GORD. The wide scale application of this approach is limited by the age at which treatment can commence as candidates must be over 18 years of age to qualify for the procedure (426, 427). Endoluminal gastroplasty is a technique developed by Bard Endoscopic technologies which is trademarked as Endocinch. This device uses sutures to create plications in the cardia in an attempt to strengthen the functioning of the LOS. However,

this procedure has been associated with complications and moderate side effects such as haemorrhaging, chest pain and dysphagia (428, 429). The effectiveness of surgery and endoscopic techniques in controlling GORD suggests that the aspiration of gastric contents in to the lungs is possibly responsible for the underlying pathophysiology linking GORD to chronic respiratory disease and not necessarily the effect of acid reflux.

7. Bile acids in the Lungs are associated with GORD-induced Lung Damage

The ineffectiveness of therapeutic controls such as PPIs and H2 antagonists and the high incidence of GORD in CF cohorts led to further investigations regarding the underlying causative factor(s) of GORD induced lung damage (430). This led researchers to investigate biomarkers of GORD in the lungs of CF. Bile acids were detected in sputum and BALF samples of CF patients as a result of these studies. Bile acids are just a biomarker of aspiration of gastric contents, including bile which is a complex mixture. Physiologically relevant concentrations of bile acids have been detected in both BALF and sputum of CF patients (324, 357-360, 431). These concentrations have been shown to be capable of inducing lung damage and inflammation in cultured lung epithelial cells (432, 433). It has been proposed that the source of these bile acids is the gastric contents which are refluxed and aspirated into the lungs during periodic episodes of GOR (324, 358, 360, 431). Evidence for this source is supported by the effectiveness of the surgery Nissen Fundoplication in slowing down progressive lung disease potentially through the control of reflux and aspiration (434, 435). However, alternative sources of bile acids in the lungs have been proposed as evidenced by transmission in the circulatory system of neonates and de-novo biosynthesis within the lungs in pulmonary arterial hypertension (346, 436). The frequency of bile aspiration is estimated to be as high as 80% in CF patients (359). The aspiration of bile has been associated with lung transplant rejection, development of

Bronchiolitis Obliterans Syndrome (BOS) and increased colonisation by *P. aeruginosa* (437, 438). Administration of the macrolide antibiotic azithromycin to lung transplant recipients has been shown to reduce the level of aspiration with an improvement in clinical outcome observed. Mechanistically, this was proposed to be due to enhanced oesophageal motility and accelerated gastric emptying, however, their role in the control of GORD and bile aspiration in respiratory disease requires further investigation (439).

7.1 Quantification and Profiling of Bile Acids in the Lungs

In order to assess patients effectively for bile aspiration, high resolution technologies for the detection of bile must be designed. Detection techniques have evolved from methods based on bile acid identification in other matrices such as blood. Currently, there is a lack of protocols in the literature describing the direct identification of bile acids from lung fluids, therefore advancements in this area are required to fully elucidate the emerging role of bile acid aspiration in chronic lung disease.

7.1.1 Enzymatic Detection

An enzymatic reaction using 3-α hydroxysteroid dehydrogenase (3-α HSD) linked to spectrophotometric analysis has been described to measure total bile acid levels present in BALF samples (440). 3-α HSD catalyses the oxidation of the hydroxyl group at position 3 of the bile acid steroid ring. In the presence of the coenzyme nicotinamide adenine dinucleotide (NAD), 3-α HSD converts bile acids into 3-keto steroids and NADH which react with nitrotetrazolium blue to form the dye formazan which can be measured spectrophotometrically at an OD_{495nm}. Thus, allowing for the calculation of total bile acids concentration. This enzymatic approach has resulted in the successful identification of aspirated bile acids in the lungs following

transplantation. These increased levels have been linked to the development of bronchiolitis obliterans syndrome (441).

7.1.2 Liquid Chromatography-Mass Spectrometry

Enzymatic assays are useful in the calculation of total bile acid levels however concentrations of individual bile acids and bile acid profiles cannot be obtained using this technique. High resolution liquid chromatography–mass spectrometry (LC-MS) is currently one of the most accurate technologies available for bile acid quantification. Most methods rely on reverse phase chromatography with a variety of flow rates and column dimensions (442, 443). LC-MS (and MS/MS) is capable of detecting bile acids from a variety of matrices including, blood serum (444), human bile (445), stool (446), and the brain (447). A highly sensitive and specific LC-MS method was developed to investigate the presence of bile acids in the lungs of paediatric CF patients and to examine its influence on chronic respiratory infection (324). This method has been successfully applied to the analysis of sputum samples from a cohort of paediatric CF patients for the detection and accurate profiling of bile acids. Bile acid profiles have also been measured in airway secretions collected from intubated and mechanically ventilated patients. The increased bile acid levels were shown to be associated with detrimental ventilator-associated pneumonia (344). Direct electrospray ionization mass spectrometry can also be utilised successfully to identify bile acids in the lower airways of adult CF patients with robust detection limits (0.01 µmol/L) (358). However, it has been suggested this method is not the most suitable for many biological matrices due to their complexity (448). Studies investigating the potential use of exhaled breath condensate for the diagnosis of bile aspiration are currently underway. However, there are limitations with regard to the sensitivity of detection and diagnostic value as

it is just an indicator of GORD (449), and therefore would not give an accurate measurement of bile acid levels in the lungs.

7.2 The Impact of Bile Acids on the CF Microbiome

The existence of a pervasive CF microbiota, along with emerging evidence for a signature COPD microbiota (450, 451) prompted a pilot microbiota study encompassing 10 paediatric patients attempting to delineate the role of aspirated bile in the lungs of CF patients (324). Stratification of paediatric patient cohorts based on detected bile acid concentrations in sputum revealed a significant reduction in biodiversity and richness with the emergence of dominant Proteobacterial pathogens such as *Pseudomonas, Stenotrophomonas* and *Ralstonia* which when present could account for up to 98% of the microbial sample in patients with high bile acid levels (324). Clinical parameters such as age, gender, antibiotics and hospitalisation did not explain the observed shift in the microbiota. It was noteworthy that, in spite of the reduced biodiversity, these patients did not exhibit a decreased FEV1. In contrast, patient samples in which bile acids were not detected exhibited increased microbial biodiversity relative to bile acid positive samples. This diversity was underpinned by species more associated with a 'healthy non-CF lung' such as the anaerobes *Veillonella* and *Prevotella* (128). More recently, evidence of an aerodigestive route to *P. aeruginosa* acquisition has been proposed, further evidence of a link between the pulmonary and gut systems (136, 452).

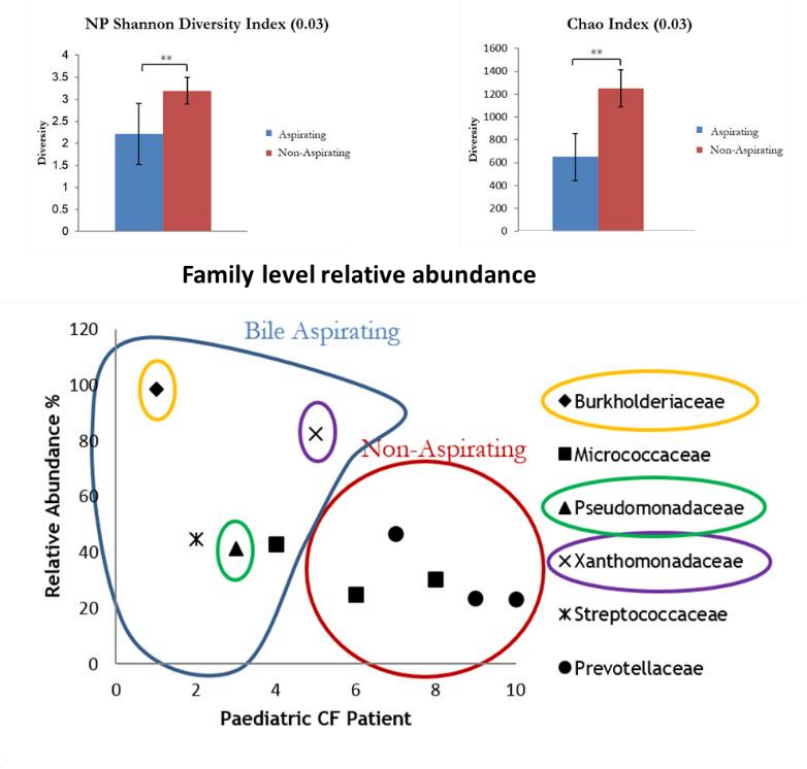


Figure 11. Aspirating patients display a reduction in both biodiversity and richness (Shannon and Chao index) with an emergence of Proteobacterial pathogens such as members of the family Burkholderiaceae and Pseudomonadaceae, adapted from Reen et al, 2014 (324).

7.2.1 Bile Triggers Respiratory Pathogens to Adopt a Chronic Lifestyle

While bile acid signalling has long been associated with a broad spectrum of diseases such as diabetes, gastrointestinal disease and obesity (453), the possibility that bile is potentially a signalling molecule in respiratory disease has not been previously considered. The strong correlation between the presence of bile acids and the emergence of dominant Proteobacterial pathogens within the CF lung microbiota suggests that bile might impact directly on the behaviour of the pathogen itself. Studies on the influence of bile on the human microbiota have been restricted to enteric pathogens associated with gastroenteritis and gut infections as well as probiotics, and have focused largely on their capacity for bile tolerance (454-457). However,

enteric bacteria would appear to employ species-specific mechanisms in response to exogenous bile. For instance, the T3SS was reduced in *Salmonella enterica* in response to bile (458) while it was increased in *Shigella flexneri*, *Vibrio parahaemolyticus* (459-461) and in the protozoan pathogen *Cryptosporidium* spp (462). Furthermore, while motility was decreased in *S. enterica* it was found to be increased in *V. cholera* (463). Therefore, the impact of aspirated bile on the respiratory microbiota in the lung may be similarly diverse. Studies on the mechanisms underpinning the response of gastrointestinal bacteria to bile have recently led to the identification of a bile 'sensor' in *Listeria monocytogenes* (464), while a role for two component systems has also been reported in *S. enterica* (465). However, until recently, no information was available on the influence of bile on respiratory pathogens, which are likely to encounter reduced, non-toxic levels of bile through aspiration. This would be analogous to the recent finding that sub-inhibitory concentrations of antibiotics elicit specific adaptive responses in pathogens, distinct from the response to higher toxic levels (466).

7.2.2 Bile and P. aeruginosa Pathogenesis

Consistent with the association between aspirated bile and Proteobacterial pathogens, bile was shown to elicit a chronic persistent biofilm lifestyle in a broad spectrum of respiratory disease pathogens. Studies on *P. aeruginosa* in particular revealed that, once exposed to physiologically relevant concentrations of bile, the pathogen adopted a chronic lifestyle, suppressing virulence systems associated with the acute phase of infection, and adopting a signal rich biofilm mode of growth (323). This switch from acute to chronic is characteristic of *P. aeruginosa* behaviour in respiratory diseases such as CF, where the chronic behaviour of this primary pathogen underlies the morbidity and mortality that underpin the pathophysiology of this disease. Indeed, chronic infection by *P. aeruginosa* has been shown to be associated with a lower FEV1 in childhood, a

faster decline in FEV1 despite optimal respiratory management, higher mortality rate and shorter median survival (467). Once *P. aeruginosa* changes its lifestyle from an acute virulent phenotype to a chronic biofilm mode of growth clinical management through antibiotic administration becomes largely ineffective (468, 469). Therefore, there is an urgent need to understand the molecular mechanisms through which this and other respiratory pathogens adopt the chronic biofilm lifestyle.

Addition of exogenous bile significantly increased biofilm formation and repressed the swarming motility of *P. aeruginosa*. In addition, production of all three *P. aeruginosa* quorum sensing (QS) signals, which have been detected in biofilms and CF sputum (470, 471), was increased in the presence of bile (323). Furthermore, promoter activity of the *tssA1* gene encoded within the chronic associated HSI-I T6SS (472, 473) was induced 3-fold in response to bile, while the acute-associated T3SS (474-477) was repressed. The ability of *P. aeruginosa* to thrive in acid suppressed stomachs of patients receiving proton pump inhibitors for the treatment of GORD is of concern due to suggestions of the existence of an aero-digestive microbiota (136, 452, 478). This is of particular relevance, as bacteria may have prior exposure to bile with the resultant aspiration allowing for the introduction of pre-adapted isolates into the lungs (136, 479). These pre-adapted isolates may therefore have an additional competitive advantage over residential members of the lung microbiota.

The impact of bile was not restricted to *P. aeruginosa* with bile also found to influence the behaviour of other respiratory pathogens such as *B. cepacia* complex, *A. baumanii*, and the emerging pathogen *Pandoraea sputonum* towards a biofilm mode of growth. In contrast, exposure to bile appeared to strongly repress biofilm formation in some isolates of *S. aureus* and *S. maltophilia* typed strains (323). These species-specific effects could further explain the

mechanism through which bile shapes the respiratory microbiota triggering the emergence of dominant Proteobacterial pathogens. It certainly supports the hypothesis that bile directly influences the lung microbiota through modulation of pathogen behaviour. Indeed, the modulation of both PQS signalling and T6SS is highly significant in light of their role in interspecies and inter-kingdom communication (241, 480-482). Induction of these compounds in the presence of bile may therefore influence the interactions between *P. aeruginosa* and competing organisms in the bile acid positive lungs of CF patients. The changes in population dynamics observed in bile aspirating patients may likely arise from the dual action of bile itself and that of the bile induced interspecies signal molecules, although these interactions are sure to be complex and difficult to define.

7.3 Bile and Inflammation

As stated earlier, cycles of infection and inflammation are key to the pathophysiology of CF and other respiratory diseases. Colonisation by pro-inflammatory pathogens in the CF lung has been shown to lead to a dysregulated and heightened pro inflammatory response by the host immune system (44, 483). This results in the recruitment of neutrophils and elevated levels of inflammatory cytokines. Hence, bile promotes the colonisation of pathogens which results in the initiation of inflammation leading to bronchiectasis and progressive loss of lung function. While a role for pro-inflammatory pathogens in instigating inflammation is well accepted, some studies suggest that airway inflammation can occur in infancy, prior to the onset of bacterial colonisation of the lung (45). Hence, in addition to the direct correlation between bile acids and chronic colonisation, the accumulation of bile acids in the lungs has been directly linked with the host inflammatory response and increased airway inflammation. Increased levels of alveolar neutrophils (359) and interleukin-8 (IL-8) (438, 441) were reported in patients with elevated

levels of bile acids. Furthermore, bile acid aspiration has also been associated with increased BALF tumour necrosis factor alpha (TNF-α) in a rodent model of chronic aspiration (484). Neutrophil dominated inflammation is a characteristic pathophysiology of CF, with bile acids shown to mediate this process in intestinal cells (485). D'Ovidio and colleagues reported a link between the aspiration of gastric reflux and the development of BOS following lung transplant. Increased levels of both neutrophil elastase and IL-8 were detected in BALF with elevated levels of bile acids. The same pattern of inflammatory marker induction in the presence of bile acids has also been described in patients with ventilator assisted pneumonia (344), where the primary bile acid chenodeoxycholic acid (CDCA) was associated with elevated levels of IL-8. Bile acids have been found to act as signalling molecules through activation of dedicated receptors such as the nuclear receptor Farnesoid X Receptor (FXR) and the membrane-bound receptor Takeda-G protein Receptor 5 (TGR5). In addition, bile acids can also activate other receptors, such as the Pregnane X Receptor which has recently been shown to mediate gut dysbiosis in response to statin therapy (486). *In vitro* reports have described how bile acids modulate production of pro-inflammatory markers, including FXR-dependent elevated levels of IL-6 production in lung epithelial cells (487-489). This suggests that bile aspiration alone is enough to drive dysregulation of the inflammatory response.

Bile acids are also capable of modulating the host immune response by targeting the Hypoxia-Inducible Factor (HIF)-1 transcription factor. This transcription factor is important in the mounting of an effective host response to infection with bile acids found to destabilise the HIF-1α subunit in an FXR and TGR-5 independent manner (489). HIF-1 has been previously shown to be required for the resolution of acute inflammation in mice (490). This destabilisation could therefore underpin the chronic inflammatory pathophysiology associated with elevated bile acids

in patients with respiratory disease. Interestingly, the bile acid induced AQ, PQS has also been shown to destabilise HIF-1α in lung epithelial cells (432) suggesting a dual targeting of this key host transcriptional checkpoint in the *P. aeruginosa* infected bile acid positive lung environment. Indeed, PQS is known to be produced at high levels in the lungs of *P. aeruginosa* positive paediatric CF patients, particularly in isolates obtained from patients under the age of three (471). In contrast, PQS could not be readily detected in adult CF sputum samples by metabolomic analysis (491). Destabilisation of HIF-1α in these patients may further contribute to the ability of *P. aeruginosa* to avoid host clearance (492)

The molecular mechanisms underpinning bile acid induced changes in the inflammatory response are yet to be fully defined, although mechanistic insights into the role of bile acids as host signals continue to emerge (485, 486). It is likely that the impact of bile acid aspiration on lung inflammation results from the dual targeting of the host cells with direct activation of inflammatory markers, as well as the indirect targeting of pro-inflammatory pathogens such as *P. aeruginosa*.

Summary and Thesis Objectives

Respiratory disease is the third leading cause of death worldwide. CF is Ireland's most common life-threatening inherited disease, characterised by chronic lung infections that are practically impossible to eradicate. *P. aeruginosa*, one of the top three global nosocomial pathogens, is the primary agent associated with morbidity and mortality in CF patients. Once established in the lung, eradication of this pathogen is almost impossible due to intrinsic antibiotic resistance and its biofilm mode of growth. Aspiration of bile into the lungs of patients with respiratory disease may be a leading cause underlying the establishment of chronic behaving dominant species, particularly *P. aeruginosa*. This has major implications for the ongoing search for more effective

therapies to combat chronic lung infections, particularly those found in CF patients. The overall aim of this thesis was to elucidate the pathways through which *P. aeruginosa* emerges as a dominant chronic pathogen in the bile positive lungs of CF patients. An integrated approach was undertaken in order to understand the impact of bile acid aspiration on the establishment and progression of chronic infections in CF. This thesis investigated the impact of bile acids on the lung microbiota, both from a cross sectional and a longitudinal perspective. Molecular approaches were employed to uncover the pathways through which *P. aeruginosa* responds and adapts to exogenous bile and bile acids, and to identify the factors underpinning the molecular mechanism. By furthering our understanding of the influence of bile on pathogen behaviour and on the population dynamics within the lungs of patients with CF there is the potential to develop increasingly smart and more effective therapeutics.

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Chapter 1

Bile Acids Detected in the Lungs of Paediatric CF Patients are Associated with Inflammation and Chronic Pathogen Microbiomes

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Abstract

The microbiology of the lower airways is a major contributor to the progression of respiratory disease in Cystic Fibrosis (CF). The acquisition and chronic colonisation of the CF lower airways occurs early in life and is associated with progressive pulmonary inflammation and bronchiectasis. The shift observed in the CF respiratory microbiota towards that of chronic pathogen-dominated microbiota, as a patient transitions to adulthood, is associated with negative clinical outcomes. However, the key host factors which mediate and facilitate the emergence of disease associated microbiota remains to be elucidated. Recent evidence suggests a role for bile acids as a host trigger of chronic respiratory infection. Therefore, a cross-sectional and longitudinal study of an Australian paediatric patient cohort was undertaken to determine whether the accumulation of bile acids in the lower airways contributes to the restructuring of the microbial communities towards that of a low diversity, pathogen dominated state. These studies revealed that the presence of bile acids was associated with increased levels of host immune factors associated with inflammation and a reduction in microbial diversity with the emergence of dominant pathogens. Collectively, these data support the hypothesis that the presence of bile acids in the lungs of paediatric patients with CF correlates with a progressive restructuring of the lung microbiota, promoting a reduction in diversity and the emergence of chronic pathogens. Therefore, the early detection and profiling of bile acids in paediatric patients could lead to more effective intervention strategies to prevent the establishment of chronic respiratory microbiota.

Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder characterised by frequent airway infections and chronic respiratory inflammation. These recurrent cycles of airway infection and inflammation lead to the development of structural lung disease, most notably bronchiectasis, bronchial wall thickening, gas trapping, and pulmonary hypo-perfusion (1). Structural lung disease, including bronchiectasis, contributes to the progressive loss of lung function and eventually results in respiratory failure and death in approximately 90% of CF patients. Recent reports have highlighted the rapid development of bronchiectasis in the first three years of life with the majority (50-70%) of children with CF already displaying signs of bronchiectasis before they enter school (2). Chronic respiratory infection is the leading cause of morbidity and mortality within CF cohorts. There has been a paradigm shift from interventions based upon the amelioration of lung disease to one that emphasises disease prevention through early intervention based on evidence from the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) and other key CF centers (3). Therefore, the identification of factors capable of initiating early, progressive, neutrophil dominant airway inflammation and contributing to the development of chronic airway infections is of critical importance in the effective clinical management of lung disease in CF (4, 5).

Though the lungs were previously considered to be a sterile environment, there has been a growing appreciation for the diverse communities of microorganisms that colonise the airways of both healthy and diseased individuals collectively referred to as the lung microbiota (6-8). A signature respiratory microbiota has been described for CF patients, with early acquisition and succession of pathogens such as *Staphylococcus aureus, Haemophilus influenzae, Moraxella catarrhalis* and *Pseudomonas aeruginosa* reported. These pathogens are associated with

bronchiectasis (3, 9-11) and exaggerated neutrophilic inflammation (9, 12). Furthermore, as patient's transition towards adulthood there is evidence for the emergence of a single dominant Proteobacterial pathogen, most frequently *P. aeruginosa.* These chronic infections are the leading cause of morbidity and mortality in CF patients due to the development of structural lung disease and progressive lung function decline. However, the factors that result in the emergence of these pathogens and trigger a transition to chronicity of infection are unknown. Identifying the factors that initiate the shift in the CF lung microbiota towards this disease associated state would underpin the development of more effective early intervention strategies. While many studies have examined the development and impact of factors such as age, inflammation and antibiotic administration on the microbiota (7, 13-22), no consensus has been reached regarding the individual contribution of each of these factors in the determination of the structure of the CF microbiota.

Bile acids have recently emerged as a key host factor promoting chronic respiratory disease, with physiologically relevant concentrations capable of inducing both an immune response and a behavioural response in pathogens (23-30). Bile acids have been shown to trigger the key CF associated pathogens *P. aeruginosa* and *S. aureus* to transition to a chronic lifestyle (24, 27). Previous cross-sectional studies have highlighted that bile acids are detected in sputum and bronchoalveolar lavage fluid samples of both paediatric and adult CF patients (23, 31-37). The presence of bile acids in paediatric patients has correlated with the emergence of dominant proteobacterial pathogens and a reduction in diversity, associated with the pervasive CF microbiota (23, 30). From the host inflammatory perspective, bile acids are associated with increased neutrophil and cytokine levels following lung transplantation (38), with

physiologically relevant µM concentrations found to destabilise HIF-1α and elicit increased levels of IL-6 in human airway cells (26, 30, 39).

The objective of this study was to investigate whether the occurrence of bile acids in the lungs of children with CF patients is associated with the establishment of chronic pathogen dominated respiratory microbiota over time. Establishing a longitudinal perspective on changes in the microbiota over time would allow stronger correlations to be made based on bile acid profiles in those samples. The elucidation of the impact that bile acids exert on the dynamics of the lung microbiota would represent a potential novel biomarker for the identification of high-risk patients facilitating earlier clinical intervention.

Materials and Methods

BALF Cohort, Sampling and Storage

Cross-sectional Study

Randomly selected BALF samples from a cohort of children (n=91) enrolled in AREST CF as part of a unique early surveillance program were collected according to the AREST-CF standard operating procedure and were available for this study (10). BALF retrieval involved lavaging of the right middle lobe (RML) with three washes of saline and one lavage of the most affected lobe determined by CT scan. To reduce the risk of contamination, the bronchoscope was applied through a laryngeal mask airway and suction was only applied once the bronchoscope tip had reached the lower airways. These patients had previously undergone surveillance bronchoscopy and chest CT starting soon after diagnosis (3-6 months), and yearly thereafter when clinically stable. Ethical approval (Ref. 1762/EPP) was previously granted to the AREST CF program by the Princess Margaret Hospital for Children, Perth ethics committee and committee and consent was obtained to participate from parents/guardians. Clinical data including culturable microbial history, antibiotic regimens and clinical symptoms and measurements were available through the ongoing multi-centre AREST CF program (40).

Longitudinal Study

Twenty patients who were enrolled in the initial cross-sectional study outlined above were selected for longitudinal analysis (n=77 BALF samples). In order to examine the potential progressive loss and/or recovery of diversity over time, ten patients with low Shannon Index (SI) diversity measurements (SI <1.8) and ten patients with high SI diversity measurements (SI >2) were selected from the cross-sectional study for further investigation. Patient samples spanned

over the course of eight years with samples ranging from 11-88 months with a minimum of two samples and a maximum of 7 samples present per patient **(Appendix Table 2)**. Based on these criteria one patient had to be excluded as only 1 sample could be provided. BALF retrieval was undertaken as outlined in the cross sectional study above. Ethical approval (Ref. 1762/EPP) was previously granted to the AREST CF program by the Princess Margaret Hospital for Children, Perth ethics committee with consent to participate obtained from parents/guardians. Clinical data including culturable microbial history, antibiotic regimens and clinical symptoms and measurements were available through the ongoing multi-centre AREST CF program

Bile Acid Profiling and Cohort Categorisation

Sample processing was performed using a method adapted from Tagliacozzi et al. (41) BALF samples were treated with equal volumes of Sputolysin (Calbiochem) and vortexed for 30 s prior to centrifugation at 5000 rpm for 15 min. The supernatant was removed into a sterile container and 250 µL removed for bile acid analysis. A 900 µL aliquot of acetonitrile (Sigma-Aldrich) was added to each 250 µL sample which was vortexed for 1 min and centrifuged at 13,600 rpm for 10 min. A 900 µL aliquot was transferred to a clean container and the sample was evaporated under nitrogen to dryness. The sample was then resuspended in 250 µL MeOH:H_2O (1:1) and subsequently analysed by LC-MS. This process has been described previously by Reen et al. (23, 42). Each BALF sample was analysed for the presence of 12 principal bile acids (Cholic acid (CA), Chenodeoxycholic acid (CDCA), Deoxycholic acid (DCA), Lithocholic acid (LCA), Ursodeoxycholic acid (UDCA), Glycodeoxycholic acid (GDCA), Taurochenodeoxycholic acid, (TCDCA), Taurodeoxycholic acid (TDCA), Taurocholic acid (TCA), Glycocholic acid (GCA), Taurolithocholic acid (TLCA) and Tauroursodeoxycholic acid (TUDCA)) compared to purified reference standards (Sigma-Aldrich). For the cross-sectional study; blind analysis was conducted

where BALF retrieval volume as a proportion of instilled volume was found not influence bile salt concentrations (r^2=0.004) suggesting variability due to dilution is unlikely to be a major factor when stratifying the population based upon levels of bile acids. For stratification of the patient cohort on the basis of bile acid concentrations the HB lower cut-off value correlated with a mean BA value of 0.0025 µM (SEM +/- 0.001), while the LB upper cut-off value correlated with a mean BA value of 0.00125 µM (SEM +/- 0.0006). The LB categorisation was chosen to reflect the lower quartile (0.01307 µM) of bile acid concentrations, while the HB lower limit was set at twice the LB cut-off. For robustness, MB samples were also included in the analysis, representing those samples that fall in between the LB and HB categories.

Bile acid profiling undertaken for the longitudinal microbiota analysis established inter patient differences with respect to the presence and concentration of bile acids. Patients were categorised based on total bile acid concentration of samples as either Bile Acid positive >0.015 µM (BA+, n=3, 11), Bile Acid negative <0.015µM (BA-, n=8, 30) or Transitioning (T, n=8, 34) if patient samples contained both BA+ and BA- samples. While a trend was observed with a reduction in the percentage of BALF retrieved as a proportion of instilled volume correlating with increased bile acid concentration, no statistical difference was observed between the transitioning cohort and the BA- cohort suggesting BALF processing may have minimal contributions to cohort stratification.

Genomic DNA extraction

The low level of infection and dilute nature of the BALF samples from the paediatric cohort has implications for downstream microbiome analysis (43, 44). BALF supernatant was centrifuged at 13,000 rpm for 10 min with genomic DNA extracted from the resulting pellet. DNA extracted using the Gentra PureGene DNA Extraction kit (QIAGEN) according to the manufacturer's

instructions. A minor modification was included in the recommended protocol consisting of an overnight incubation with proteinase K to ensure optimal and unbiased representation of DNA from all the bacterial groups present in BALF. Genomic DNA was re-suspended in sterile water with the gDNA concentration and quality (260/280) recorded via a nanodrop spectrophotometer. Quantification of the concentration and quality (260/280) of gDNA was also recorded by Qubit fluorometer. The extracted gDNA was stored at -20^{o}C.

Next Generation Sequence (NGS) Analysis

PCRs were commenced running a pre-amplification for 20 cycles using GM3 and 1061R. One µL of this PCR was transferred to the second 20µl PCR which was done using the standard primers and was run for another 20 cycles. Subsequent PCRs included 5 ng of DNA extract, 15 pmol of each forward primer 341F 5'-NNNNNNNNNNTCCTACGGGNGGCWGCAG and reverse primer 785R 5'- NNNNNNNNNNTGACTACHVGGGTATCTAAKCC in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µL of BioStabII PCR Enhancer (Sigma). This provided coverage of the V3-V4 region for microbiome analysis. For each sample, the forward and reverse primers had the same 10-nt barcode sequence. PCRs were carried out for 30 cycles using the following parameters: 2 min 96°C pre-denaturation; 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. DNA concentration of amplicons of interest was determined by Gel electrophoresis. About 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mis-priming products, followed by an additional purification on MinElute columns (Qiagen). Each purified amplicon pool DNA (100 ng) was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by

preparative Gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina).

Microbiome profiling and population analysis

All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (45, 46). Reads were aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008)) (47) against the SILVA SSU rRNA SEED and quality controlled (46). All reads shorter than 350 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. In addition, putative contaminations and artefacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis.

Following this, identical reads were identified (dereplication), the unique reads were clustered (OTUs) on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering was done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (48) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. Classification of each hit was performed by local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 123; http://www.arb-silva.de) using BLASTN (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard default settings applied (49). The classification of each OTU reference read was then mapped, yielding quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as, multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function "(% sequence identity + % alignment coverage)/2" did not exceed the value of 93, were considered unclassified

(50). Sequence data from the microbiome community table were rarefied to 1502 sequences per sample using the R package vegan., comparable to recent studies on paediatric BALF from patients with CF (51). Negative control samples were processed using the same DNA extraction method in parallel with the test samples yielded 1-295 sequence reads.

Statistical Analysis

All clinical and diversity data were analysed using Prism version 5.0 (GraphPad, San Diego, CA, USA), or the R statistical package, for significance. Linear regression analysis was performed using GraphPad. Mann Whitney and one way ANOVA with post-hoc corrective testing were applied as appropriate and in all cases differences <0.05 were considered statistically significant. Experimental data presented is the average of at least three independent biological replicates. Statistical analysis was performed by paired student's t-test (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$).

Table 1; An outline of strains utilised in this study; a combination of clinical isolates recovered from paediatric patients attending Cork University Hospital (CUH) and typed lab strains.

Strain/Plasmid	Description	Source
***Staphylococcus aureus* NCDO949**	Typed strain isolated from pleural fluid	Shinfield, UK
***Staphylococcus aureus* CUH-E**	Paediatric clinical isolate from CUH	This study
***Staphylococcus aureus* CUH-T**	Paediatric clinical isolate from CUH	This study
***Staphylococcus haemolyticus* CUH-M**	Paediatric clinical isolate from CUH	This study
***Staphylococcus haemolyticus* CUH-D (LB)**	Paediatric clinical isolate from CUH	This study
***Staphylococcus epidermidis* CUH-D (MS)**	Paediatric clinical isolate from CUH	This study
***Staphylococcus epidermidis* CUH-K (LB)**	Paediatric clinical isolate from CUH	This study
***Ralstonia* CUH-242**	Pigmented paediatric clinical isolate from CUH	This study
***Ralstonia* CUH-229**	Non pigmented paediatric clinical isolate from CUH	This study
***Stenotrophomonas maltophilia* CUH-B (PIA)**	Paediatric clinical isolate from CUH	This study
***Stenotrophomonas maltophilia* CUH-C (PIA)**	Paediatric clinical isolate from CUH	This study
PA14 WT	Wild Type	(52)
***Pseudomonas aeruginosa* CUH-A (PIA)**	Paediatric clinical isolate from CUH	This study
***Pseudomonas aeruginosa* CUH-B (McC)**	Paediatric clinical isolate from CUH	This study

Biofilm assays

Overnight cultures were adjusted to an $O.D_{600nm}$ 0.05 in TSB in the presence and absence of bile or bile acids. Aliquots (1 mL or 200 µL) were transferred in to 24-well plates or 96 well plates respectively and incubated at 37˚C overnight. Biofilm formation was measured by removing culture by pipetting. Wells were washed with water by pipetting to remove any unattached biofilm. Attached biofilm was measured by staining for 30 min with 1 mL/ 200 µL of 0.1% (w/v) crystal violet. 100% (v/v) ethanol was used to solubilise the crystal violet followed by a measurement of the absorbance at a wavelength of 595nm.

Results

Sample preparation for bile acid profiling and genomic DNA extraction

A batch of 91 BALF samples was received for the cross-sectional study and a batch of 77 samples was received for the longitudinal analysis. These were prepared for bile acid profiling (assisted by David Woods) and genomic DNA extraction as outlined in Figure 1 below.

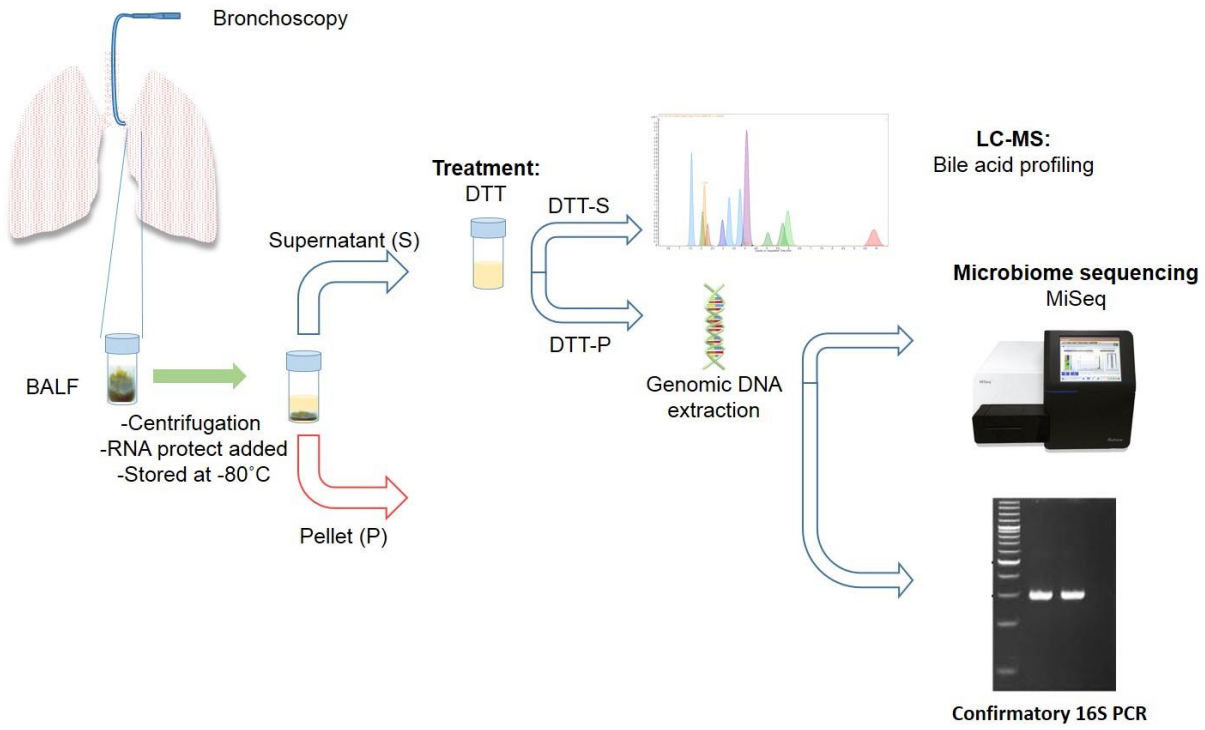

Figure 1. Method of sample preparation of Australian BALF samples. Samples were prepared for bile acid profiling and gDNA extraction as outlined in the materials and methods. Bile acid profiling was conducted using LC-MS analysis with extracted gDNA sent for sequencing.

Bile acid profiling and genomic DNA extractions was successfully undertaken for the 91 cross sectional samples with gDNA yields (ng/µl) and quality (260/280nm) outlined in Table 2.

Table 2; gDNA concentrations from cross sectional BALF samples.

Sample	ng/µl	260/280	**Sample**	ng/µl	260/280
1	13.7	2.05	**45**	6.8	2.13
2	7.2	1.97	**46**	25.6	1.84
3	1.3	2.56	**48**	4.6	2.03
4	3.5	2.31	**49**	2.6	2.22
5	7.3	2.16	**50**	113.4	1.34
6	6.2	1.75	**51**	8.7	1.43
7	4.7	2.70	**52**	5.1	1.07
8	6.1	2.55	**53**	4	1.32
9	4.8	2.26	**54**	27.9	1.87
10	10.5	1.56	**55**	4.2	1.55
11	7.2	1.12	**56**	6.6	1.53
12	7.6	1.30	**57**	7.8	1.34
13	19.7	1.57	**58**	9.2	1.45
14	11.8	1.39	**61**	3.2	1.07
15	15.3	1.46	**62**	4	1.34
16	4	1.53	**63**	4.5	1.09
17	16.7	1.00	**64**	7.5	1.48
18	16.4	0.86	**65**	5.3	2.12
19	43.1	1.52	**66**	6.8	1.94
20	46.4	1.12	**67**	4.8	1.51
21	22	1.03	**68**	1.1	0.67
22	3.2	0.79	**69**	5.7	1.86
23	1.5	0.40	**70**	9.5	1.86
24	2.8	3.79	**71**	15.4	1.92
25	1.7	0.40	**72**	23.7	1.28
26	4.9	2.28	**73**	1.4	1.27
27	107.6	1.14	**74**	10.6	1.87
28	6.3	1.91	**75**	8	1.76
29	6.8	2.86	**76**	5.7	2.36
30	3.9	3.47	**77**	25.3	1.87
31	5.2	2.57	**78**	5.9	2.04
32	6.9	1.52	**79**	12.7	1.90
33	4.9	3.30	**80**	7.2	1.47
34	4.8	1.82	**81**	17.3	1.73
35	7.3	2.15	**82**	7.5	3.01
36	11.3	1.48	**83**	56.4	1.50
37	6	2.05	**84**	7.3	2.19
38	4.8	2.05	**85**	5.2	2.02
39	9.1	2.15	**86**	10.3	1.86
40	17.2	1.86	**87**	10.8	1.56
41	3.8	3.69	**88**	5.5	0.66
42	8.2	1.66	**89**	15.1	2.04
43	7.7	1.92	**90**	8.7	1.96
44	7.7	1.97	**91**	8.6	1.47

The patient cohort ranged in age from 2-5 years and were representative of the AREST CF cohort with regards demographic and clinical features (**Appendix Table 1**) (51, 53). There was a technical fault in the processing of samples 47, 59 and 60 so these samples were no longer available for analysis. Bile acid profiles of twelve individual bile acids generated through LC-MS analysis are illustrated in Figure 2. The concentrations of bile acids detected, although lower than those detected by our group and others in sputum or saliva (23, 37), were in line with previous reports from BALF (31). Glycodeoxycholic acid (GDCA) and Glycocholic acid (GCA) were particularly abundant bile acids in this cohort. It was clear however that inter-patient differences existed in terms of the concentrations of bile acids detected.

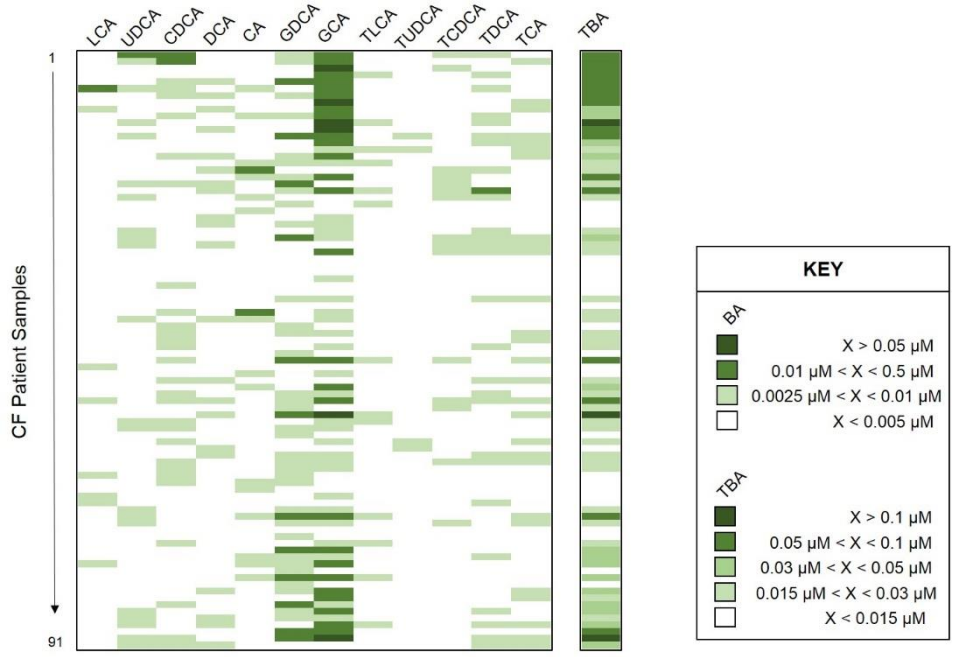

Figure 2. Bile acid (BA) profiling for 88 patients with key denoting both individual bile acid (BA) and total bile acid (TBA) concentrations. Each BALF sample was analysed for the presence of 12 principal bile acids; Cholic acid (CA), Chenodeoxycholic acid (CDCA), Deoxycholic acid (DCA), Lithocholic acid (LCA), Ursodeoxycholic acid (UDCA), Glycodeoxycholic acid (GDCA), Taurochenodeoxycholic acid, (TCDCA), Taurodeoxycholic acid (TDCA), Taurocholic acid (TCA), Glycocholic acid (GCA), Taurolithocholic acid (TLCA) and Tauroursodeoxycholic acid (TUDCA).

Bile acids correlate with a reduction in microbial diversity in CF lung microbiomes

Lower respiratory infections, even from as early as the first weeks of life, are strongly associated with the development of pulmonary inflammation and bronchiectasis. Furthermore, changing dynamics of the lung microbial community structure is emerging as a critical factor in the pathophysiology of CF. Microbial profiles of the paediatric BALF samples were established based on the V3-V4 region of 16S rDNA with 5 samples excluded as microbiome analysis could not be completed. Shannon Index (SI) as a measure of lung biodiversity was determined for each sample with lower SI indices appearing to correlate with the presence of bile acids **(Fig 3A).** The SI values and bile acid concentrations for each patient are outlined in Appendix Table 2.

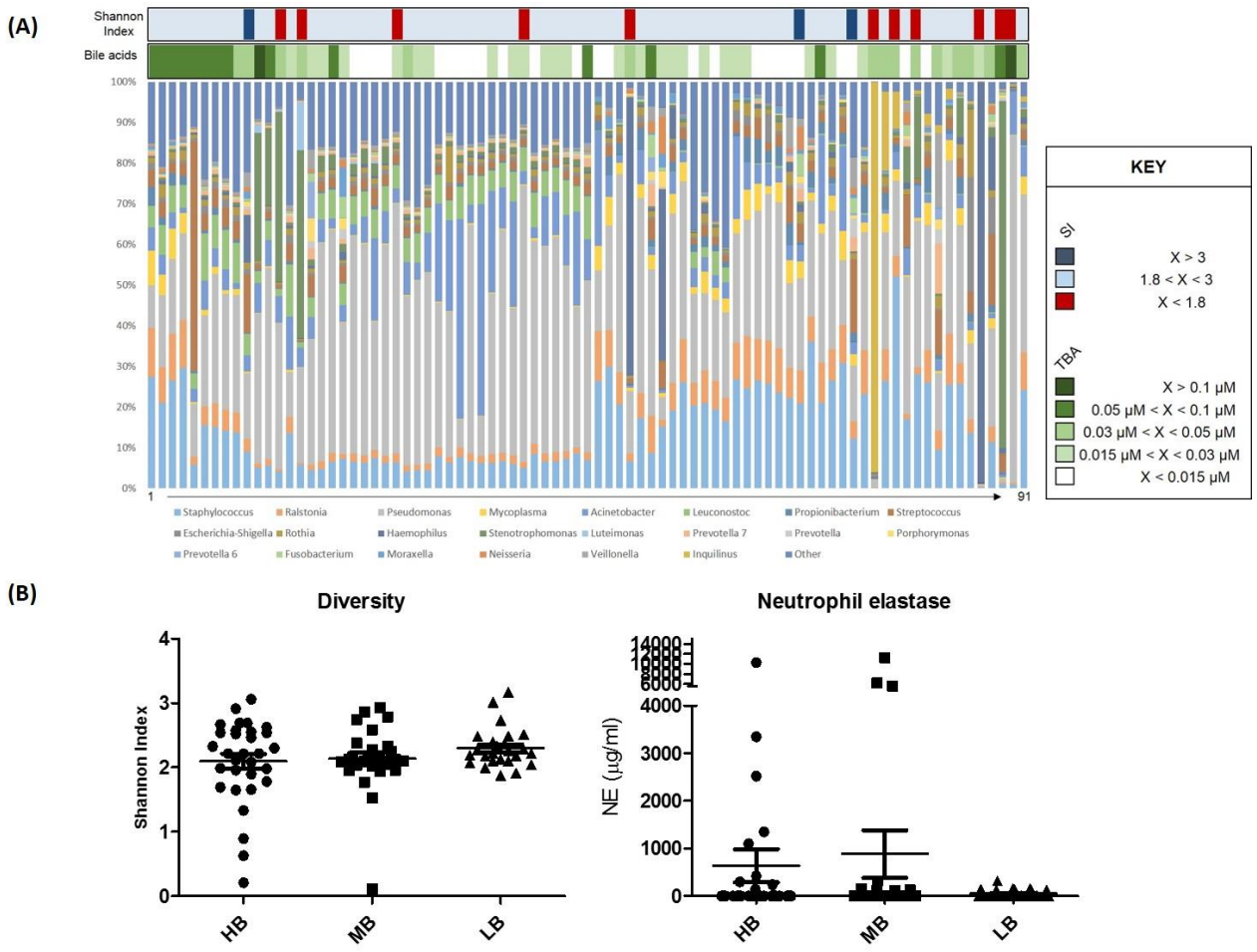

Figure 3. (A) Stacked chart analysis of the lung microbiota of the 88 patient samples with genera >1% identified. Analysis is overlaid with diversity of the microbiota as measured by SI and concentrations of bile acids present highlighting a correlation between the presence of bile acids and a reduction in biodiversity. (B) Stratification of the patient cohort on the basis of bile acid concentrations detected into high bile (HB >0.03 µM), moderate bile (MB 0.015-0.3 µM) and low bile (LB <0.015 µM) further highlights the correlation between the presence of bile acids and the reduction in diversity with HB trending towards a statistically significant difference to LB.

Stratification of the patient cohort into three categories on the basis of quartile bile acid concentrations; high bile (HB >0.03 µM, upper quartile), moderate bile (MB 0.015-0.3 µM) and low bile (LB <0.015 µM, lower quartile) further highlights the correlation between the presence of bile acids and the reduction in SI with the difference between the HB and MB categories to the LB category approaching statistical significance (one tailed t-test p=0.0776 and p=0.0846

respectively) **(Fig 3B).** Inflammation of the CF lung is dominated by neutrophils that release oxidants and proteases, particularly elastase. Neutrophil elastase (NE) in the CF airway secretions precedes the appearance of bronchiectasis, and correlates with lung function deterioration and respiratory exacerbations. Stratification of the cohort based on bile acid status revealed a correlation with NE levels, with no NE detected in LB samples (**Figure 3B**). Although HB (one tailed t-test, p=0.0702) and MB (one tailed t-test, p=0.0567) samples trended towards higher levels of NE when compared with LB samples the difference between the groups were not statistically different. Importantly, these three categories were indistinguishable based on age, gender and antibiotic regimens (Appendix Figure 1).

The previous observation that BAs in the lungs correlate with changes in the microbiology of the lung led us to investigate whether the presence of BAs in patients could explain the shift in the dynamics of the microbiota over time. In order to investigate whether the progressive loss and/or recovery of diversity over time is associated with the accumulation of bile acids in these patients, 10 patients with the lowest SI (<1.8 with the exception of two samples 5 and 36) values (5, 14, 16, 36, 40, 51, 77, 87, 89, 90) and the same number of patients with the highest SI (>2) values (6, 9, 10, 15, 22, 41, 50, 70, 75, 76) were enrolled from the cross-sectional study for longitudinal analysis. 77 BALF patient BALF samples were obtained which spanned over the course of eight years with samples ranging from 11-88 months with a minimum of two samples and a maximum of 7 samples present per patient (Appendix Table 3). Based on these criteria one patient had to be excluded as only 1 sample could be provided. Bile acid profiling, gDNA extraction and microbiome analysis was undertaken as described above. On the basis of low read values (Table 3), the following samples were excluded from analysis; 5e, 7c, 13d, 16e, 18e as well as one sample obtained from patient 18 which was found to be a duplicate sample.

Table 3; gDNA concentrations from longitudinal BALF samples.

ID		ng/µl	260/280	**Reads**	**ID**		ng/µl	260/280	**Reads**
1	C	5	1.42	120029	**11**	A	0.4	0.36	3918
	D	316.3	1.84	219713		C	22	1.89	4807
	F	19.9	1.84	263960	**12**	B	1.1	0.70	36780
	G	292.1	1.87	195093		C	3.8	1.27	54919
2	B	2.3	1.58	1807		D	1.4	1.61	3908
	C	3.8	1.56	3619		E	3.5	1.32	6668
	G	3.6	2.25	135513		F	3.6	2.26	5878
3	B	14.8	1.91	10985		G	2.1	1.05	9174
	C	1.7	1.41	10919	**13**	C	2	1.04	4942
	E	14	2.16	251502		D	0.9	1.19	697
4	A	32.5	1.63	126434		E	2.2	1.98	2099
	D	38	1.96	243494		F	15	2.23	20828
	E	22.4	2.07	293621		G	27.9	1.91	9557
5	B	1	2.44	46963		H	8	2.08	16773
	C	3.4	1.88	14668	**14**	D	2.2	1.64	15858
	D	7.5	1.37	155175		E	2.5	0.78	17342
	E	0.8	-0.89	327	**15**	B	0	0	35340
	F	15.6	1.91	71908		E	4.8	2.42	19336
	G	14	2.09	14223	**16**	C	13.4	2.08	19045
6	B	3.1	3.09	35567		D	2	3.64	55950
	D	29	1.70	60616		E	2.4	2	625
	E	48.8	1.74	15450		F	1.5	1.16	25691
	F	7	1.90	273565		G	3.6	1.03	6828
	G	20.6	1.83	163260	**17**	B	3.2	1.70	2905
	H	96.6	1.82	157699		C	0.8	0.51	1934
	I	64.7	1.74	137271		D	156.9	1.24	5341
7	B	0.8	1.28	8086		E	4.8	2.63	1979
	C	0	0.16	459		F	0.5	1.51	1216
	D	11	1.75	279670	**18**	C	0.4	2.41	2995
8	C	5.7	1.09	63475		C	0.2	0.30	22101
	D	2.6	1.50	11806		D	1.3	2.04	1507
	E	2	1.19	11504		E	3.6	1.85	948
	F	9.3	1.42	5291	**19**	D	0.4	0.27	46775
9	D	11.1	2.22	2876		E	4.4	2.08	5991
	E	7.8	2.22	166964		F	96.7	1.91	27151
	F	45.7	1.66	4485	**20**	C	2.5	0.86	15570
10	B	2.3	1.99	13638					
	C	12.2	1.88	73715					
	D	7.8	1.37	98326					
	E	4.7	2.92	1691					
	F	2.5	2.24	15					

Following on from previous studies and the initial cross sectional which demonstrated that BAs are present in both BALF and sputum of paediatric patients, BA profiling of the longitudinal cohort was conducted. BALF samples were profiled for the presence of twelve individual bile

acids with patients categorized as Bile Acid+ (BA+) if bile acids were consistently present in all patient samples (TBA >0.015 μM), Bile Acid- (BA-) if patient samples had low to no bile acids present (TBA <0.015 μM) and transitioning patients having bile acids present intermittently (patient samples contained a mixture of BA+ and BA- samples) (**Fig. 4**). Glycocholic acid (GCA) and Taurocholic acid were abundant in BA+ patients. While there was no significant difference in age and antibiotic administration between the three groups, there were more female patients in the BA+ category compared to the BA- category. This could be attributed to the overrepresentation of females in the initial cross-sectional study (**Appendix Figure 1**).

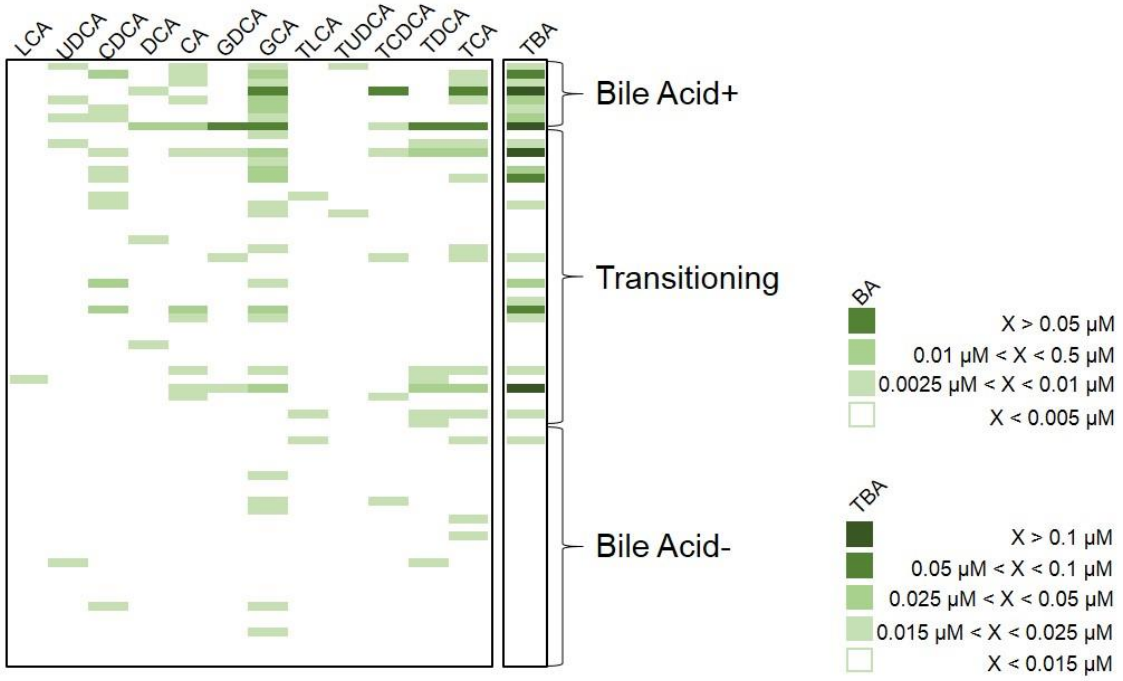

Figure 4. Bile acid profiling of 77 longitudinal patient samples with a minimum of 2 samples per patient and a maximum of 7. As such 1 patient had to be excluded from further analysis as only 1 sample could be provided. Patients were categorised based on the presence of bile acids over time with patients designated BA+ consistently having bile acids present, transitioning patients having bile acids present intermittently and BA- patients not having bile acids present.

Age-associated reductions in biodiversity

Microbiome analysis of the paediatric BALF samples was undertaken based on the V3-V4 region of 16S rDNA with SI measurements completed for each patient sample. Characterisation of patients on the basis of SI measurements over time revealed three broad categories: (i) patients in which the microbiome consistently remained stable over time (2.035 +/ 0.439), (ii) patients in which the microbiome fluctuated between high diversity and low diversity samples over time (1.371 +/- 0.791), and (iii) patients whose microbiome crashed over time with no recovery in diversity suggesting the emergence of a dominant organism (1.178 +/- 0.881) (**Figure 5**).

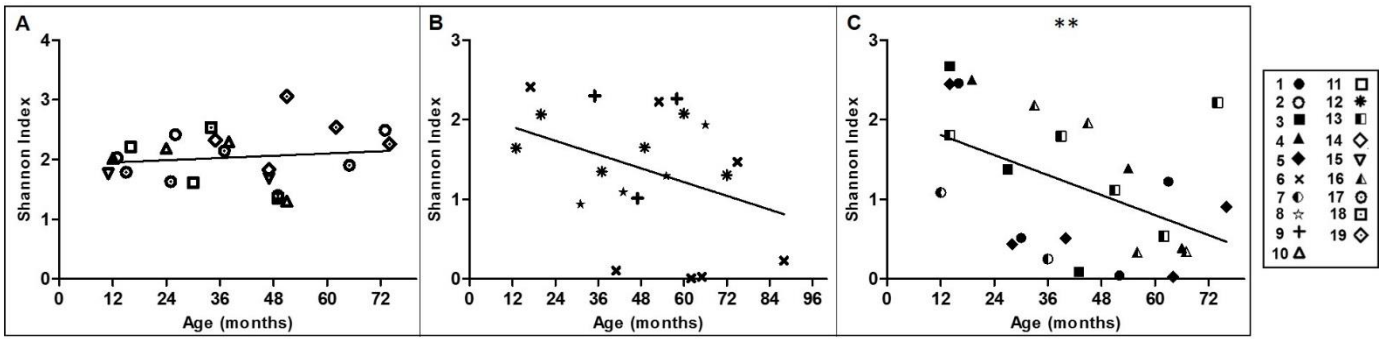

Figure 5. Longitudinal analysis of the diversity of the microbiomes of 19 patients' revealed patients could be categorised into three broad categories; (A) Highly diverse, stable microbiomes (B) Fluctuating diversity microbiomes and (C) Crashed microbiomes.

Interestingly, while an age associated decline in the diversity of microbial communities has been described in CF patients, this did not apply to category A where the patient's lung microbiota remained relatively diverse and stable over time. This raised the question as to what factors underpinned the differences in SI observed between groups of patients, which cannot solely be explained by or attributed to age.

Bile acid positivity promotes changes in the CF paediatric microbiome

Principal component analysis based on the microbial profiles of the total patient cohort revealed the presence of two primary clusters. Correlation analysis with the SI categories described in Figure 5 revealed that cluster 1 was predominantly a composite of samples from the crashed microbiome category while cluster 2 was predominantly composed of samples from the highly diverse stable microbiomes. There was a potential third cluster present, however this cluster was found to not separate completely from cluster 1 and 2. This suggested that, apart from the diversity score, the signature taxonomic profile was being shaped by some host or environmental factor.

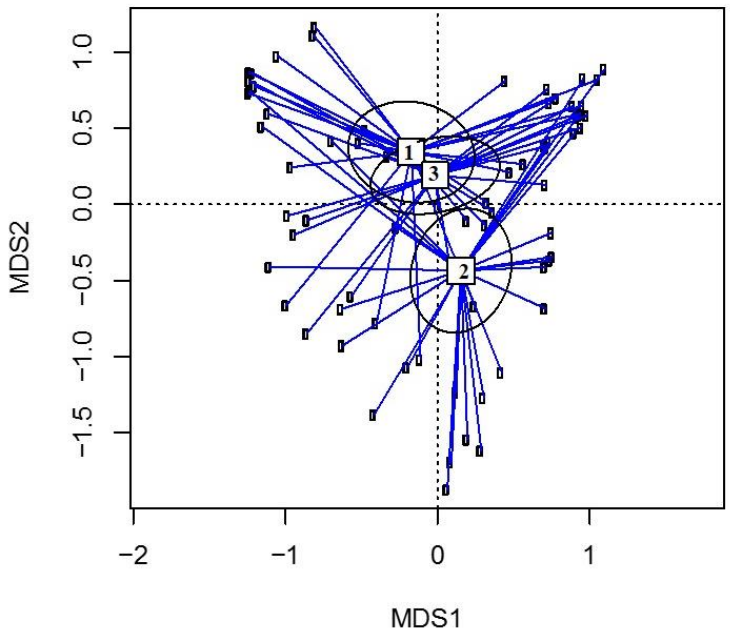

Figure 6. PCA of the global microbiome from the total patient cohort revealed separation into two primary clusters with patient samples from the crashed microbiome category predominantly located in cluster 1 while patient samples from the highly diverse, stable microbiome found in cluster 2.

Correlation of the PCA analysis with BA profiling revealed cluster 1 to be dominated by samples from the BA+ category, while cluster 2 was dominated by samples from the BA- category.

Samples from the transitioning cohort were found to be distributed between the two clusters. This was further supported by hierarchical clustering using the Bray Curtis dissimilarity Index in which two primary clusters; 1 (A & B) and 2, were once again observed (**Fig. 7**). The samples contained within cluster 1 were predominantly BA+ while samples in cluster 2 were primarily BA-. Clustering was found to be independent of either age or augmentin regimes (Appendix Figure 2).

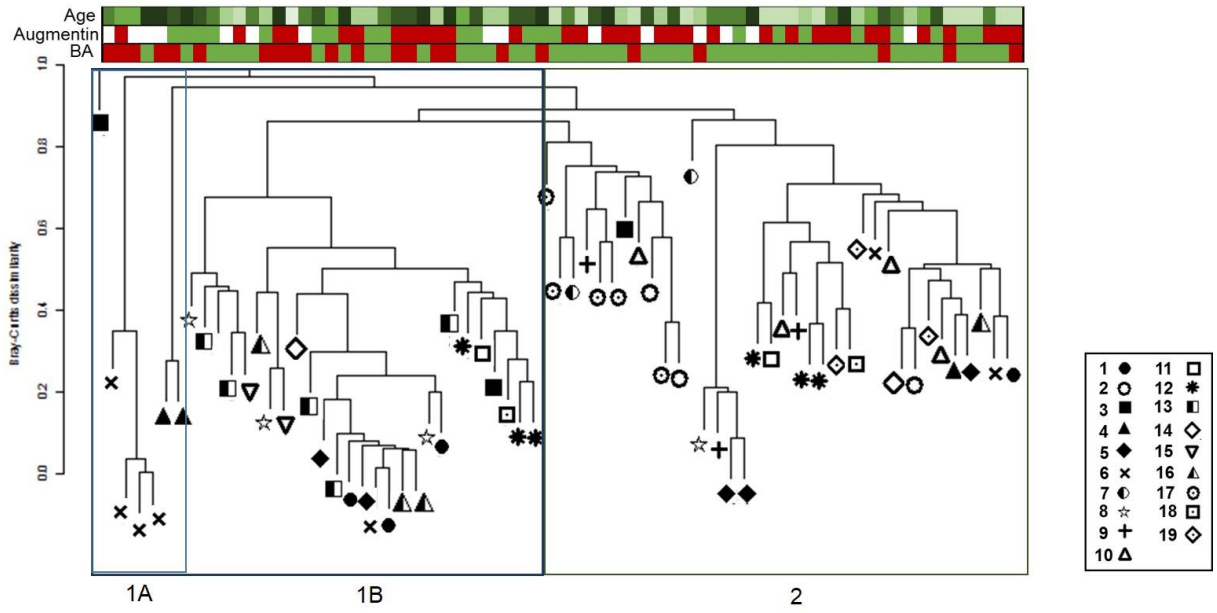

Figure 7. Hierarchical clustering based on the bray Curtis dissimilarity index highlighting bile acids underpin microbial community remodelling.

Bile acids are linked to a progressive reduction in diversity

The previously reported transition towards low microbial diversity over time was not evident in all patients tested in this study **(Fig. 5)**. Therefore, the central question to be addressed here was whether the presence of bile acids underpinned the age associated reduction in diversity. Global linear regression analysis of the total patient cohort revealed a significant decrease in Shannon Index and Simpson Index over time with an accompanying significant increase in Berger-Parker

Index (pathogen dominance). However, the stratification of the total patient cohort into the assigned bile acid categories highlighted that these age associated effects are only evident in the transitioning patients shifting towards BA positivity (**Fig. 8**). In contrast, the BA- cohort maintained a highly diverse microbiomes over time while the BA+ cohort trended towards low diversity and increased pathogen dominance though was not significant. This was potentially due to the consistently high concentrations of bile acids over time, resulting in the prior establishment low diversity scores. The reduction in diversity was significant in the transitioning cohort where the accumulation of bile acids correlated with a reduction in diversity indexes.

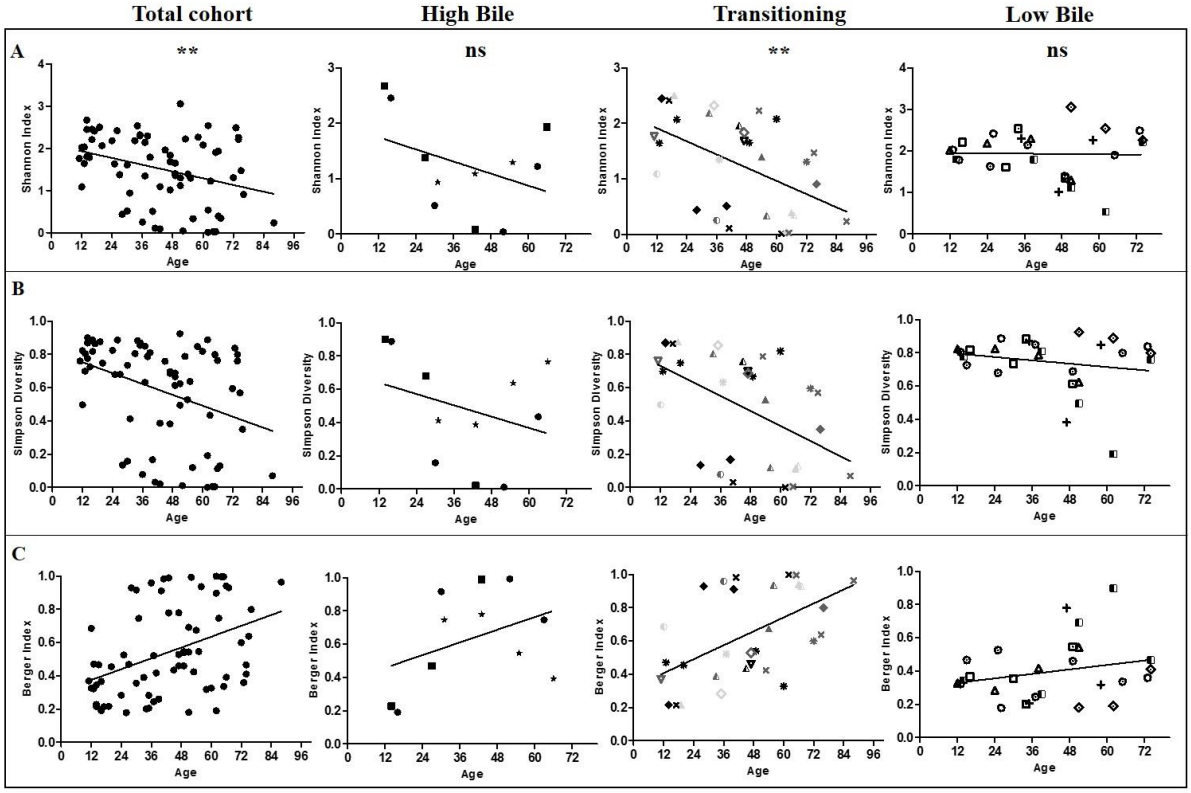

Figure 8. Diversity measurements of the total patient cohort and stratification into BA+, T and BA- cohorts. (A,B) Significant reduction in Shannon Index and Simpson Index diversity measurement over time in total patient cohort, only evident in the T cohort. (C) Significant increase in Berger-Parker pathogen dominance Index over time in total cohort, only observed in T cohort.

Bile acids promote pathogen acquisition and chronic colonisation

The presence of bile acids is associated with the persistent infection by the pathogenic organisms *Pseudomonas, Inquilinus* and *Haemophilus* in this paediatric cohort. Once acquired by patients, these pathogens become established and dominate the microbiota of the BALF samples analysed. In general, the earlier samples from all patients in this study displayed a relatively diverse microbiota populated by *Veillonella, Neisseria* and *Streptococcus* (**Figure 9A**). In patients that transition towards BA+, the subsequent acquisition and establishment of pathogenic organisms such as *Pseudomonas, Staphylococcus, Haemophilus, Stenotrophomonas* and *Achromobacter* is evident. This is in direct contrast to BA- patients which retain a highly diverse microbiota including the health associated organisms such as *Neisseria, Veillonella* and *Prevotella.* The organisms *Propionibacterium* and *Sphingomonas* are also found to be more abundant in this cohort of patients. In instances where a pathogen emerges such as *Pseudomonas* in patient 13 or *Haemophilus* in patient 9, these do not appear to persist or become the dominant member of the microbial community. In patient 13 though the relative abundance of *Pseudomonas* initially increases to 69% and 89%, the following year this is reduced to 47%. Hence, even though the bug is still present, it has not retained its position strongly as dominant organism. In patient 9, though *Haemophilus* is acquired and is detected at a relative abundance of 78%, the following year the organism has completely cleared and is not detectable in the patient samples. In a bile negative environment the data suggests that the infection is capable of being successfully cleared or controlled either by the host immune system or clinical intervention.

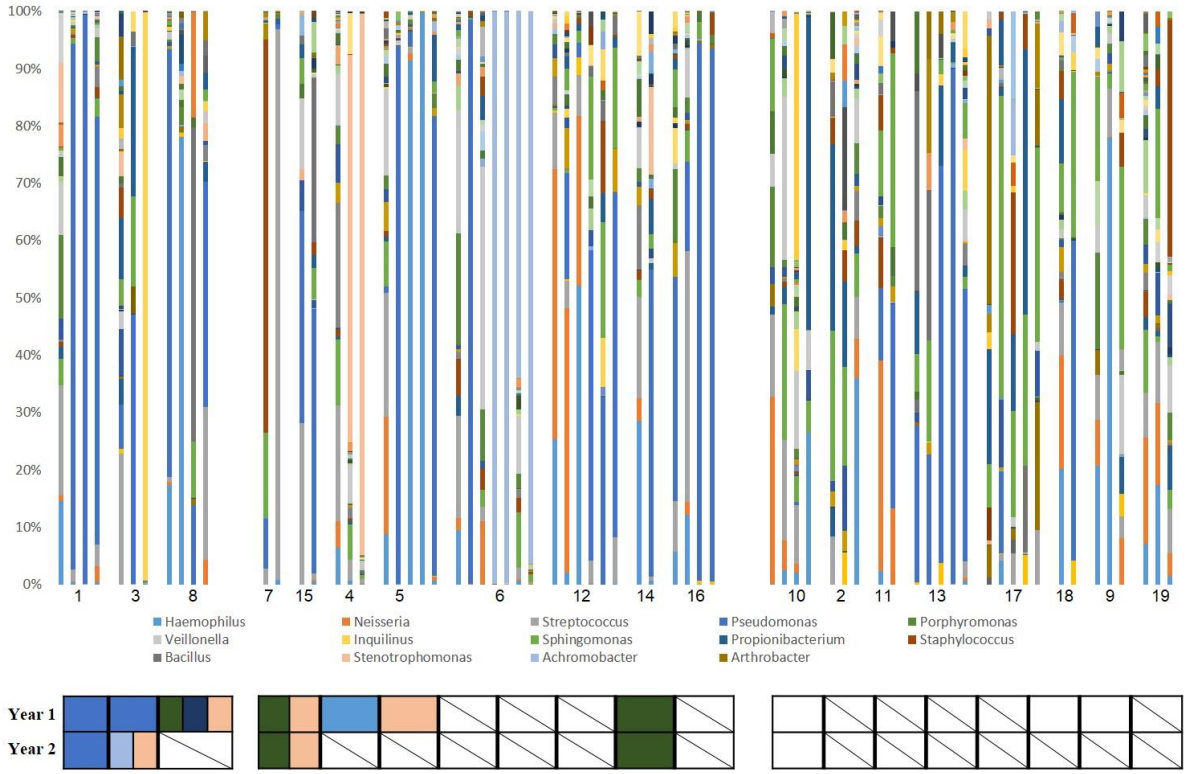

Figure 9. (A) Stacked chart denoting the microbial composition present in BA+, T and BA- patient samples. BA+ and T samples display a reduction in biodiversity and increased levels of dominance of the microbiota by individual genera. In contrast, BA- samples are generally more diverse. (B) Culturable data from the clinical data of samples obtained in the two years post-microbiome study confirms the hypothesis that the presence of pathogens is associated with the presence of bile acids as can be seen in BA+ and T patients.

Furthermore, analysis of culturable data from the AREST clinical database in the two years post sampling for microbiome analysis demonstrates that pathogenic organisms are more routinely cultured in BA+ and transitioning patients (**Figure 9B**). This is consistent with the hypothesis of persistent colonisation by CF associated pathogens in the presence of bile acids. However, this analysis must be viewed in the context of limited availability of follow-on patient samples. Interestingly, although *Pseudomonas* was present in the microbiome of BA- patient 18, a follow-on sample was found to be culture negative, suggesting the patient may have successfully cleared

the infection. This contrasts to BA+ patients where colonisation of the lung with a pathogen is persistent and cannot be resolved.

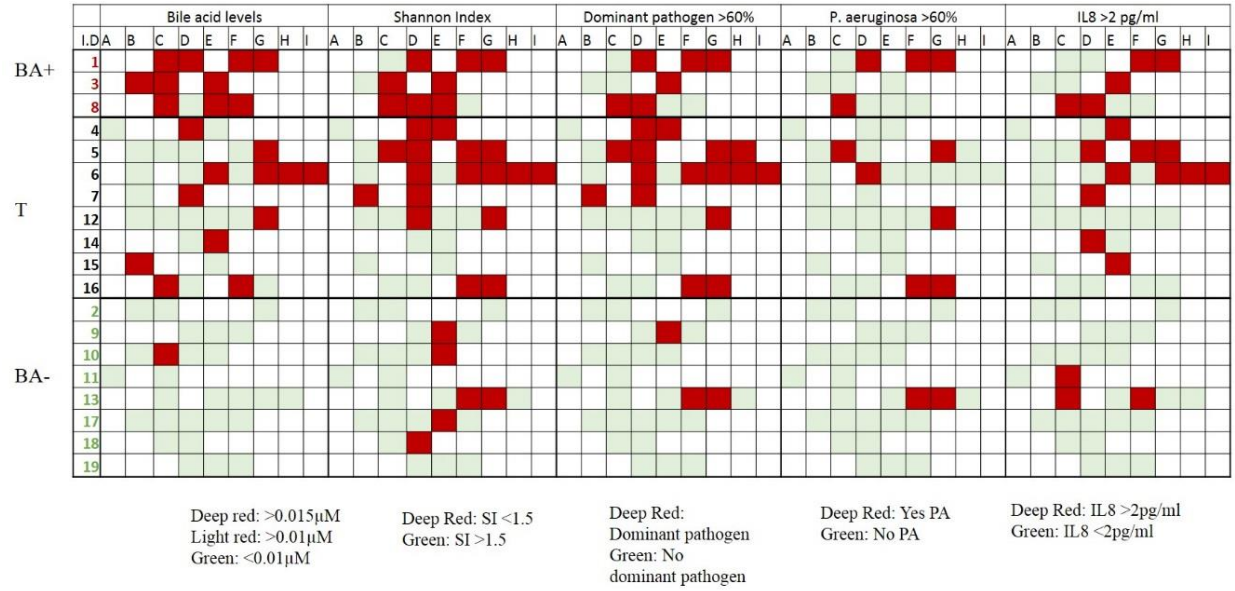

Figure 10. A summary overview of the longitudinal study. BA+ patients display elevated bile acid levels, reduction in diversity as measured by SI, increased pathogen dominance of the microbiota and elevated levels of the pro-inflammatory cytokine IL-8. In contrast, BA- patients displayed reduced bile acid levels, generally higher levels of diversity, reduced pathogen dominance and lower levels of the cytokine IL-8. The transition category in which patient samples consist of both BA+ and BA- samples is an intermediate between the two categories in terms of the parameters assessed.

In conclusion, BA+ patients were found to display elevated levels of bile acid with a reduction in diversity as measured by SI, increased pathogen dominance of the microbiota and elevated levels of the pro-inflammatory cytokine IL-8 also observed. In contrast, BA- patients were found to display reduced bile acid levels, generally higher levels of diversity, reduced pathogen dominance and lower levels of the cytokine IL-8. The transition category in which patient samples consist of both BA+ and BA- samples was found to be an intermediate between the bile

acid categories **(Fig. 10).** This is the first longitudinal study that points to the association between bile acids and the restructuring of the lung microbial communities.

Phenotypic profiling of clinical isolates of a range of respiratory pathogens

The growing appreciation for the extent of species phenotypic heterogeneity within the CF lung led us to further investigate whether the response of lung microbiota associated microbial species to bile is uniform. Therefore, a range of bacterial isolates, outlined in Table 1, were tested in order to investigate whether bile is capable of modulating the behaviour of clinical isolates in the same manner as previously investigated typed strains. The strains tested were a combination of clinical isolates recovered from paediatric patients attending Cork University Hospital previously collected in the BRC and typed lab strains. Interestingly, not only was there a differential response to bile between the typed lab strains and clinical isolates, there was apparent variation in the bile response between clinical isolates. As mentioned above, this is unsurprising in light of reports describing phenotypic heterogeneity of strains residing in the CF lung (54-56). In *Staph aureus,* the most prevalent early coloniser in paediatric patients with CF, initial studies reported that bile repressed biofilm formation in the typed strain NCDO949 (24). However, in a marked contrast, it was found to stimulate biofilm formation in the two clinical isolates (CUH-T and CUH-E) **(Fig. 11A)**. In *Staph haemolyticus,* one of the isolates (CUH-M) tested did not exhibit any change in biofilm in response to bile, while a slight but significant reduction in biofilm formation was observed in the other *Staph haemolyticus* isolate (CUH-D LB) **(Fig. 11B)**. In the two *Staph epidermidis* strains tested (CUH-D MS & CUH K LB) bile significantly increased biofilm formation. In the emerging respiratory pathogen *Ralstonia,* a dark brown pigmented isolate (CUH-242) significantly increased biofilm formation in response to bile. In contrast, a non-pigmented *Ralstonia* isolate (CUH-229) was found to be a non-responder **(Fig. 11C)**. The

two *Stenotrophomonas maltophilia* strains tested (CUH-B PIA & CUH-C PIA) were non responsive to the presence of bile, again in contrast to the typed strain tested previously (24). In *P. aeruginosa*, the biofilm response to bile was conserved relative to the typed lab strain in one of the clinical isolates tested (CUH-A PIA) with an apparent hyper response to bile. However, biofilm formation was not significantly altered in another clinical isolate tested (CUH-B McC) further highlighting phenotypic heterogeneity amongst isolates **(Fig. 11D)**. This data confirms that phenotypic diversity with regards to bile responsiveness is present in our clinical isolate collection. This suggests that potential strain specific adaptations may occur in a bile positive CF lung environment, a hypothesis which will be further explored in chapter 3. In order to confirm this, further investigations would be required encompassing bile acid profiling and the isolation of microbes from the same clinical sample.

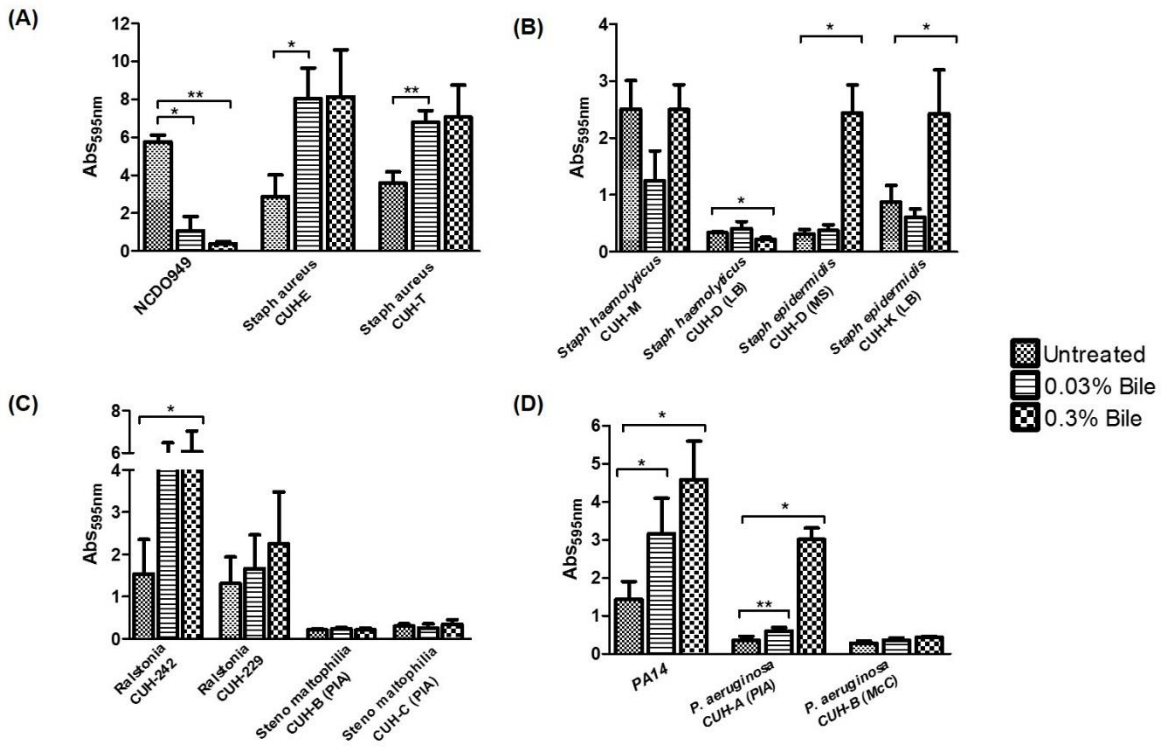

Figure 11. Biofilm formation in clinical isolates and typed strains of a range of respiratory pathogens. (A) *Staph aureus*, (B) *Staph haemolyticus* and *Staph epidermidis*, (C) *Ralstonia* and *Stenotrophomonas maltophilia* and (D) *P. aeruginosa* highlighting phenotypic heterogeneity regarding their response to bile.

Discussion

The CF lung has been shown to harbour distinct microbial communities with dysbiosis evident throughout disease progression characterised by a shift towards a low diversity, pathogen rich microbiota (13-15, 57-59). The polymicrobial nature of CF infections has highlighted the importance of community structure and interactions between members and their contribution to the pathogenesis of respiratory disease. Of particular importance, is the knowledge of host factors which shape these microbial communities facilitating the establishment of chronic infections; the primary cause of morbidity and mortality in the CF population. The aim of the clinical studies outlined in this chapter was to undertake a cross-sectional/longitudinal analysis of the microbiology of the lower respiratory tract of paediatric patients with CF, and to establish if correlations existed between bile acids and pathogen dominated microbiomes.

The cross-sectional study encompassing 91 patient samples revealed inter-patient differences in both the concentrations of bile acids and in the microbial profiles and diversity present. This initial study indicated a potential correlation between the presence of bile acids and reshaping the microbial communities present. This evidence appeared to position bile acids as a key player in the modulation of the respiratory microbiome towards a low diversity, chronic pathogen dominated state commonly observed in end stage lung disease. In order to further investigate this hypothesis 20 patients were selected from the cross-sectional analysis for a longitudinal study. Several reports have described a reduction in microbial biodiversity of the CF lung as patient's transition into adulthood (7, 13, 14, 22, 60) . However, the factors underpinning this age associated decline remain unclear. Additionally, the fact that not all patient's exhibit these decreases in diversity is worthy of investigation. Within our cohort of 19 patients, 8 patients retained stable, highly diverse microbiotas while the diversity of 4 patient's microbiotas

fluctuated over time and the diversity of 7 patient's microbiotas crashed over time. PCA analysis on the global microbiota of the total patient cohort revealed separation into two primary clusters. As recent findings have identified a role for bile acids in the progression of chronic lung disease (28, 35-37, 61), we wanted to investigate whether the presence of bile acids in these paediatric patients could explain this separation.

A hierarchical clustering tree based on the Bray Curtis dissimilarity matrix revealed that the clustering could be attributed to the presence of bile acids. The majority of BA+ samples were found in cluster 1 and the majority of BA- samples were found in cluster 2. While a selection of patient samples were found clustered together and certain early year samples clustered together, this was not the case for the majority of the samples and could not explain the global clustering. The administration of the antibiotic augmentin did not affect clustering. These results support the association between bile acids and the reshaping of microbial communities towards low diversity populations. Analysis of three commonly utilised diversity measurements Shannon Index, Simpson Index and Berger-Parker Index confirmed the age associated reduction in diversity and age associated increase in dominance of organisms in the total patient cohort (7, 13, 14, 22, 60). However, only patients transitioning towards bile acid positivity display this signature CF effect. This firmly supports the hypothesis that the appearance of bile acids in the lungs triggers a programmed switch in the microbiota whereby theirs is a reduction in diversity and an overgrowth of single dominant organisms.

From a clinical perspective, understanding how bile acids transition to the lungs of these patients is crucial. A role for gastro-oesophageal reflux (GERD) and aspiration has been proposed in recent years underpinned by evidence from *in vitro* and *in vivo* studies of microbial pathogenesis, inflammation and lung community profiling (23, 24, 27, 37, 39, 42, 62, 63). Furthermore, an

association between aspiration and airway inflammation in children with CF is evident from examination of bronchoalveolar fluid (64). Importantly, only low concentrations of bile salts are required to change microbial communities and phenotypes (23, 24, 27, 42), and therefore micro-aspiration rather than overt GERD may be sufficient to significantly shape the lung microbiome in early CF (43). Both chronic cough and asthma have also been reported to result in increased bile acid levels in sputum (37), and studies are ongoing to determine the incidence of reflux and aspiration in these cohorts. Alternatively, de-novo synthesis of bile acids has been speculated to occur in pulmonary arterial hypertension (65), and to reach the lung by the circulatory system in neonates (28). Therefore, further studies will be needed to address the complexity of how bile acids are able to accumulate in the lungs of patients with CF.

The in-depth characterisation of the microbiota revealed the presence of several key CF-associated pathogenic organisms in BA+ and transitioning patients. This included *Pseudomonas, Haemophilus, Stenotrophomonas* and *Staphylococcus.* Interestingly though it is not yet known if bile acids influence the acquisition of these organisms, upon emergence of these organisms within the microbiome bile acids influenced the ability of these organisms to chronically colonise and persist in the lung with pathogens persisting for several years. In contrast, the microbiota of BA- patients were highly diverse containing members of the healthy associated microbiota including *Neisseria*, *Prevotella* and *Veillonella.* Interestingly, where acquisition of a pathogen such as *Pseudomonas* was observed in the BA- cluster, microbiome analysis of subsequent samples revealed the pathogen did not become a dominant member of the microbial community hence no evidence of chronic colonisation was observed. This suggests a potential enhanced resolution of infection in the patient in a bile acid negative environment. This further

support the hypothesis of a centralised role for bile acids in facilitating chronic colonisation of the CF lung by *P. aeruginosa* and other Proteobacterial pathogens.

This study is the first paediatric longitudinal study examining a role for bile acids in the onset of the disease associated microbiota in CF. However, this and other respiratory microbiota studies should be carefully interpreted with consideration given to the numerous limitations that exist with regards to sample collection, processing and sequencing. The primary issue when dealing with paediatric BALF samples is the risk of low bacterial load thereby increasing the risk of background contamination, particularly from DNA extraction kits (66-68). This should be kept in mind with respect to this study in which there was both low volumes of BALF available and low bacterial load. The collection and processing of such low bacterial load samples is therefore critical with proactive measures required to ensure that no exogenous DNA is introduced throughout the handling of samples. The inclusion of negative controls during processing is essential in the monitoring of background contamination levels, with several negative controls including DNA kit controls and water controls in place during the processing of these samples (69). The lack of a bronchoscope control in this study is not ideal, a gap which should be addressed going forward as any background contamination introduced during sample collection could be misinterpreted as residents of the respiratory microbial community. Sequencing of such controls leads to the identification of background contamination facilitating their exclusion from data analysis with numerous exclusion strategies outlined by Marsh et al. (70). The choice of DNA extraction kit employed has also been shown to significantly impact the bacterial DNA yields obtained, with different extraction kits reported to harbour contaminating microbiomes referred to as the "kitome" (67, 68). In this study, increased DNA concentrations were yielded when employing the QIAGEN Blood Core Kit B. However, the low quality of the DNA

(260/280) obtained using this extraction method could impact upon downstream analysis. However, the internal quality controls of LGC deemed these samples of sufficient quality for sequencing. Even after employing an optimised DNA extraction method, there may still be a problem in the generation of sufficient amplicons for sequencing. In these instances, such as for this study, nested PCR reactions may be used to enhance amplicon yield for downstreaming sequencing (71). However, use of nested PCR requires the additional liquid handling steps which increases the risk of the introduction of exogenous DNA (70). Enhanced PCR cycles may also increase amplification errors and increase the detection of background contamination (68). The exclusion of low read sequencing data, as applied in this study, which may not be reliable should be undertaken in order to not confound the interpretation of the data or the potential biological significance. Hence, while this study has provided some valuable insights into the impact of bile acids on the paediatric lung microbiota, they must be viewed in the context of the challenges encountered in the processing and sequencing of the samples.

In conclusion, this study establishes that the presence of bile acids in the lung is accompanied by a loss of diversity, emergence of a dominant pathogen which is often *Pseudomonas* and from a clinical perspective higher levels of the pro-inflammatory cytokine IL-8 (**Figure 10)**. While this study aims to establish a causal role for bile acids in the progression of respiratory disease, the limited number of samples available restrict the outputs to strong associations. Further comprehensive longitudinal analysis, including potential animal models, will be required to make a convincing case for causality. These findings provide new knowledge on what could represent both a potential biomarker for the identification of high risk patients and a potential therapeutic target for the development of novel therapeutics, providing an opportunity for effective early clinical intervention.

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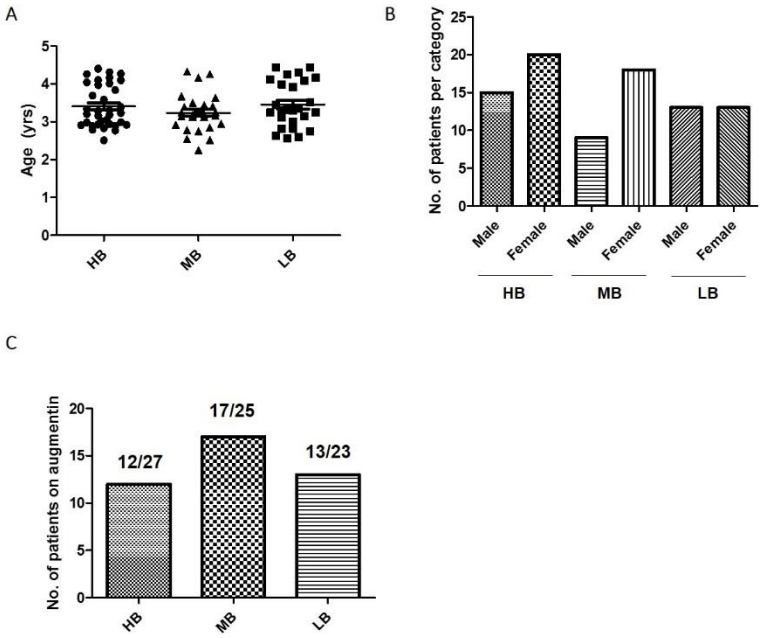
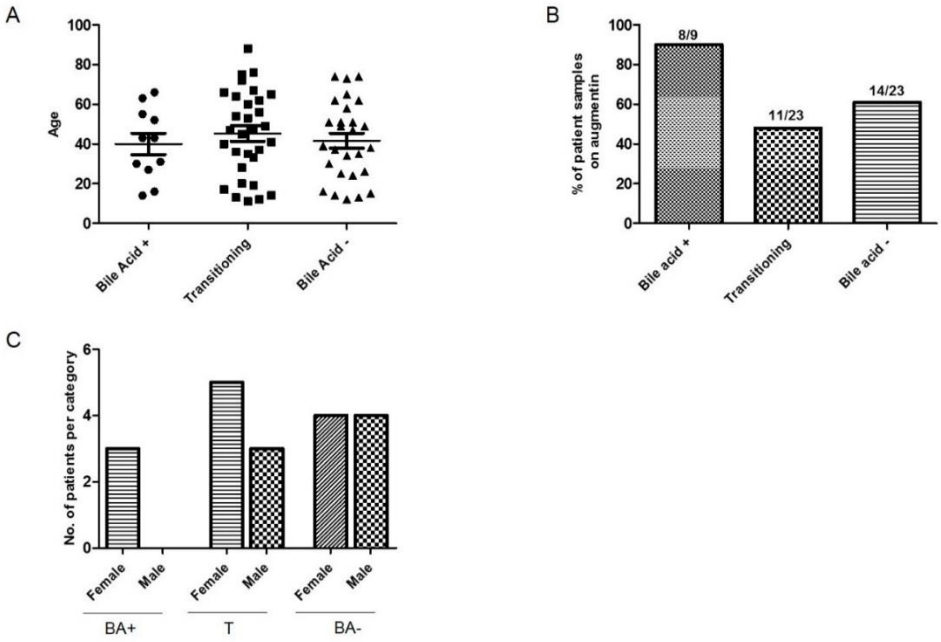
Appendix



Appendix Figure 1. Comparison of age, patients on augmentin and gender across the HB, MB and LB categories.



Appendix Figure 2. Comparison of age, patients on augmentin and gender across the BA+, T and BA- categories.

Appendix Table 1; Clinical Dataset for AREST CF Cross-sectional Paediatric Cohort BALF Samples.

ID	G	Age (yr)	Allele2[¥]	M	Mut2	H[#]	Aug[^]
1	2	2.94	c.2657+5G>A	I	V		
2	1	4.10	R117H	I	IV		
3	2	2.88	*	I			0
4	1	3.16	F508del	I	II		
5	2	3.02	F508del	I	II		
6	1	3.02	W1282x	I	I		
7	1	4.01	*	I			
8	2	2.63	*	I			
9	1	4.10	F508del	I	II		1
10	2	2.99	*	I			
11	2	3.05	G551D	I	III		0
12	1	2.91	F508del	I	II		0
13	1	4.16	F508del	I	II		1
14	2	3.20	F508del	I	II		0
15	2	2.55	c.3367G?Cp.gly	I			
16	1	2.92	*	I			0
17	2	3.14	F508del	I	II		1
18	1	2.92	F508del	I	II		1
19	2	3.97	F508del	I	II		1
20	1	2.83	F508del	I	II		0
21	2	2.89	F508del	I	II		0
22	2	4.33	F508del	I	II		0
23	2	3.92	F508del	I	II		
24	2	2.81	F508del	I	II		1
25	1	3.11	G551D	I	III		
26	2	3.24	c,1585-1G>A	I	I		1
27	2	2.95	G551D	I	III		1
28	2	3.24	c,1585-1G>A	I	I		1
29	2	3.18	T663RfsX8	I	I		
30	2	4.26	R117H	I	IV		0
31	1	3.24	F508del	I	II		1
32	2	3.51	*	I			1
33	1	3.29	2657+5G>A	I	V		1
34	1	4.30	F508del	I	II		0
35	1	3.00	F508del	I	II		
36	2	2.60	I336SfsX28	I	I		1
37	2	2.83	Arg117Leu	I	IV		0
38	2	3.31	F508del	I	II		1
39	1	4.26	G551D	I	III		1
40	2	2.52	L259LfsX1	I	I		1
41	1	2.75	D1152H	I	IV		0
42	2	2.85	F508del	I	II		1
43	1	2.75	F508del	I	II		0
44	2	3.13	R334W	I	IV		0
45	2	3.30	c.2657+5G>A	I	II		1
46	2	3.28	F508del	I	II		1
48	1	2.63	F508del	I	II		1
49	1	2.82	R117H	I	IV		0
50	2	3.27	K447RfsX2	I	I		0
51	1	3.34	F508del	I	II		0
52	2	3.50	F508del	I	II		1
53	2	3.39	F508del	I	II		1

54	2	3.39	F508del	I	II		1
55	1	3.30	R560T	I	III		1
56	2	3.67	P67L	I	IV		0
57	1	3.20	c.3121G-A	I			1
58	2	4.44	G551D	I	III		1
61	2	3.13	R347P	I	IV		0
62	1	2.56	F508del	I	II		1
63	1	3.25	1585-1G>A	I	I		1
64	1	3.63	F508del	I	II		0
65	1	3.44	F508del	I	II		1
66	2	3.99	F508del	I	II		0
67	2	3.35	F508del	I	II		1
68	1	4.16	L475P	I			0
69	1	3.45	F316fs	I	I		0
70	2	4.25	*	I			0
71	1	4.17	F508del	I	II		1
72	1	4.04	F508del	I	II	17	1
73	2	2.78	F508del	I	II	0	0
74	2	3.25	K684fs	I	I		0
75	1	3.14	R117H	I	IV		1
76	2	4.09	c.2657+3insA	I			0
77	2	4.40	F508del	I	II		1
78	2	3.23	F508del	I	II		0
79	2	3.69	R1162X	I	I		0
80	2	4.12	W1282X	I	I		1
81	2	4.31	F508del	I	II	0	0
82	1	4.44	P67L	I	IV	23	0
83	1	3.84	c.441delA	I	I	46	0
84	2	3.30	F508del	I	II	0	1
85	1	2.93	F508del	I	II	0	1
86	2	2.25	F508del	I	II	0	1
87	1	3.16	F508del	I	II		1
88	2	3.16	*	I		16	1
89	2	3.58	S341SfsX29	I	I	0	0
90	2	2.5	G551D	I	III	56	1
91	2	3.41	F508del	I	II		1

* Unknown, [#] H – Hospital Days, [^] Aug – Augmentin [~] G – Gender (1 – Male, 2 – Female)

[¥] All patients were F508del for Allele 1

Appendix Table 2; Summary of Shannon index values and bile acid concentrations in the cross sectional cohort.

Sample	Bile acids	Shannon Index	Reads	Sample	Bile acids	Shannon Index	Reads
1	0.0841	2.623736	14905	**56**	0.0257	2.123768	24809
2	0.0562	2.690581	18227	**57**	0.0222	2.120983	9372
3	0.0657	2.54197	9813	**58**	0.0148	2.281479	15682
4	0.0549	2.466574	5719	**61**	0.0212	2.749276	32042
5	0.0707	1.893592	33983	**62**	0.0134	2.511719	17134
6	0.0749	2.669317	23420	**63**	0.0185	2.379834	24658
7	0.0548	2.536743	32484	**64**	0.0208	2.135887	19088
8	0.062	2.5502	34347	**65**	0.0184	2.24259	29916
9	0.0396	2.579203	18191	**66**	0.0113	2.192011	17492
10	0.048	3.063137	44222	**67**	0.0139	2.216605	13230
11	0.1071	1.989198	33787	**68**	0.0063	2.333679	5094
12	0.0599	1.958435	28376	**69**	0.0105	2.735136	23815
14	0.0438	1.648071	26791	**70**	0.0125	3.014657	14318
15	0.0196	2.789375	30466	**71**	0.027	2.164757	13720
16	0.0435	1.658508	37536	**72**	0.0601	2.084145	26359
17	0.0196	2.862336	63354	**73**	0.0229	2.275316	10014
20	0.0237	2.045524	11366	**74**	0.0038	2.254727	9261
21	0.0644	1.983253	13610	**75**	0.0112	3.172943	36965
22	0.0202	2.930159	38301	**76**	0.0159	2.588906	11294
23	0.0116	2.067256	31750	**77**	0.046	0.207159	13975
24	0.0128	2.097885	6523	**78**	0.0326	2.112447	8781
25	0.0098	2.484482	26335	**79**	0.0318	1.781911	12691
26	0.0146	2.040025	18196	**80**	0.0044	2.363968	49242
27	0.017	1.772314	30343	**81**	0.0389	1.691005	28280
28	0.0421	2.302747	19449	**82**	0.0124	2.486593	28464
29	0.0192	2.104598	44622	**83**	0.0413	2.915503	13726
30	0.0294	2.039099	17278	**84**	0.0227	2.024782	7268
31	0.0037	2.175337	32023	**85**	0.032	2.215801	24689
32	0.0032	2.218581	31839	**86**	0.0344	2.21365	22775
33	0.0035	2.128452	13359	**87**	0.0184	0.112218	103426
35	0.0076	1.911904	38646	**88**	0.0421	2.522394	44816
36	0.0046	1.997317	40075	**89**	0.0513	0.895432	15589
37	0.0229	2.102168	11943	**90**	0.1528	0.629684	11482
38	0	1.87854	7937	**91**	0.0401	2.212557	20140
39	0.0248	2.123431	34390				
40	0.018	1.524188	32592				
41	0.011	2.104476	7281				
42	0.0231	1.939326	7748				
43	0.0152	1.957913	38184				
44	0.016	2.109537	32331				
45	0.007	2.274865	8180				
46	0.0996	2.328738	22260				
48	0.0099	2.178856	7552				
49	0.0114	2.393698	11890				
50	0.0206	2.086208	7694				
51	0.0374	1.331204	19667				
52	0.0181	1.959752	13926				
53	0.0644	2.690778	22992				
54	0.0173	2.33584	57661				

Appendix Table 3; Clinical Dataset for AREST CF Longitudinal Paediatric Cohort BALF samples.

CS ID	L ID		G	Age	Allele 2	Mut2	Symptoms	H	Augmentin
BA+ (>0.015µM)									
90	1	C	2	16	Gly551Asp	III	Yes	140	1
		D		30			Yes	56	1
		F		52			Moist cough	4	1
		G		63			Moist cough	19	1
77	3	B	2	14	Phe508del	II	Frequent cough & occassional sputum	0	1
		C		27			Yes	13	
		E		43					
36	8	C	2	31	Ile336SerfsX28	I	Cough		1
		D		43			Cough		1
		E		55			No		0
		F		66					1
Transitioning									
89	4	A	2	19	Ser341SerfsX29	I	No	31	0
		D		54			Yes	0	0
		E		66			No	0	0
51	5	B	1	14	Phe508del	II	No		0
		C		28			No		0
		D		40			No		0
		F		64					
		G		76			No		0
40	6	B	2	17	Leu259LeufsX1	I	No		1
		D		41			No		0
		E		53			Occasional moist cough		1
		F		62			No		
		G		65					
		H		75			Cough due for admission		1
		I		88					
5	7	B	2	12	Phe508del	II			1
		D		36					
76	12	B	2	13	c.2657+3insA		No	0	1
		C		20			No	0	1
		D		37			No	2	1
		E		49			Frequent cough		0
		F		60			No		0
		G		72			No		0
10	14	D	2	35	Unknown				
		E		47					
6	15	B	1	11	Trp1282x	I			1

		E		47					
41	16	C	1	33	Asp1152His	IV	Nonproductive mild cough		0
		D		45			No		1
		F		56			No		1
		G		67			No		1
BA- (>0.015µM)									
87	2	B	1	13	Phe508del	II	No		0
		C		26			No		1
		G		73			No		1
16	9	D	1	35	Unknown		No		1
		E		47			Slight moist cough		0
		F		58					
14	10	B	2	12	Phe508del	II	No		
		C		24			No		1
		D		38			No		1
		E		51					0
15	11	A	2	16	c.3367G?Cp.gly1123?arg		No		0
		C		30					
50	13	C	2	14	Lys447ArgfsX2	I	No		
		E		39			No		1
		F		51			No		0
		G		62					0
		H		74			No		1
75	17	B	1	15	Arg117His	IV	No	0	0
		C		25			No		0
		D		37			No		1
		E		49			Cough and runny nose	1	1
		F		65			Slight cough		1
9	18	C	1	34	Phe508del	II	No		
		D		49					0
70	19	D	2	51	Unknown		No		1
		E		62			No		0
		F		74			Cough for few days	1	1

CS; Cross sectional, L; Longitudinal, [#] H – Hospital Days, [^] Aug – Augmentin [~] G – Gender (1 – Male, 2 – Female)

[¥] All patients were F508del for Allele 1

Chapter 2

Elucidating the Molecular Mechanism Underpinning Bile Induced Chronic Behaviour in *Pseudomonas aeruginosa*

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Abstract

The ability of *Pseudomonas aeruginosa* to colonise and persist in the Cystic Fibrosis (CF) lung has hindered the successful clinical management of CF. Chronic colonisation by this nosocomial pathogen is a leading cause of morbidity and mortality in the CF population. Therefore, understanding the environmental and host factors which contribute to the pathogens ability to persist are of utmost importance. Gastro-oesophageal reflux (GOR) and subsequent pulmonary aspiration has emerged as a major comorbidity in CF and a range of other respiratory conditions. The finding that bile acids are present in the lungs of paediatric patients, a potential consequence of GOR derived aspiration, and can cause *P. aeruginosa* to adopt a chronic lifestyle, was intriguing. In order to uncover the molecular mechanism through which bile modulates the behaviour of this pathogen, a combination of global transcriptomic and phenotypic analyses was undertaken. Bile responsive pathways responsible for virulence, adaptive metabolism, and redox control were identified, with macrolide and polymyxin antibiotic resistance increased significantly in the presence of bile. *P. aeruginosa* could utilise bile as a sole carbon source, with metabolic rewiring of the cell thought to contribute to the pathogens response to bile. Bile acids, and chenodeoxycholic acid (CDCA) in particular, elicited chronic biofilm behaviour in *P. aeruginosa*. Together, these data suggest that the capacity of *P. aeruginosa* to respond to bile may underpin its emergence as a dominant member of the lung microbiota contributing to the progression of this chronic respiratory disease.

Introduction

The opportunistic gram negative pathogen *Pseudomonas aeruginosa* is the leading cause of morbidity and mortality in the Cystic Fibrosis (CF) cohort, with the airways of up to 70% of adult CF patients being colonised by this organism (1). Recent evidence has implicated a role for bile and bile acids in the progression of CF associated lung disease with reports that bile can modulate the behaviour of *P. aeruginosa* and other clinical pathogens (2-7). Reen and colleagues conducted a panel of phenotypic assays in order to elucidate the impact of physiological concentrations of bile (ranging from 0.003% to 0.3% (w/v) complex bovine bile) in two of the most widely utilised model strains of *P. aeruginosa*; PA14 and PAO1. PAO1, initially isolated from a wound, is moderately virulent in contrast to the PA14 burn wound isolate (8-10). However, the response to bile was found to be conserved in both isolates (7). Reen and colleagues found that *P. aeruginosa* exhibits an increase in biofilm formation and type six secretion in the presence of bile, consistent with the pathogens switch to a chronic lifestyle. Conversely, the repression of acute virulence associated systems such as type three secretion and swarming motility was also observed (7). The ability of *P. aeruginosa* to transition towards this chronic lifestyle is associated with a reduction in the effectiveness of antimicrobial treatment plans. Furthermore, a reduction in the quality of life of affected patients due to progressive loss of lung function also occurs following chronic colonisation, a consequence of increased lung inflammation and lung damage (11, 12). Therefore, the mechanistic understanding of how this key respiratory pathogen emerges as a dominant member of the lung microbial community and adopts a chronic mode of growth in the presence of bile is crucial in facilitating the design of more effective clinical management strategies.

Bile and bile salts have been shown to have antimicrobial properties through its impact on bacterial cell membranes, proteins, DNA integrity and through its iron and calcium chelation properties (13-19). The *in vitro* tolerance of micro-organisms to bile is routinely tested with animal derived bile. Porcine bile is reported to most closely resemble human bile with respect to the relative proportions of bile salts, phospholipids, cholesterol and the ratios of glycine to taurine bile salts. However, its relatively high toxicity has precluded it from being widely used experimentally. Therefore, bovine bile which is known to be less toxic than porcine bile is more routinely used (20-22).

Whilst not much is known regarding the bile tolerance capabilities of respiratory tract organisms, there is a wealth of knowledge regarding the ability of gastrointestinal tract microbes to survive and persist in the presence of bile (Results Table 4). The gram negative bacteria *Salmonella enterica*, *Escherichia coli* and *Vibrio* spp have been shown to display a higher intrinsic resistance to bile when compared to gram positive bacteria (23-26). The lipopolysaccharide (LPS) moiety located in the outer membrane has been shown to be important in this increased resistance to bile with the *tolQRA* operon proposed to aid in the maintenance of membrane integrity (15, 27-29). Multidrug efflux pumps such as the AcrAB (also known as CmeABC) and EmrAB (also known as VceAB) pumps also contribute to bile tolerance and are involved in the removal of bile that permeates the outer membrane (15, 24, 30-32). In addition to the role of efflux pumps, porins present in the outer membrane of *E. coli* and *Vibrio cholerae* have been shown to play a determining role in bile tolerance, with modifications to porin structure influencing the ability of bile salts to penetrate the membrane (24, 33). Additionally, bile salt hydrolases required for the degradation of bile acids have been shown to play a vital role in the bile tolerance of *Listeria, Clostridium, Bacteroides, Lactobacillus, Bifidobacterium* and *Enterococcus* species (34-40).

From a transcriptional perspective, bile acids have been demonstrated to induce both a generalised and oxidative stress response in a variety of organisms including *E. coli* and *Enterococcus faecalis* strains (41, 42).

The mechanisms through which bacteria sense and respond to bile and/or bile acids in the environment is not fully understood. However, it has been proposed to involve classical signal transduction systems including two component systems such as PhoPQ (43) and transcriptional activators such as RpoS and SigB (44, 45). Though *P. aeruginosa* has been shown to contain the PhoPQ two component system, the structural differences present in the sensor kinase domain infer that it may not have the same function in bile signal transduction as in enteric organisms (46). Alternatively, bacterial cells may indirectly transduce the bile response through detection of alterations in the cell membrane. Bile and/or bile acids have been shown to induce a wide range of responses in target organisms. In *Salmonella,* bile and/or bile acids are utilised as an integral environmental signal resulting in the repression of motility, induction of biofilm formation and invasion in to epithelial cells until conditions become more favourable (47-49). Bile salts have also been shown to increase the invasiveness of *Campylobacter jejuni* through an activation of Cia protein activity (50). In *Vibrio parahaemolyticus,* bile acids have been shown to increase virulence factor production and adherence to epithelial cells. Modulation of intracellular calcium concentrations is thought to mediate this response (51, 52). In contrast, bile acids have been shown to decrease virulence factor production in *V. cholerae* whilst inducing biofilm formation, which has been shown to be linked to increased levels of intracellular cyclic-di-GMP (53-55).

This chapter aimed at deciphering the molecular mechanisms controlling the response of *P. aeruginosa* to bile, ultimately resulting in the switch to chronicity. This involved conducting a range of phenotypic assays including screening for the impact of bile on antibiotic resistance,

cyclic di GMP levels and redox status in the cell. In order to gain an insight into the gene systems underpinning the phenotypic response to bile a combination of transcriptome profiling and transposon mutant library screening was conducted. This screening process was of particular importance in identifying genetic elements mediating the bile response with this crucial lifestyle switch known to involve the action of several inter-connected, signal-mediated, regulatory systems. Bile is a complex mixture therefore the organism's response to bile may represent a combinatorial response to the individual components of bile with bile acids central to the induction of biofilm formation. It is hoped that these findings may translate into enhanced clinical control of *P. aeruginosa* infections.

Materials and Methods

Bacterial strains and plasmids

Strains and plasmids used in this study are outlined in Table 1 with primers outlined in Table 2. All cultures of *P. aeruginosa* were routinely grown in Tryptic Soy Broth (TSB) media or Luria-Bertani broth (LB) with shaking at 180 rpm at 37˚C. Strains were maintained on Tryptic Soy Agar (TSA) or Luria-Bertani agar (LBA). For the purpose of antibiotic resistance assays Mueller-Hinton (MH) agar or broth was used. The following antibiotics were added to the growth media where appropriate; 50 µg/mL tetracycline. Bovine bile was selected due to its non-toxic nature with concentrations of 0.3% or 0.03% (w/v) added to media prior to autoclaving at 105˚C for 30 min. Complex bile salts and individual bile salts were prepared in either water or methanol and were added to sterile media after filter sterilising with a 0.2 µ filter to a final concentration of 50 µM or 200 µM.

Table 1; An outline of strains and plasmids utilised in this study.

Strain/plasmid	Description	Reference/source
PAO1	Wild type	Holloway collection
PA14	Wild type	(56)
PA14::*siaD*	PA14 harboring mariner Tn7 transposon insertion in PA14_02110 (*siaD);Gm*^{R}	(56)
PA14::*mexA*	PA14 harboring mariner Tn7 transposon insertion in PA14_05530 (*mexA);Gm*^{R}	(56)
PA14::*mexB*	PA14 harboring mariner Tn7 transposon insertion in PA14_05540 (*mexB);Gm*^{R}	(56)
PA14::68110	PA14 harboring mariner Tn7 transposon insertion in PA14_68110*;Gm*^{R}	(56)
PA14::68120	PA14 harboring mariner Tn7 transposon insertion in PA14_68120*;Gm*^{R}	(56)
PA14::68130	PA14 harboring mariner Tn7 transposon insertion in PA14_68130*;Gm*^{R}	(56)
PA14::68140	PA14 harboring mariner Tn7 transposon insertion in PA14_68130*;Gm*^{R}	(56)

PA14::21210	PA14 harboring mariner Tn7 transposon insertion in PA14_21210;Gm^R	(56)
PA14::*dctB*	PA14 harboring mariner Tn7 transposon insertion in PA14_68230 (*dctB);*Gm^R	(56)
PA14::*dctD*	PA14 harboring mariner Tn7 transposon insertion in PA14_68250 (*dctD);*Gm^R	(56)
PA14::*dctA*	PA14 harboring mariner Tn7 transposon insertion in PA14_49130 (*dctA);*Gm^R	(56)
PA14::*dctP*	PA14 harboring mariner Tn7 transposon insertion in PA14_68260 (*dctP);*Gm^R	(56)
PA14::*dctQ*	PA14 harboring mariner Tn7 transposon insertion in PA14_68280 (*dctQ);*Gm^R	(56)
PA14::*dctM*	PA14 harboring mariner Tn7 transposon insertion in PA14_68290 (*dctM);*Gm^R	(56)
PA14::30840	PA14 harboring mariner Tn7 transposon insertion in PA14_30840;Gm^R	(56)
PA14::*gtrS*	PA14 harboring mariner Tn7 transposon insertion in PA14_22960 (*gtrS);*Gm^R	(56)
PA14::*gltR*	PA14 harboring mariner Tn7 transposon insertion in PA14_22940 (*gltR);*Gm^R	(56)
PA14::*glcB*	PA14 harboring mariner Tn7 transposon insertion in PA14_06290 (*glcB);*Gm^R	(56)
PA14::*aceA*	PA14 harboring mariner Tn7 transposon insertion in PA14_30050 (*aceA);*Gm^R	(56)
TY5021	*P. aeruginosa* clinical isolate from Zonguldak Karaelmas University Hospital	(57)
PA14 pbbr1MCS4	PA14 WT containing the empty pbbr1MCS4 plasmid;Cb^R	This study
PA14::*glcB* pbbr1MCS4	PA14::*glcB* containing the empty pbbr1MCS4 plasmid;Cb^R	This study
PA14::*glcB* complemented	PA14::*glcB* containing the *glcB*-pbbr1MCS4 plasmid;Cb^R	This study
pbbr1MCS4	Empty cloning vector; Ap^R, Cb^R	(58)
pbbr1MCS4-*glcB*	Cloning vector ligated to the *glcB* gene; Ap^R, Cb^r	This study
PA14 pMP220	PA14 WT containing the empty pMP220 plamid; Tc^R	This study
PA14 pMP220-*cdrA*	PA14 WT containing the pMP220-*cdrA* reporter fusion	This study
pMP220	Transcriptional reporter; Tc^R	(59)
pMP220-*cdrA*	*cdrA-lacZ* fusion in pMP220; Tc^R	This study
pMP220-*mexAB*	*MexAB*-lacZ fusion in Pmp220;Tc^r	BRC
prk2013	Helper plasmid; Km^R	BRC
CH3-blue competent cells	*E.coli* strain for transformation	BIOLINE

Table 2; Primers utilised in this study.

Primer Name	Sequence 5'-3'	Reference
Kpn1-*glcB* F	GGggtaccCCGTCCAGAGCTGGTCTAGAGC	This study
BamH1-*glcB R*	CGggatccCGGCAGAACGGTTGGGACAGCA	This study
Kpn1-*cdrA* F	CGGggtaccCCGGATCGGCGCCTTGTTGCTGA	This study
Xba1-*cdrA* R	TGCtctagaGCATGGCTATCCGGACGGACCAT	This study
Taq-proC F	CTTCGAAGCACTGGGTGGAG	This study
Taq-proC R	TTATTGGCCAAGCTGTTCG	This study
Taq-*hmgA* F	CCAACATCGACTTCGTGATCT	This study
Taq-*hmgA* R	GGACGGAAGGTGTTCTCG	This study
Taq-*fahA* F	AACGTCGGCAAGCTGTTC	This study
Taq-*fahA* R	GATGGGCACGTGCTTGTA	This study
Taq-*maiA* F	GACATCCACCCGTTGCAC	This study
Taq-*maiA* R	ACTGCCGGACCTGCTCTT	This study
Taq-*psrA* F	CGTCTGATCACCAGCAAGG	This study
Taq-*psrA* R	CCTTCTTCGAACCGAAGTGA	This study
Taq-*lasI* F	CCGCACATCTGGGAACTC	This study
Taq-*lasI* R	CCAGCGTACAGTCGGAAAAG	This study
Taq-*pqsA* F	CCTCGATTGGAGTGCCTTC	This study
Taq-*pqsA* R	GAACCCGAGGTGTATTGCAG	This study
Taq-*phzS* F	CTGGGCTGGTTCGACATC	This study
Taq-*phzS* R	CGGGTACTGCAGGATCAACT	This study
Taq-*dctA* F	GTTCTTCTCCGTGCTCTTCG	This study
Taq-*dctA* R	AACTCGAACACCGGCTTG	This study
Taq-*dnr* F	GGTGCGCTACCTGCTGAC	This study
Taq-*dnr* R	ATTTCCACCCGGCAGTTC	This study
Taq-*pslB* F	TACTTCCGATGCGCTGCT	This study
Taq-*pslB* R	GTCCTTCAACCGCTGCAC	This study

RNA isolation and transcriptional analysis

Three independent replicates of *P. aeruginosa* strain PAO1 were cultured in TSB in the presence and absence of 0.3% (w/v) bovine bile. Samples were inoculated at an $O.D._{600\ nm}$ 0.025 with shaking at 180 rpm at 37˚C. Cells were harvested for RNA extraction at O.D. 0.8 at which point 500 µL culture was treated with 1 mL of RNA protect Bacteria Reagent (QIAGEN). Total RNA was extracted according to manufacturer's instructions of the RNAeasy kit (QIAGEN) and DNase treated using TURBO DNase (Ambion). Isolated RNA was sent to ATLAS Biolabs

(Germany) for Affymetrix 3' Expression service analysis. The quality and quantity of the RNA was measured by Nanodrop and Agilent Bioanalyser 2100. ATLAS Biolabs undertook biotin-labelling of cRNA, hybridisation, washing and scanning of the Affymetrix GeneChip (*Pseudomonas aeruginosa* Genome Array). The software GeneSpring GX was used for raw data analysis in order to identify changes in gene expression in the presence of bile. A student's paired ttest ($p \leq 0.05$) on robust multiarray average normalised data from the GeneSpring software provided a list of altered gene expression >1.5.

Validation of the microarray was conducted on cDNA derived from extracted RNA. A mix of AMV reverse transcriptase, RNasin (100 U/µL), random primers (0.5 µg/µL) and 10 mM dNTPs (all obtained from Promega) were used to generate cDNA. Real time primers to gene targets of interest were designed using the Universal Probe Library Assay Design Center (Roche). RealTime PCR was conducted on a Chromo4 Continuous Fluorescence Detector (MJ Research) using FastStart TaqMAN Probe Master and universal probes. The housekeeper gene *proC* was used in all instances for measurements of relative gene expression levels.

In order to move from transcriptional changes resulting from secondary affects arising from long term cellular metabolism, RNA was extracted from cells grown to early log phase. Three independent replicates of *P. aeruginosa* strain PA14 were cultured in the presence and absence of 0.3% (w/v) bovine bile. Samples were inoculated at an $O.D._{600\ nm}$ 0.025 with shaking at 180 rpm at 37˚C. Cells were harvested for RNA extraction at O.D. 0.3 with the protocol for RNA extraction and cDNA generation as described above.

Bile transcriptome comparison analysis

A database consisting of all available transcriptomes from *P. aeruginosa* comprising a total of 250 datasets was available in BRC. XLSTAT software was used to generate a Pearson Correlation matrix. (www.xlstat.com). The pheatmap software package (R 3.21) was used to generate an association tree of the relationship of the bile transcriptome to the other transcriptomes in the database, based on the Pearson correlation matrix.

Elastolysis assay

A standard Elastin Congo Red (ECR) assay with modifications was used to measure elastolytic activity. Cells were harvested from mid-log phase PTSB (5% (w/v) peptone, 0.25% (w/v) TSB) cultures were washed and re-suspended in PTSB at an $O.D._{600nm}$ 0.05 in the presence and absence of 0.3% (w/v) bile. Cultures were incubated for 21 h at 37˚C with shaking at which point the supernatant was filtered with a 0.45 µM filter. A 50 µL sample of the filtered supernatant was added to tubes containing 20 mg of ECR and 1 mL of (0.1M Tris [pH 7.2], 1Mm $CaCl_2$] buffer. Tubes were incubated for a further 18 h at 37˚C with rotation and subsequently placed on ice after the addition of 100 µL of 0.12 M EDTA. Centrifugation at 13,000 rpm for 5 min removed insoluble ECR, with the supernatant collected and measurement of the $O.D_{495nm}$ conducted. Absorption due to *P. aeruginosa* pigment production was corrected for by subtracting the $O.D._{495nm}$ of each sample incubated in the absence of ECR.

Pyocyanin assay

Overnight cultures of PA14 WT were adjusted to an OD_{600nm} 0.05 in LB with and without 0.3% (w/v) bovine bile and incubated at 37˚C with shaking at 180 rpm. After 8 h and 16 h, to obtain a cell free supernatant 10 ml of culture was centrifuged at 5000 rpm for 15 min. 3 mL of

chloroform was added to the cell free supernatant, vortexed and centrifuged for 5 min at 5000 rpm. The bottom blue phase was transferred to a tube containing 0.2 M hydrochloric acid, vortexed and centrifuged at 5000 rpm for 5 min. The absorbance of the top pink phase was read at an $OD_{520nm.}$

Congo red binding assay

Polysaccharide production of *P. aeruginosa* was measured by culturing at an O.D. $_{600nm}$ 0.05 in 2 mL of PI medium (20 g peptone, 10 g K_2SO_4, 1.4 G $MgCl_2.6H_2O$, 25 mg triclosan, glycerol 20 mL /L) in the presence and absence of 0.3% (w/v) bile for 48 h at 37˚C without shaking. Polysaccharides were collected by centrifugation and resuspended in 1 mL of 20 mg/mL Congo red suspended in PI media and. Tubes were incubated for 90 min with shaking. Polysaccharides and bound congo red were removed by centrifugation at 13,000 rpm for 5 min where the supernatant was collected and the $O.D._{490nm}$ measured. The total congo red percentage left in the supernatant was measured relative to the un-inoculated media control.

Promoter fusion construction

The region of the PA14 gene *cdrA* beginning at -500 to +20 relative to the translational start site was amplified by PCR with the addition of *Kpn*I and *Xba*I restriction sites. This gene of interest and pMP220 plasmid were double digested with restriction enzymes *Kpn*I and *Xba*I, ligated together and transformed into CH3-blue chemically competent *E. coli* cells (BIOLINE). The successful promoter fusion was transformed into PA14 WT.

Promoter fusion analysis

P. aeruginosa cultures containing the *mexAB*-pMP220 promoter fusion plasmid were grown in TSB supplemented with tetracycline. Cells were adjusted to an $O.D_{600nm}$ 0.05 in 20 ml TSB in

the presence and absence of 0.3% and 0.03% (w/v) bile and incubated at 37˚C with shaking at 150 rpm. A time course assay was conducted with sampling at the logarithmic and stationary phase of growth. At each sampling timepoint, a 1 mL aliquot was taken where the $O.D._{600nm}$ was recorded and 80 µL of permeabilisation buffer was added to 20 µL of culture. A β-galactosidase assay from the *lacZ* promoter fusions was performed according to a modified protocol first described by Miller (Miller JH, 1972). Samples and substrate solution were incubated at 30˚C for 30 min prior to commencement of the assay. 600 µL of substrate solution was added to the samples noting the time it takes for a yellow colour to develop at which point 700 µL of stop solution was added. After centrifugation for 13,000 rpm for 5 min, the $O.D_{420nm}$ is recorded. A *cdrA*-pMP220 promoter fusion was constructed to indirectly assay the levels of cyclic di-GMP in the cell. *P. aeruginosa* cultures containing the *cdrA*-pMP220 promoter fusion were grown either in TSB supplemented with tetracycline. Cells were adjusted to an $O.D_{600nm}$ 0.02 in the presence and absence of bile with a time course assay was conducted for the WT

Miller units calculation: $(O.D_{420nm}$ X 1000)/($O.D_{600nm}$ X 0.02 X Time)

Permeabilisation buffer was made up using 100 mM dibasic sodium phosphate, 20 mM potassium chloride, 2 mM magnesium sulphate, 0.8 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate and 5.4 µL/mL beta-mercaptoethanol. Substrate solution consists of 60 mM dibasic sodium phosphate, 40 mM monobasic sodium phosphate, 1 mg/mL ONPG and 2.7 µL/mL β-mercaptoethanol. Stop solution is solution a 1M sodium carbonate.

Biofilm assays

Overnight cultures were adjusted to an $O.D_{600nm}$ 0.05 in LB/TSB in the presence and absence of bile or bile acids. Aliquots (1 mL or 200 µL) were transferred in to 24-well plates or 96 well

plates respectively and incubated at 37˚C overnight. Biofilm formation was measured by removing culture by pipetting. Wells were washed with water by pipetting to remove any unattached biofilm. Attached biofilm was measured by staining for 30 min with 1 mL/ 200 µL of 0.1 % (w/v) crystal violet. 100% (v/v) ethanol was used to solubilize the crystal violet followed by a measurement of the absorbance at a wavelength of 595nm.

Antibiotic resistance assay

Overnight cultures of *P. aeruginosa* were adjusted to 0.5 MacFarland units in MH broth. MH agar plates supplemented with or without 0.3% or 0.03% (w/v) bile were uniformly swabbed with culture. Antibiotic discs or E-strips (Thermo Scientific) were placed on to the surface of the agar manually or using a disc dispenser. Plates were left to incubate at 37 ˚C overnight after which the zone of inhibition was measured. The following antibiotics were tested by disc assay; amikacin, tetracycline, azithromycin, chloramphenicol, ciprofloxacin, erythromycin, piperacillin-tazobactam and gentamicin with the following were tested by e-strip; polymyxin B, colistin and erythromycin. Time-course growth kinetic assays were performed on a BioScreenC plate reader at 37˚C. Overnight cultures of *P. aeruginosa* were adjusted to $O.D._{600nm}$ 0.05 in MH supplemented with increasing concentrations of polymyxin B or erythromycin in the presence and absence of bile.

Bioinformatic analysis

The KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway database was used for further analysis of the bile transcriptome. The KEGG database (freely available from http://www.genome.ad.jp/kegg/) is a repository of annotated genomic information used for the systematic analysis of gene function. KEGG facilitates pathway analysis via graphical

representations of key cellular processes including central metabolism and signal transduction. Genes altered in the presence of bile were mapped using the KEGG pathway database to identify global changes and pathways of interest.

Redox and cellular respiration analysis

Overnight cultures of *P. aeruginosa* were adjusted to $O.D_{600nm}$ 2.0 with 200 µL of adjusted cultured added to 25 mL of TSB supplemented with 0.01 mg/mL of tetrazolium violet (Sigma Aldrich). Cultures were incubated at 37˚C shaking at 180 rpm for 24 h. Formazan production was measured by centrifuging 5 mL of culture at 5,000 g for 5 min. The supernatant was discarded with the pelleted cells re-suspended in 1.2 mL of dimethyl sulfoxide and centrifuging again at 5,000 g for 5 min. The $O.D_{510nm}$ was recorded of the cell free supernatant.

Growth screen of the PA14 NR transposon mutant library on complex

The complete PA14 non redundant transposon mutant library was screened for the ability to grow on 1X M9 minimal media (10X M9 minimal media; 1L H_2O, 72g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl and 10g NH_4Cl) supplemented with 1.5% (w/v) bile as a sole carbon source with $MgSO_4$ and FeCl provided as cofactors. The QPix2-XT colony picker was used to transfer the library for the growth screen into 96 well plates with plates left to incubate overnight at 37˚C. Following incubation, the $O.D_{600nm}$ of the 96 well plates was recorded. Candidates from the global screen were selected by reading the $O.D_{600nm}$ where anything less than 0.2 was considered to have abolished or a reduction in growth on bile when compared to the WT which reached $O.D_{600nm}$ 0.5. The global screen yielded 638 candidates which were subsequently further tested where a reading less than 0.1 was selected as a candidate. This yielded 390 candidates which were included in one final round of screening with testing on sodium citrate as a positive control.

Complementation of mutants

Transposon mutants from the Harvard collection were complemented in trans by cloning of the coding sequence of the respective genes into the pBBR1MCS-4 plasmid system. Primers used for amplification of each loci are described in Table 2. Following amplification by PCR, samples were loaded on an agarose gel and the appropriate fragment was excised and purified using the QIAGEN Gel Extraction kit (QIAGEN). Restriction sites engineered into the primers facilitated direct cloning in to similarly digested pBBR1MCS-4 plasmid. Ligations were performed overnight and transformation into *E. coli* was subsequently achieved using chemically competent CH3 Blue cells (Bioline). Conjugation into the mutant strains was performed by tri-parental mating using the pRK2013 helper plasmid with selection on M9 media supplemented with citrate for exclusion of donor *E. coli*. As controls, plasmid alone was also transformed into each strain.

Motility assays

Swarming motility was measured on 0.6% (w/v) Eiken agar in the presence and absence of bile/bile salts. Sterile toothpicks were used to inoculate to gently inoculate a single colony onto the surface of the Eiken agar with minimal pressure. Plates were incubated overnight for 1-2 days with degree of motility visualized and recorded.

Alkyl-quinolone extraction

Overnight cultures of *P. aeruginosa* were adjusted to an OD_{600nm} 0.02 in 20 mL TSB with or without 0.3% (w/v) bovine bile and incubated at 37˚C with shaking at 180 rpm for 8 h. Culture was centrifuged at 5000 rpm for 15 min to obtain a cell free supernatant. Alkyl quinolones were extracted by addition of 10 mL of acidified ethyl acetate followed by vortexing and centrifugation for 5 min at 5000 rpm. The top clear phase was transferred to a fresh tube and

stored at -20˚C overnight. Rotary evaporation was completed to remove the solvent with extracts re-suspended in 1 mL of methanol for analysis by thin layer chromatography.

Biofilm screen of the PA14 NR transposon mutant library

The complete PA14 non redundant transposon mutant library encompassing 6000 mutants was screened for biofilm formation in LB supplemented with 0.3% (w/v) bile. The original library consisted of 5800 mutants representing mutations in 4600 genes. During replication of the library empty wells were included as controls to monitor library contamination and a selection of mutants were re-picked due to poor growth. The PA14 non redundant library was created using the Mar2XT7 transposon with a subset of mutants created using the Tn5 based transposon. The QPix2-XT colony picker was used to transfer the library for the biofilm screen. The biofilm assay was processed as described above. Candidates were selected from the initial screen based on an $O.D_{595nm}$ readout of the neat plate between 1 and 2.5 yielding 508 candidates of interest. These 508 candidates underwent three further independent rounds of testing in LB supplemented with 0.3% (w/v) bile where 246 isolates were selected for a final round of screening if the $O.D_{595nm}$ value was between 1 and 2.5 on the neat plate or between 0.2-0.8 in a 1 in 10 dilution. These 246 genes of interest were tested for biofilm formation in both the presence and absence of bile with the final list of targets consisting of strains that exhibited less than a 1.5-fold increase in biofilm formation in the presence of bile. A separate biofilm screen of the PA14 NR library of a subset of 150 sensory and response regulator mutants was conducted to identify potential signal transduction components involved in the biofilm response to bile.

Growth on bile salts

Growth of *P. aeruginosa* PA14 WT on 1X M9 minimal media (10X M9 minimal media; 1L H_2O, 72g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl and 10g NH_4Cl) supplemented with a range of complex bile salts concentrations (ranging from 1 μM-5 mM) as a sole carbon source with $MgSO_4$ and FeCl provided as cofactors was tested as follows. An overnight culture was centrifuged at 3000 rpm for 10 min, the supernatant was removed, and cells were re-suspended in 1 mL of PBS. To ensure all spent media was removed, cells were re-centrifuged at 3000 rpm for 10 min with the supernatant removed and cells re-suspended in 1 mL of PBS. A starting O.D. of 0.025 was added to 20 mL of M9 minimal media with a range of bile salt concentrations added. A visual inspection of conical flasks was carried out after 24 h of growth.

Statistical Analysis

Data presented is the average of at least three independent biological replicates. Statistical analysis was performed by student's t-test (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$).

Results

Transcriptional and phenotypic analysis of *P. aeruginosa* response to bile

Determining the global transcriptional response of Pseudomonas aeruginosa to physiological concentrations of bile

In order to elucidate the molecular mechanism underpinning the phenotypic response of *P. aeruginosa* to bile, transcriptome profiling was conducted in the presence and absence of 0.3% (w/v) bile. This revealed a bile responsive gene expression signature in which 367 genes were altered, 120 of which exhibited increased gene expression and 247 of which displayed decreased gene expression when compared to the untreated control (fold changes ranged from 41.0 to -59.4-fold). Genes which were greater than 5-fold up/down regulated are outlined in Table 4 with the complete list of altered gene expression outlined in Appendix Table 1.

Table 3; Most highly expressed and repressed genes in the presence of bile categorised by gene function according to COG analysis.

Upregulated genes in the presence of bile

Gene	Name	Fold Change	Gene Description
Energy production and conversion			
PA0195	*pntA*	5.698029	putative NAD(P) transhydrogenase, subunit alpha part 1
PA0196	*pntB*	6.072907	pyridine nucleotide transhydrogenase, beta subunit
PA0521		5.02969	probable cytochrome c oxidase subunit
PA1183	*dctA*	5.316506	C4-dicarboxylate transport protein
PA2634	*aceA*	8.60934	isocitrate lyase
PA2953		5.112918	electron transport flavoprotein-ubiquinone oxidoreductase
PA3092	*fadH1*	10.94138	2,4-dienoyl-coA reductase
PA4770	*lldP*	6.846915	L-lactate permease
PA4771	*lldD*	6.366787	L-lactate dehydrogenase
Lipid transport and metabolism			
PA0506		12.92649	probable acyl-CoA dehydrogenase
PA0508		41.03524	probable acyl-CoA dehydrogenase
PA1748		5.433264	probable enoyl-CoA hydratase/isomerase
PA3013	*foaB*	8.261979	fatty-acid oxidation complex beta subunit

PA3014	*faoA*	8.045702	fatty-acid oxidation complex alpha subunit
PA3925		6.362694	probable acyl-coA thiolase
Inorganic ion transport and metabolism			
PA0524	*norB*	11.78624	nitric-oxide reductase subunit B
PA1051		6.853995	probable transporter
PA2662		6.388695	conserved hypothetical protein
Post translational modification, protein turnover and chaperones			
PA1596	*htpG*	5.018658	heat shock protein
Unknown			
PA2663	*ppyR*	8.42526	*psl* and pyoverdine operon regulator

Downregulated genes in the presence of bile

Gene	Name	Fold Change	Gene Description
Energy production and conversion			
PA1984	*exaC*	59.43127	NAD+ dependent aldehyde dehydrogenase
PA2247	*bkdA1*	11.76501	2-oxoisovalerate dehydrogenase alpha subunit
PA2248	*bkdA2*	8.665485	2-oxoisovalerate dehydrogenase beta subunit
PA2249	*bkdB*	8.010507	branched chain alpha keto acid dehydrogenase lipoamide
PA2250	*lpdV*	10.54123	lipoamide dehydrogenase-Val
Lipid transport and metabolism			
PA0493		12.16488	probable biotin-requiring enzyme
PA0494		12.53772	probable acyl-CoA carboxylase subunit
PA0887	*acsA*	8.322072	acteyl-coenzyme A synthetase
PA1999	*dhcA*	31.30977	dehydrocarnitine coA transferase subunit A
PA2013	*liuC*	12.64843	putative 3-methylglutaconyl-CoA hydratase
PA2014	*liuB*	14.03458	methylcrotonyl-CoA carboxylase, beta subunit
PA2015	*liuA*	10.92419	putative isovaleryl-CoAdehydrogenase
PA2552		10.12445	probable acyl-CoA dehydrogenase
PA2553		17.72097	probable acyl-CoA thiolase
PA2555		14.54166	probable AMP-binding enzyme
PA5174		10.50413	probable beta-ketoacyl synthase
Carbohydrate transport and metabolism			
PA2114		12.58743	probable major facilitator superfamily transporter
PA3187		37.64074	probable ATP binding component of ABC transporter
PA3190		37.49556	probable binding protein component of ABC transporter
PA3195	*gapA*	11.15306	glyceraldehyde 3-phosphate dehydrogenase
Amino acid transport and metabolism			
PA0129	*bauD*	7.690926	amino acid permease
PA0495		14.14879	hypothetical protein
PA0865	*hpd*	9.271919	4-hydroxyphenylpyruvate dioxygenase
PA1565	*pauB2*	8.70774	FAD-dependent oxidoreductase

PA2110		9.982603	hypothetical protein
PA2111		11.69932	hypothetical protein
PA4024	*eutB*	9.765597	ethanolamine ammonia-lyase large subunit
PA4502		5.121125	probable binding protein component of ABC transporter
Secondary metabolites biosynthesis, transport and catabolism			
PA2008	*fahA*	34.14261	fumarylacetoacetase
PA2009	*hmgA*	54.90419	homogentisate 1,2-dioxygenase
Cell wall/membrane/envelope biogenesis			
PA2113	*opdO*	15.40829	pyroglutamate porin
PA3186	*oprB*	5.547807	glucose/carbohydrate outer membrane protein precursor
Coenzyme transport and metabolism			
PA1905	*phzG2*	5.702814	probable pyridoxamine 5'-phosphate oxidase
PA2112		10.56511	conserved hypothetical protein
PA4217	*phzS*	5.672542	flavin-containing monooxygenase
Transcription			
PA2016	*liuR*	7.376461	regulator of *liu* genes
PA5380	*gbdR*	9.957009	putative amidotransferase
Nucleotide transport and metabolism			
PA3516		8.26889	probable lyase
Post translational modification, protein turnover and chaperones			
PA2069		5.337103	probable carbamoyl transferase
Unknown			
PA0099		5.363333	hypothetical protein
PA0131	*bauB*	5.099461	beta-alanine biosynthetic protein
PA0132	*bauA*	9.548833	beta-alanine:pyruvate transaminase
PA0492		9.769155	conserved hypothetical protein
PA0730		20.26402	probable transferase
PA0852	*cbpD*	5.277362	chitin-binding protein
PA2109		13.239	hypothetical protein
PA2358		7.733476	hypothetical protein
PA2554		7.59388	probable short chain dehydrogenase
PA3234		5.911297	probable sodium:solute symporter
PA4211	*phzB1*	8.585621	probable phenazine biosynthesis protein

In order to further investigate the significance of this bile gene expression profile, genes were characterized based on functionality according to gene ontology and KEGG pathway analysis **(Fig. 1)**. This highlighted an induction of genes involved in energy production and conversion (*PA0195*, *PA0196*, *PA2634*) whilst amino acid metabolism (*PA0495*, *PA2111*, *PA0865*), intracellular

trafficking and secretion associated genes were repressed in the presence of bile (*PA0078-PA0096*). Consistent with a switch towards a chronic lifestyle transcriptional changes were evident in genes associated with virulence traits, biofilm production and antibiotic resistance.

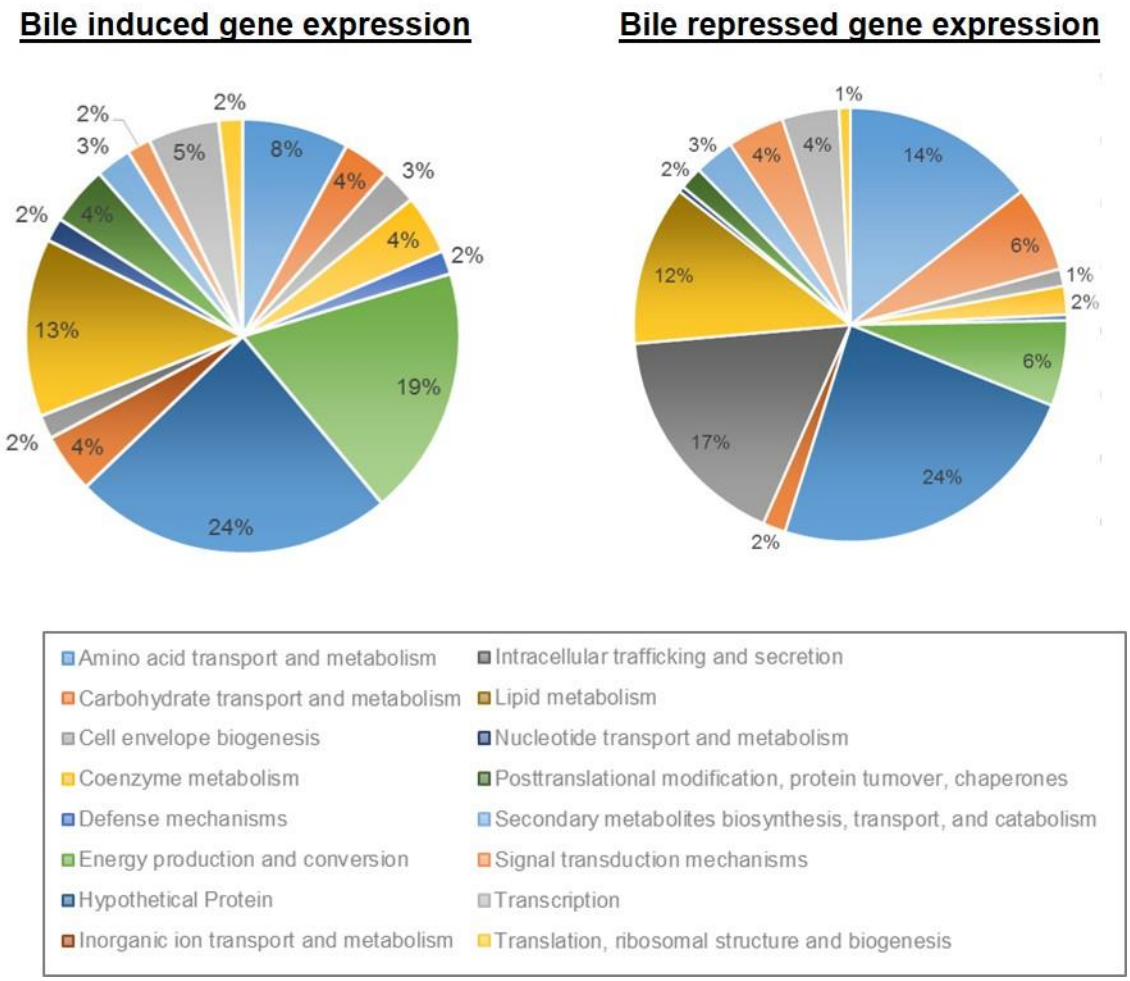

Figure 1. Categorization of the bile transcriptome according to the functionality of genes with altered expression in the presence of bile according to gene ontology. Genes associated with energy production and conversion are induced in the presence of bile while genes associated with intracellular trafficking and secretion are repressed in the presence of bile.

A unique bile specific transcriptional signature exists

In order to assess whether the bile transcriptome displays a unique gene expression response and to more comprehensively elucidate the transcriptional mechanism through which bile elicits its response, the bile transcriptome was compared to a database consisting of 250 publicly available *P. aeruginosa* transcriptomes available in the BIOMERIT Research Centre. Analysis of the database using a Pearson correlation matrix heatmap facilitated the measurement of relatedness of the bile transcriptome to these other transcriptomes (**Fig. 2A**). The bile transcriptome was found to be most similar to a PA14 vs *phhR* mutant (Pearson score; 0.282). The phenylalanine (PhhR) catabolic regulon including *phhC, hpd, hmgA, maiA* and *fahA* were found to be repressed in the presence of bile and validated by RT-PCR **(Fig. 2B)**. The bile transcriptome was also found to be similar a *gtrS* mutant vs PAO1 (0.143). The GtrS-GltR two component system is involved in the regulation of glucose metabolism and the type three secretion system, both of which are altered in the bile transcriptome. The bile transcriptome was inversely related to aromatic amino acids vs serine in synthetic CF sputum media (-0.203) and the *pycR* mutant vs PAO1 (-0.135). PycR is a LysR transcriptional regulator which has been demonstrated to be essential for *P. aeruginosa* chronic infection.

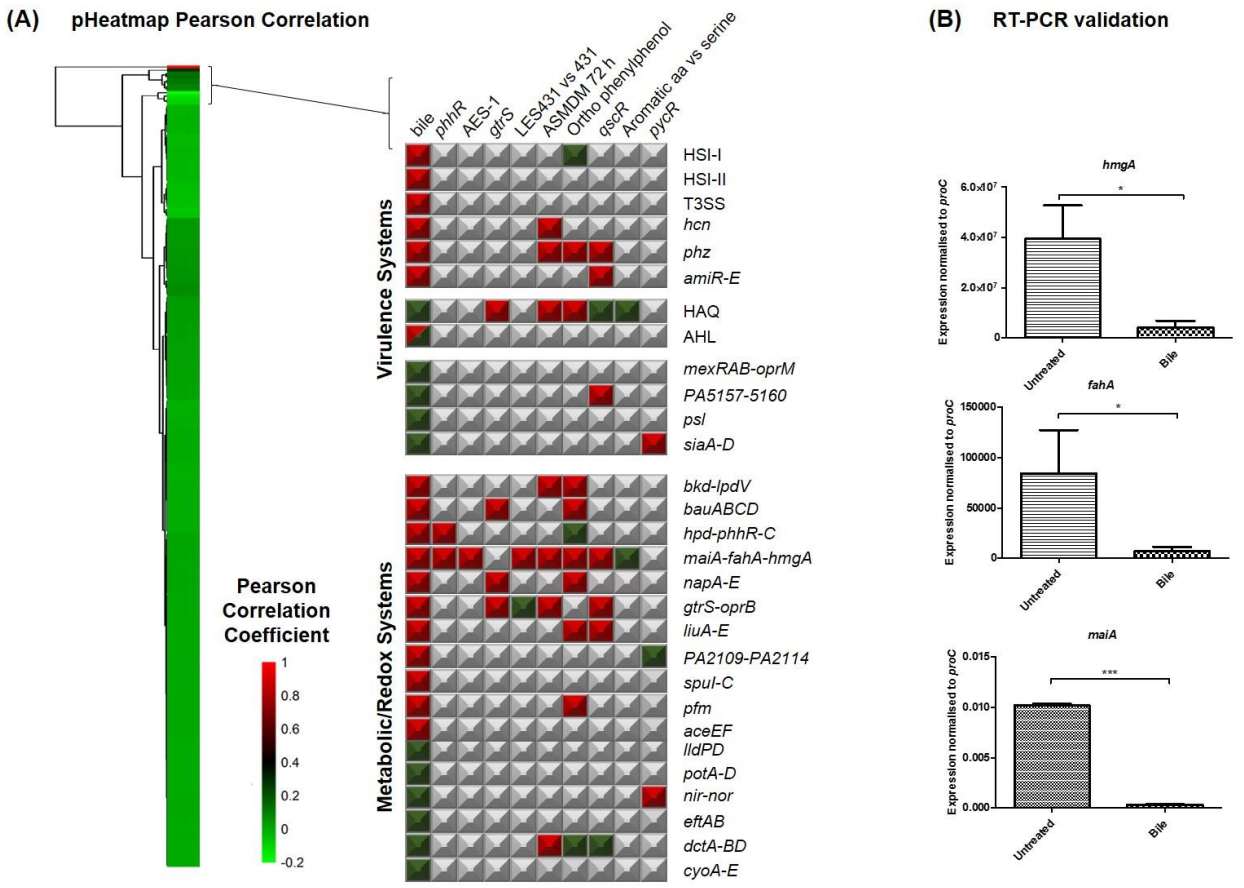

Figure 2. (A) Pearson correlation heatmap matrix demonstrating a unique bile specific response distinct to that of the most closely clustered transcriptomes. (B) RT-PCR validation of selected genes from the PhhR catabolic regulon; *hmgA*, *fahA*, *maiA* confirm the findings of the global bile transcriptome.

Importantly, comparison of the bile transcriptome to the transcriptomes it clustered closely with revealed that the gene expression profile displayed in the presence of bile was distinct to that of similar transcriptomes, confirming that the response to bile is not simply due to a generalized stress response. Though there is crossover in the global gene expression profiles of these transcriptomes, the bile specific signature encompasses changes in quorum sensing, secretion systems, biofilm formation, antibiotic resistance, and redox control. Within the transcriptomes that clustered most closely with bile the combination of genes associated with acute virulence

(T3SS, *hcn*, *phz*) and the chronic lifestyle (*psl*, *mexAB-oprM* and *siaA-D)* which were altered in the bile transcriptome were not affected in the same manner in any of the clustered transcriptome. The global gene expression profile elicited by exposure to bile is consistent with the phenotypic switch to a chronic lifestyle previously reported (7).

Quorum Sensing

Quorum sensing is a complex, hierarchical, cell density regulated cell-cell signalling network controlling virulence and biofilm formation in *P. aeruginosa.* In addition to the classical regulation of QS by increased population cell density, recent evidence has shown environmental signals contribute to QS control. Upon reaching a threshold cell density there is a release of chemical signals resulting in the induction of an array of genes. Three QS systems are well described in *P. aeruginosa;* the AHL based systems Las and Rhl and the *P. aeruginosa* AQ system PQS, whilst an emerging role for IQS has been described. It has been demonstrated by promoter fusion (*lasR, rhlR and pqsA)* that bile increases the expression of these three QS systems. The transcriptional changes observed in the presence of bile are consistent with these findings indicating an earlier activation of QS with a 1.82 fold induction of *lasI* and a 1.61 fold induction of *pqsA* **(Fig. 3A)**. LasI and PqsA are involved in synthesis of the autoinducer proteins required for activation of their respective QS systems with the latter being the first gene of a five gene alkylquinolone biosynthetic operon *pqsA-E*. Interestingly, elevated levels of the LasI signal 3-oxo-C12-HSL in the cell is associated with biofilm maturation and differentiation (60).

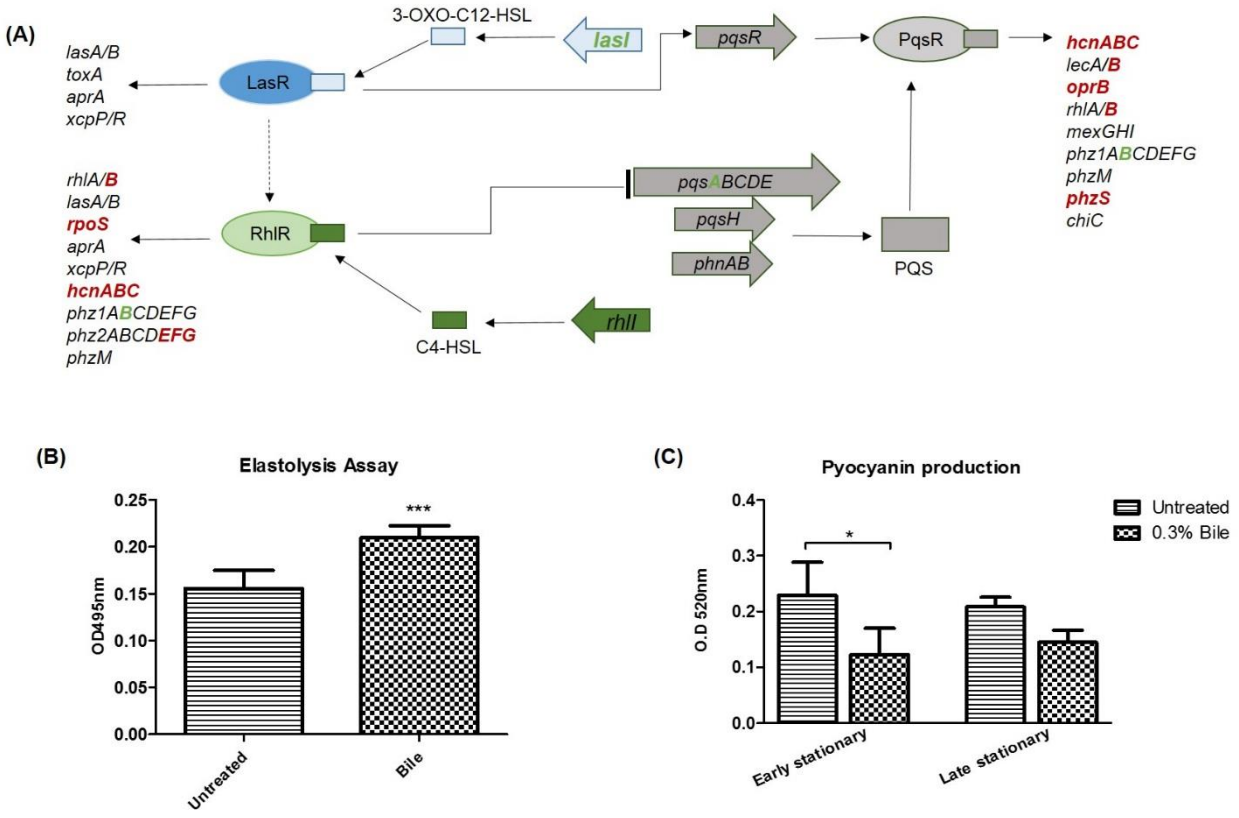

Figure 3. (A) Impact of bile on the QS circuitry in *P. aeruginosa* in the presence of bile. Upregulated genes are highlighted in green, downregulated genes are highlighted in red and unaltered genes are highlighted in black. (B) Increased elastolytic activity in bile treated samples as evidenced by increased levels of Congo red present in bile treated culture supernatant (C) Downregulation of pyocyanin production in LB supplemented with bile in early stationary phase with no significant difference in late stationary phase.

However, in silico transcriptome analysis of downstream QS target genes revealed that in some cases rather than the classical activation of genes, expression was unaltered or repressed. This could be explained by the involvement of genes in multiple pathways which are often under the control of multiple regulatory networks. The impact of bile on the expression of these QS systems and downstream effectors may be more pronounced in later stationary phase. An elastolysis assay was conducted and revealed a significant increase in elastase activity in the presence of bile **(Fig. 3B)**. This suggests an induction of the *lasA/lasB* genes not evident in the transcriptome and

supports the hypothesis of induction of downstream effectors as the cell enters stationary phase. Pyocyanin production was reduced in early stationary phase with no significant difference in late stationary phase in the presence of bile which is consistent with the repression in expression of the *phzS* gene, the final enzyme of the pyocyanin biosynthetic pathway **(Fig. 3C).**

Type Three and Type Six Secretion Systems

The Type three secretion system (TS33) and the type six secretion system (T6SS) are a complex, highly regulated apparatus utilized by *P. aeruginosa* to inject effector proteins into both host cells and neighbouring bacteria. Using a needle-like machinery similar to bacteriophage *Pseudomonas* can manipulate cells with which it interacts with. The T3SS is encoded by 36 genes located on 5 operons with at least 6 additional genes encoding effectors and chaperone proteins (61). There is transcriptional repression of both the translocation apparatus, effectors and regulatory proteins in the presence of bile **(Fig. 4)**. This is in line with chronic isolates of *P. aeruginosa* from CF patients who gradually lose the ability to secrete type three effector proteins. Hence whilst this system is important for acute infection, it is less so in the persistence of *P. aeruginosa* throughout infection. However, there has been a growing appreciation for the role that T3SS may play in infection as it has been reported that T3SS is induced under microaerobic conditions. These conditions would be more reflective of the CF lung environment. In contrast, the T6SS which is responsible for the delivery of toxins to other competing organisms is found to remain functional in chronic isolates. T6SS are encoded by three distinct gene clusters; Hcp1 secretion island (HSI)-I, HSI-II and HSI-III. Whilst transcript levels of HSI-I and HSI-II were found to be down regulated in the presence of bile, a previous study reported up-regulation of the tssA1 promoter (HSI-I system) **(Fig. 4)**. The difference between these findings are potentially due to the transcriptome being conducted at an earlier time point whilst

the promoter fusion assay was conducted using stationary phase cells which are potentially more reflective of *P. aeruginosa* chronic infection.

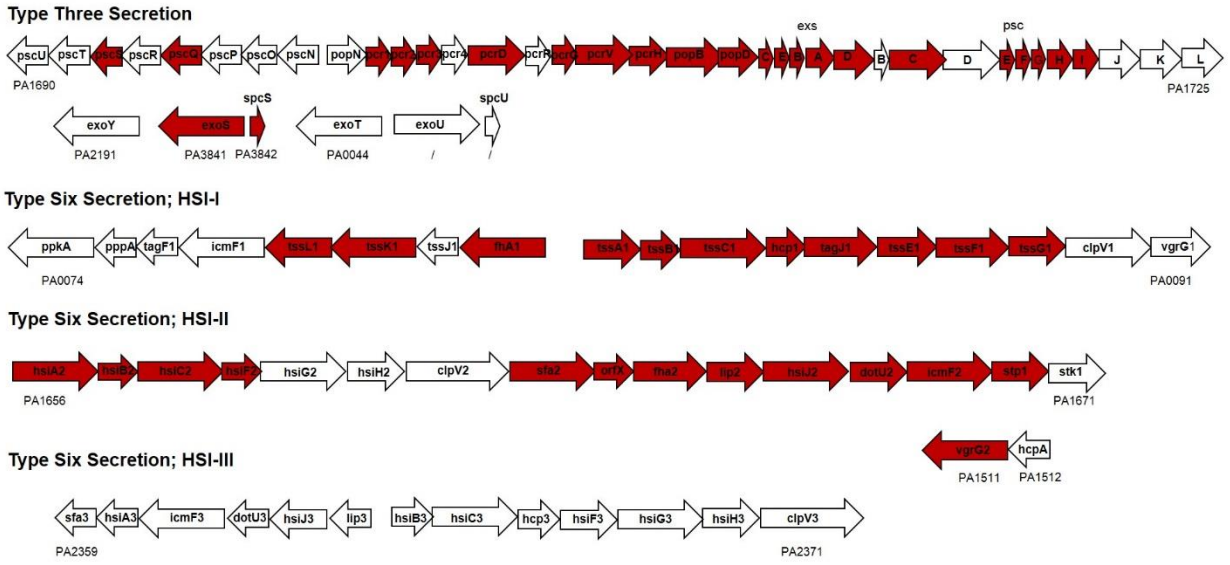

Figure 4. Transcriptome analysis of T3SS highlighting a significant transcriptional down-regulation of genes within the operon. Transcriptional analysis of the T6SS operons shows bile causes a significant transcriptional down-regulation of the HSI-I and HSI-II operons with no impact observed on the HIS-III operon.

Biofilm Formation

P. aeruginosa is known to exist in a biofilm; a multicellular community protected by a matrix of extracellular polymeric substances, in the lungs of CF patients. In *P. aeruginosa* there are three primary polysaccharides contributing to the formation of a biofilm; alginate, Pel and Psl. In addition to these polysaccharides, extracellular DNA, flagella, type IV pili and fimbriae have also been shown to be important components of the biofilm matrix. The primary polysaccharide utilized by PAO1 is Psl. However, PA14 has been shown to lack a complete *psl* locus and hence primarily utilizes Pel (62). The induction of biofilm formation in *P. aeruginosa* in the presence of bile was found to be comparable in both lab strains. Analysis of the transcriptome revealed that while the

pel and alginate genes were unaltered, the *psl* operon (A,B,E,G) was upregulated. Psl has been shown to be a major contributor to biofilm formation with a crucial role in the initiation of biofilm development and the maturation into a mature biofilm. Hence, the up-regulation of this system in PAO1 in the presence of bile is significant. The increased expression of Psl was supported by an increase in polysaccharide production in the presence of bile as measured by a Congo red binding assay. The Congo red binding assay is an inverse assay hence the lower the Congo red measurement indicates increased polysaccharide production **(Fig. 5A)**. Furthermore, the transcriptional regulator PsrA which is linked to the positive regulation of expression of the *psl* operon and repression of T3SS was also found to be upregulated. This was confirmed by RT-PCR analysis **(Fig. 5B)**.

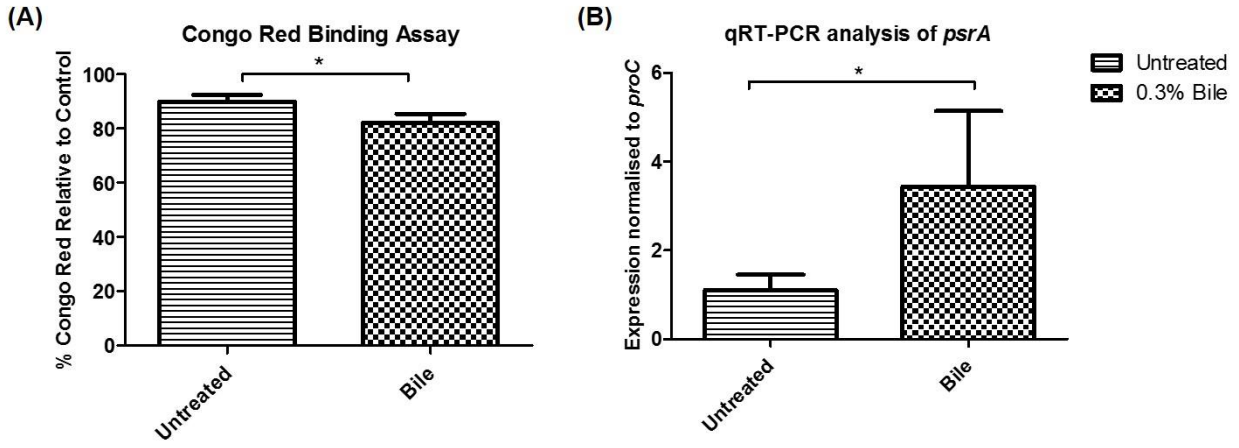

Figure 5. (A) Increased polysaccharide production in the presence of bile as measured by the inverse Congo red binding assay hence polysaccharide production is increased in the presence of bile. (B) Confirmation of induction of the *psrA* gene by RT-PCR analysis.

The intracellular accumulation of the second messenger c-di-GMP has been associated with the switch towards a biofilm lifestyle. The levels of c-di-GMP in the cell is determined by the activity of diguanylate cyclases which synthesise c-di-GMP and phosphodiesterases which break down the

molecule. In *P. aeruginosa* 41 proteins involved in c-di-GMP synthesis/degradation have been identified, with the GGDEF domain containing diguanylate cyclase *siaD* (PA0169) found to be upregulated in the presence of bile. The localised pools of c-di-GMP in the cell control the reciprocal regulation of bacterial motility and biofilm formation (63). This led to investigations of the impact of bile on c-di-GMP levels in the cell. A *cdrA*-pMP220 promoter fusion was constructed and conjugated into PA14 WT allowing for the indirect measurement of cyclic di-GMP levels in the cell. This revealed increased levels of cyclic di GMP in the presence of bile at all time-points tested **(Fig. 6A).** This increase is consistent with the findings that bile induces biofilm production. Furthermore, this increase in biofilm formation is abolished in the transposon mutant diguanlyate cyclase *siaD* indicating the importance of cyclic di-GMP in the biofilm response to bile **(Fig. 6B)**

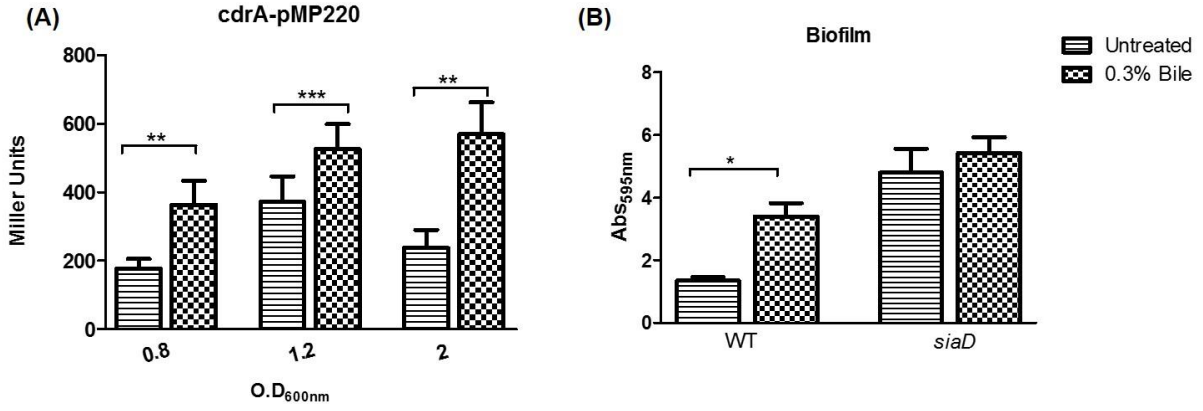

Figure 6. (A) Increased cyclic di GMP levels in the presence of bile as measured by *cdrA*-pMP220 promoter fusion at three timepoints; log, early stationary and late stationary (B) Crystal violet attachment assay showing loss of the biofilm response to bile in the diguanylate cyclase *siaD* mutant.

Antibiotic resistance

The ability of organisms to produce a biofilm is associated with an increase in both tolerance and resistance to antibiotics further impeding the clinical management of bacterial infections. In addition to bile induced biofilm formation, there was a transcriptional induction of several multi-drug resistance systems in the presence of bile. This included increased expression of the *mexAB-oprM* system as well as *mexR* the transcriptional repressor of this system. However, repression by *mexR* may be overcome through oxidation. MexAB-oprM has been shown to confer resistance to a range of antibiotics including quinolones, macrolides, tetracycline, chloramphenicol, novobiocin and the majority of β-lactams. Increased expression of *PA3310,* a gene recently proposed to play a role in polymyxin resistance and *PA5157-5159* encoding components homologous to the EmrAB efflux system in *E. coli* were also observed with the latter shown to confer resistance to nalidixic acid. Therefore, the impact of bile on antibiotic resistance of *P. aeruginosa* to a panel of clinically relevant antibiotics was investigated. Antibiotic susceptibility was measured using a combination of antibiotic discs and E-strips. Disc assay analysis revealed increased resistance to chloramphenicol and the macrolides azithromycin and erythromycin in the presence of bile **(Fig. 7)**. Erythromycin and azithromycin are members of the macrolide class of antibiotics which target bacterial translation. Azithromycin in particular is routinely used in the treatment of CF as in addition to its antimicrobial properties, it has also been found to exhibit anti-inflammatory properties. The increased resistance observed was found to be independent of the *PA5157-5160* systems **(Fig. 7)**. However, in the clinical isolate TY5021 overexpressing the MexAB pump, no further increase in macrolide resistance (azithromycin and erythromycin) was observed in the presence of bile. The TY5021 strain was resistance to the same degree as the bile induced wild-type strain for azithromycin and completely resistant to

erythromycin. This suggests that activation of MexAB may potentially contribute to bile induced macrolide resistance. Upon testing chloramphenicol the mutants derived from the PA14 NR library were found to be completely resistant irrespective of bile treatment and so this antibiotic was not investigated any further.

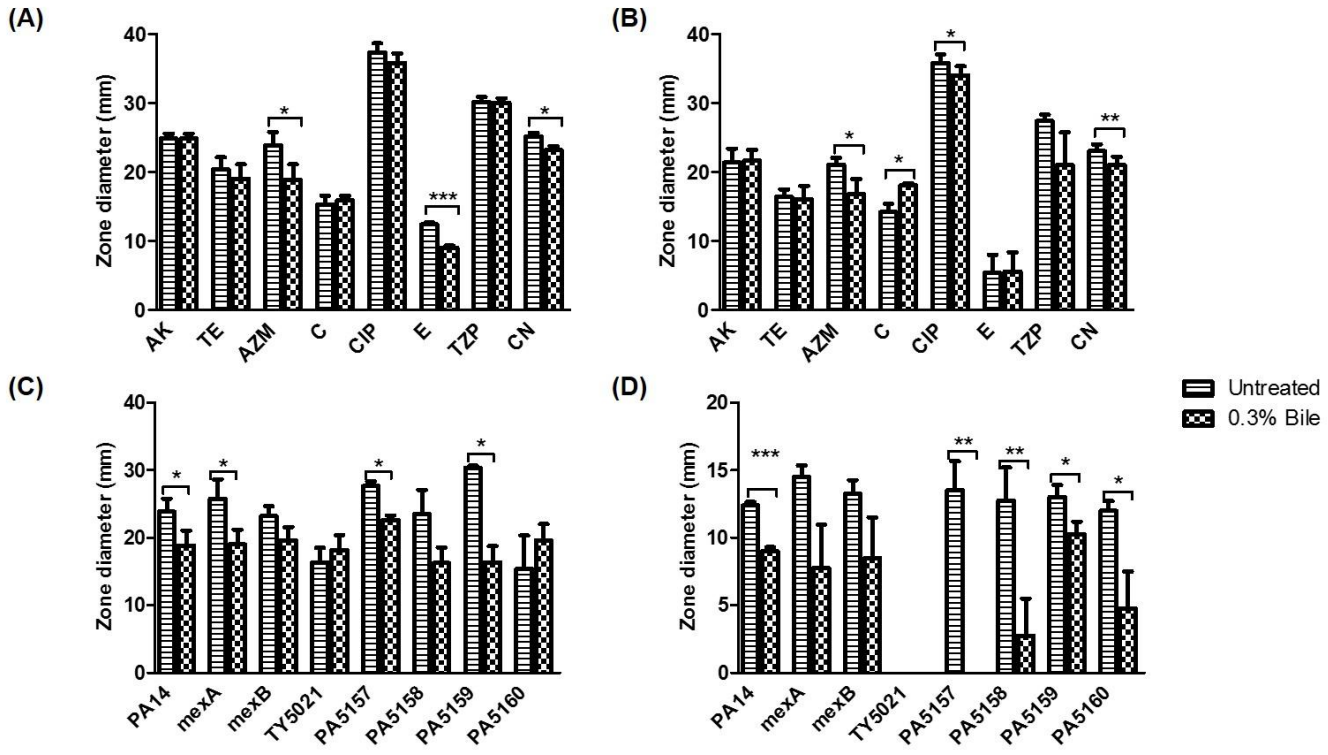

Figure 7. Antibiotic susceptibility disc assay in (A) PA14 WT and (B) PA01 WT for the following antibiotics amikacin 30 (AK), tetracycline 30 (TE), azithromycin 15 (AZM), chloramphenicol 30 (C), ciprofloxacin 5 (CIP), erythromycin 15 (E), piperacillin/tazobactam 110 (TZP) and gentamicin 10 (CN). Increased azithromycin resistance (C) and erythromycin resistance (D) occur independently of the MexAB and PA5157-5160 efflux pumps.

E-strip analysis confirmed increased resistance to erythromycin, as well as an increase in resistance to the polymyxin antibiotics colistin (also known as polymyxin E) and polymyxin B **(Fig. 8)**. The antibiotics vancomycin and oxacillin were also tested but PA14 was found to be completely resistant irrespective of bile treatment (data not shown) and so no further testing was carried out.

The polymyxin class of antibiotics target the permeability of cell membranes and are considered a last resort antibiotic for the therapeutic control of *P. aeruginosa* due to the high level of toxicity. Resistance to polymyxins has been shown to derive from modification of the lipid A group of LPS with a potential role for *PA3310* (*PA14_21210*) in this process. Bile induced resistance, however, was shown to be independent of this gene **(Fig. 8C)**.

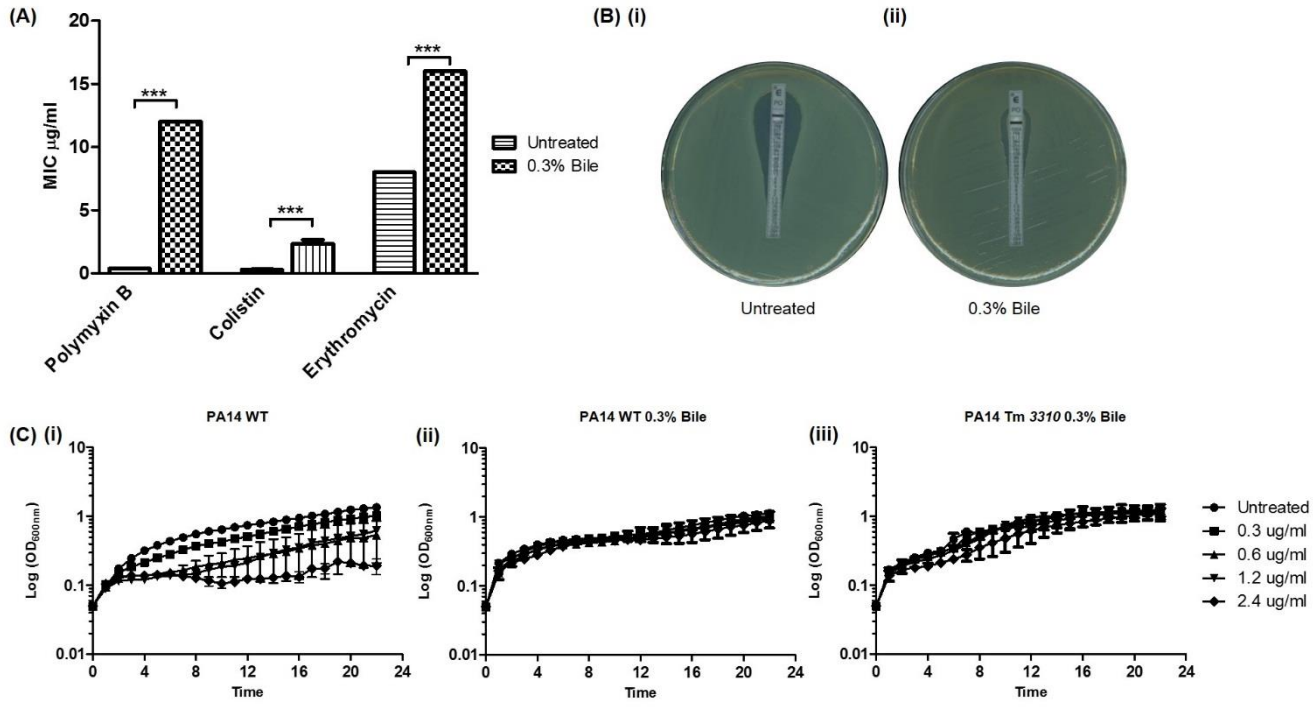

Figure 8. (A) E-strip antibiotic resistance assay highlighting increased resistance to polymyxin B, colistin and erythromycin in the presence of bile. *P. aeruginosa* was completely resistant to the antibiotics vancomycin and oxacillin in both the presence and absence of bile. (B) Representative plates of the polymyxin B e-strip assay (i) untreated and (ii) bile treated. (C) Growth kinetic assays in (i) PA14 WT in MH broth (ii) PA14 WT in MH broth supplemented with 0.3% (w/v) bile and (iii) A *PA14 Tm 3310* all in concentrations of polymyxin ranging from 0.3 µg/ml to 2.4 µg/ml. Bile increases the resistance of PA14 WT to polymyxin in a manner independent of the *PA3310* gene.

Central metabolism

There is an ever-growing appreciation of the link between central metabolism to virulence in pathogens (64). With this in mind and to gain a further understanding of the molecular mechanism potentially underpinning the phenotypic response to bile, detailed KEGG pathway analysis of central metabolism and pathways feeding into central metabolism was undertaken **(Fig. 9)**. This highlighted a transcriptional induction of the early stages of the citric acid cycle also known as the tricarboxylic acid cycle (TCA) and a branch point within the TCA known as the glyoxylate shunt (*acnB, aceA* and *glcB)* which is known to facilitate utilization of fatty acids and acetate. Further supporting a role for the glyoxylate shunt in the bile response was the repression of genes in the later stage of the TCA cycle (*idh)* and though *fumC2* expression was not altered the subsequent gene in the operon was down regulated. Additional repression at the C4-dicarboxylate branch points of the TCA cycle including succinate, fumarate and malate were also observed. This finding is consistent with recent reports from in situ transcriptomics and metabolic flux analysis of *P. aeruginosa* in the CF lung (65, 66).

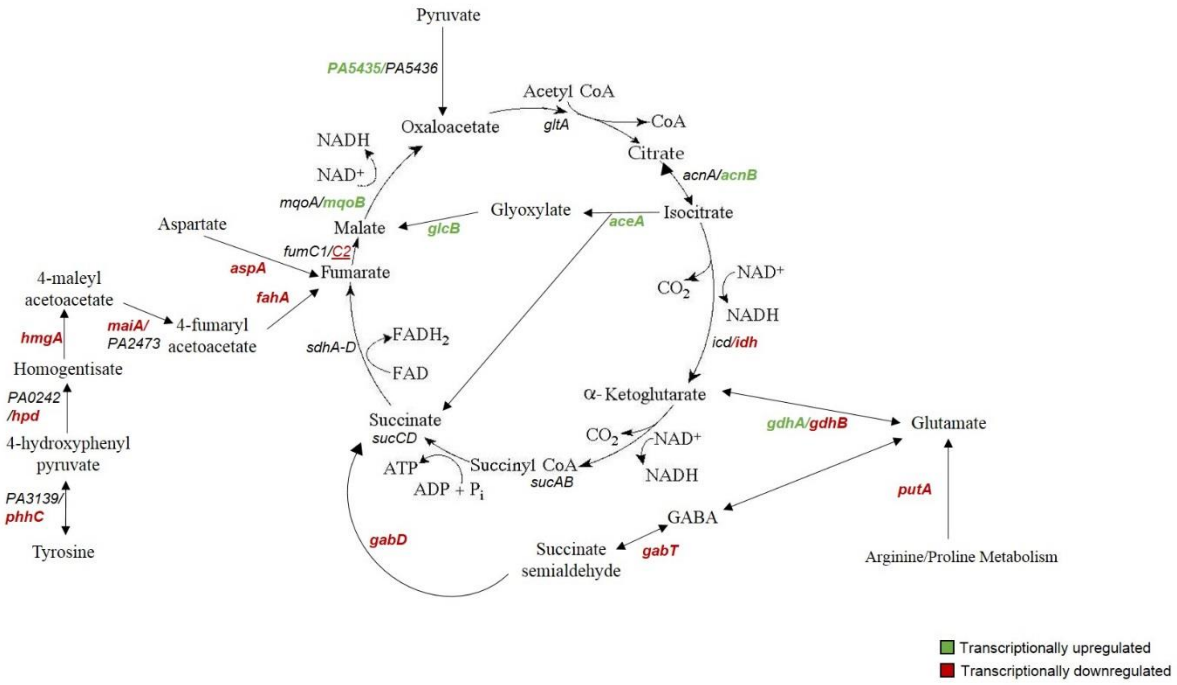

Figure 9. KEGG pathway analysis of the TCA cycle with genes found to be upregulated in bile denoted in green and genes found to be downregulated in bile denoted in red. Mapping identified a transcriptional upregulation of the glyoxylate pathway with a transcriptional repression of the later stages of the TCA responsible for the generation of the electron carrier NADH.

Upon establishing the role of the TCA cycle, the role of three other central carbon pathways (the Emden-Meyerhof pathway also known as glycolysis, the pentose phosphate pathway and the Entner-Doudoroff pathway) were investigated by mapping to KEGG **(Fig. 10)**. This analysis showed repression of genes involved in glucose metabolism of the Entner-doudoroff pathway (*glk, zwf, pgl, edd)* and an upregulation of genes involved in the pentose phosphate pathway (*rpe, tktA)*. An integrated, global figure of the impact of bile on all the pathways relating to central carbon metabolism is in the Appendix Fig. 1.

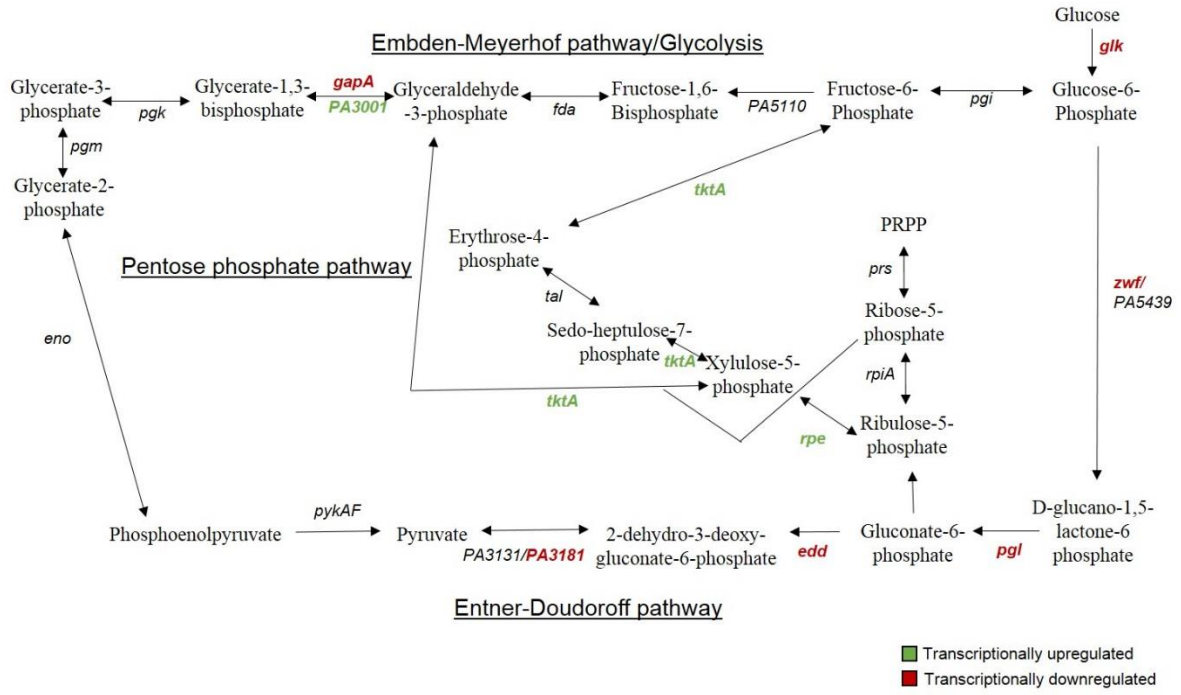

Figure 10. KEGG pathway analysis of the central carbon pathways; Embden-meyerhof/glycolysis pathway, pentose phosphate pathway and the Entner-Doudoroff pathway reveals altered carbon flux away from the Entner-Doudoroff pathway and through the pentose phosphate pathway.

The observed transcriptional changes indicating a rewiring/reorganisation of central metabolism could be expected to manifest and have consequences on the redox potential within the cell. In order to test this hypothesis a tetrazolium violet reduction assay was conducted. This assay measures the reduction of tetrazolium violet to a colored formazan and hence is an indirect measurement of the NAD^+/NADH redox modulating couple in the cell. Redox carriers including NAD^+/NADH form the basis of cellular metabolism with redox homeostasis essential to the survival of the cell. A significant suppression of cellular respiration was observed in the presence of both 0.3% and 0.03% (w/v) bile suggesting an increased amount of NAD^+ in the cell in the presence of bile **(Fig. 11A)**. These redox changes were further supported by the morphological changes observed in *P. aeruginosa* colonies grown on TSA supplemented with bile **(Fig. 11B)**. The wrinkly formation of bile treated colonies is characteristic of an altered redox state. This is

not evident on untreated plates highlighting that bile may elicit its effect on *P. aeruginosa* through redox. The alteration of numerous systems relating to cellular metabolism, as well as changes in redox potential in the cell suggests that the response of *P. aeruginosa* to bile may potentially involve a form of adaptive metabolism.

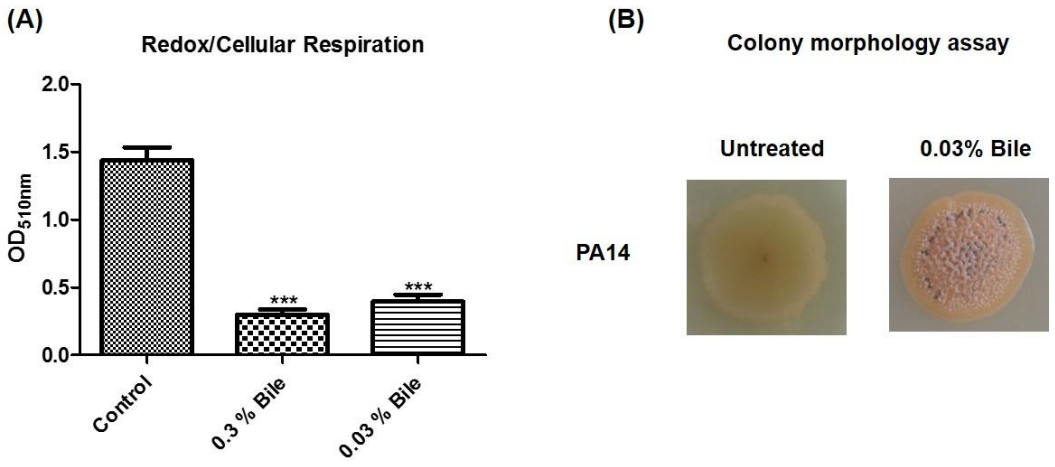

Figure 11. (A) Redox suppression in the presence of bile as shown by tetrazolium violet reduction assay. (B) Colony morphology assay on TSA with wrinkling phenotype in the presence of bile indicating redox changes within the cell in the presence of bile.

Investigating the direct response of *P. aeruginosa* to bile

The transcriptome implicated a metabolic signature potentially mediating the response to bile. In order to determine the immediate transcriptional response to bile and decipher direct responses from possible secondary effects, an earlier transcriptional profile conducted at an $O.D._{600nm}$ 0.3 was conducted. The following down-regulated genes were selected for analysis; *hmgA*, *dnr* and *phzS* while the following upregulated genes were selected; *lasI*, *pqsA*, *psrA*, *pslB* and *dctA* **(Fig.**

12). The significant transcriptional changes observed in the transcriptome and validated by RT-PCR were not evident at this early log timepoint. The only gene significantly altered at this earlier timepoint was *psrA*, a transcriptional regulator known to respond to long chain fatty acid signals. Therefore, the up-regulation of this gene could be in response to the presence of fatty acids in complex bile. Though *pqsA* was also upregulated in the presence of bile, this was not statistically significant (p=0.055). Though this trend towards increased expression indicates that quorum sensing may play a fundamental role in the direct response to bile as a signal.

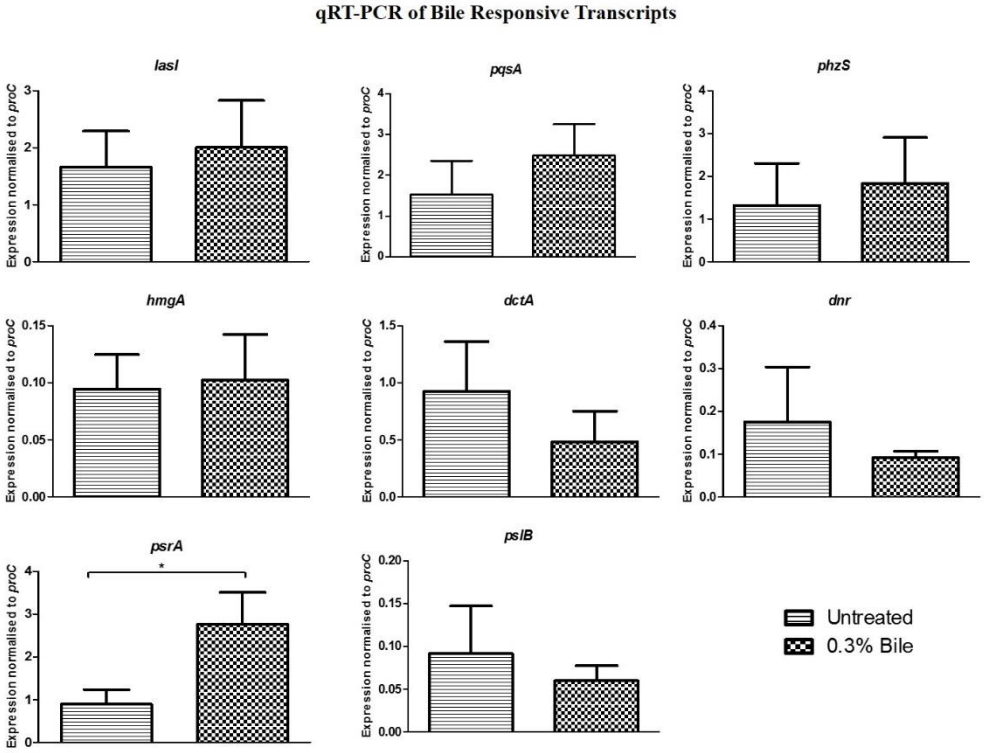

Figure 12. RT-PCR analysis of RNA extracted from *P. aeruginosa* cells grown to early log phase (O.D 0.3). A selection of genes found to be altered in the presence of bile at O.D. 0.8 were selected for analysis. However, the only gene from the panel of genes that was tested found to be significantly altered was the transcriptional regulator *psrA*, which is known to respond to fatty acids present in complex bile. Therefore, there does not appear to be a direct transcriptional response to bile. Data presented is the mean of three independent biological replicates. Statistical analysis was performed by Student's t-test ($p < 0.05$).

Global screen of the PA14 NR library for loci involved in the growth on bile

As mentioned previously, the signal transduction system(s) mediating the response of *P. aeruginosa* and other respiratory tract organisms to bile had not yet been identified. However, the bile tolerance capabilities of enteric organisms has been well characterised in recent years, perhaps a reflection of its importance in the carriage of these organisms in the gastrointestinal tract (Table 4). The bile tolerance responsiveness of these enteric organisms involves a combination of efflux pumps, porins, outer membrane modifications, bile salt hydrolases and classical signal transduction components.

Table 4; Mechanisms of bile and bile acids tolerance strategies in a range of enteric bacteria.

Organism	Proposed Mechanism	Genes	References
Salmonella spp	Membrane integrity	*orf1-tolQRA*	(27, 28)
	Signal transduction/Two component system	*phoPQ*	(43)
	Efflux pump	*acrAB*	(30, 67)
	Transcritpional regulator/DNA binding protein	*marRAB*	(67)
	Enterobactrial common antigen biosynthesis	*wecA/wecD*	(68)
	LPS O antigen structure	*dam*	(29, 69)
	LPS modification	*rfaB*	(70)
	Quorum sensing	---	(71)
Escherichia coli	Efflux pump	*acrAB, emrAB*	(24)
	LPS structure	*rfa*	(15)
	Multidrug transporter	*mdtABCD, mdtM*	(72, 73)
	Signal transduction/Two component system	*baeSR*	(72)
	Porin	*ompC*	(24)
	Toxin/Antitoxin system	*mqsR/A*	(74)
	SOS gene	*dinF*	(75)
Vibrio spp	Porin	*ompU, ompT*	(33, 76)
	Transcriptional regulator	*toxR*	(33)
	LPS structure	*galU, galE, waaF, wavB*	(16, 77)
	Efflux pump	*vceAB, tolC*	(25, 31)
	Transcriptional regulator	*rpoS*	(44)
Campylobater jejuni	Efflux	*cmeABC*	(32, 78)
Enterococcus faecalis	General stress response	*dnaK, groEL*	(42, 79)
Listeria monocytogenes	General stress response	*groESL*	(80)
	Bile salt hydrolases	*bsh, pva, btlB*	(81, 82)
	Transcritpional regulator	*sigB*	(45)
	Transport protein	*opuC, betL, btlA*	(83, 84)
	DNA repair protein	*uvrA*	(85)
Lactobacillus spp	Bile salt hydrolases	*bsh*	(20)
	Oligopeptide binding protein	*oppA*	(86)
	Metabolic changes	---	(87-91)
	Transport proteins	*LBA0552, LBA1429, LBA1446, LBA1679*	(92) (93)
	Signal transduction/Two component system	---	
Lactococcus lactis	Bile salt hydrolases	*bsh1, bsh2*	(94)
Bifidobacterial spp	Transport proteins	*Bbr_0838/0832/1756/0406-0407/1804-1805/1826-1827*	(95, 96)
	Heat shock protein	*hsp-20*	(97)
	Metabolic changes	---	(98, 99)
	Energy production	F(1)F(0)-ATPase	(100)
	Bile salt hydrolases	*bsh*	(101)
Klebsiella pneuomonia	Transcriptional regulator/Transport proteins	*cadCBA, tdcABCDE*	(102)
	Efflux pump	*eefABC*	(103)
Propionibacterium freudenreichii	Metabolic changes	---	(104)

The distribution of some of these key bile tolerance systems present in a selection of gastrointestinal tract organisms and *P. aeruginosa* are outlined below. The presence of these systems in respiratory tract organisms may point to a unified response to bile. However, these systems may display structural and hence functional differences.

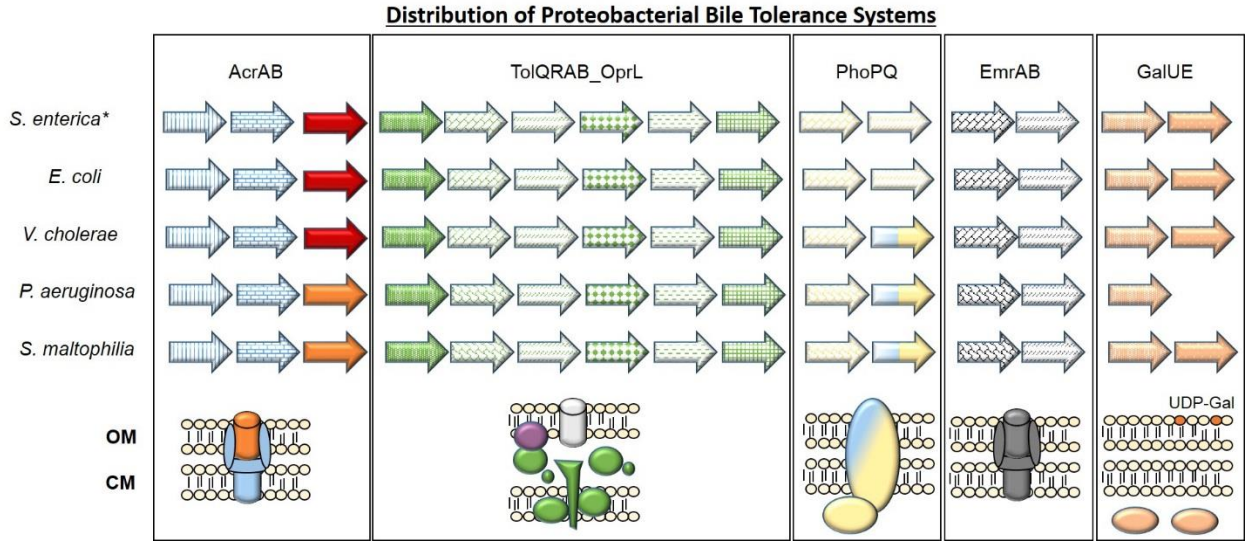

Figure 13. Distribution of bile tolerance systems, the efflux pumps AcrAB and EmrAB, membrane associated systems TolQRAB and GalUE and the two component system PhoPQ in a range of Proteobacterial organisms based on sequence homology.

In order to investigate the systems underpinning the response of *P. aeruginosa* to bile, screening of the PA14 NR transposon mutant library was undertaken. Two screens of the PA14 mutant library were conducted; one screening for the inability to grow on minimal media with bile as a sole carbon source and the second for mutants that no longer displayed enhanced biofilm in the presence of bile **(Fig. 14)**. The transcriptomic analysis revealed a programmed metabolic shift in the presence of bile. *P. aeruginosa* was found to be able to utilise bile as a sole carbon source when supplemented into M9 minimal media, however it was unable to grow on bile acids as a sole carbon when supplemented into M9 minimal media. Therefore, the regulatory systems governing the

utilisation of bile as a carbon source may be connected to the signal transduction pathways mediating the response to bile with a growing body of research implicating adaptive metabolism to virulence control in a range of species. We therefore first wanted to identify the genetic elements underpinning the growth of *P. aeruginosa* on bile as a sole carbon source.

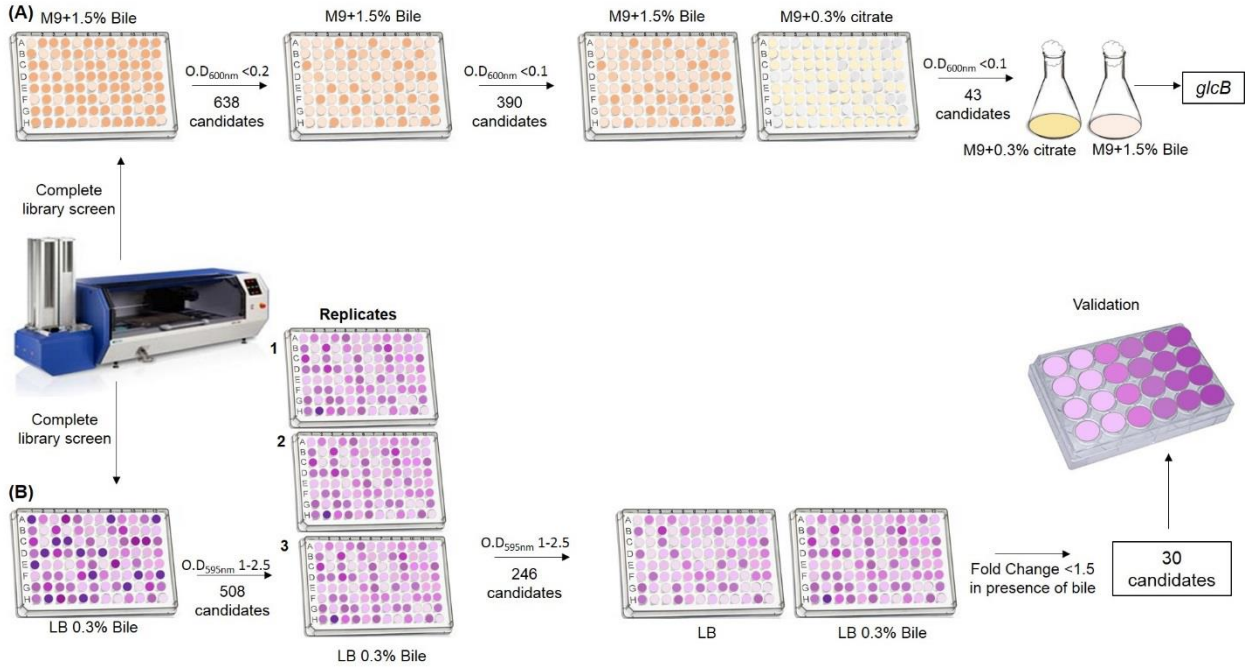

Figure 14. Outline of the processing of two functional screens of the PA14 NR library (A) Growth screen on M9 minimal media supplemented with 1.5% (w/v) bile with growth being recorded spectrophotometrically (B) Biofilm screen in LB supplemented with 0.3% (w/v) bile processed by a crystal violet assay.

Screening of the PA14 transposon mutant library for mutants defective for growth on bile as a sole carbon source led to the identification of a single mutant auxotrophic for growth on bile; *glcB*. The growth defect on bile in this mutant was confirmed not to be due to a generalised growth defect on all carbon sources. Complementation of this mutant in trans restored the ability of this mutant to grow on bile **(Fig. 15)**

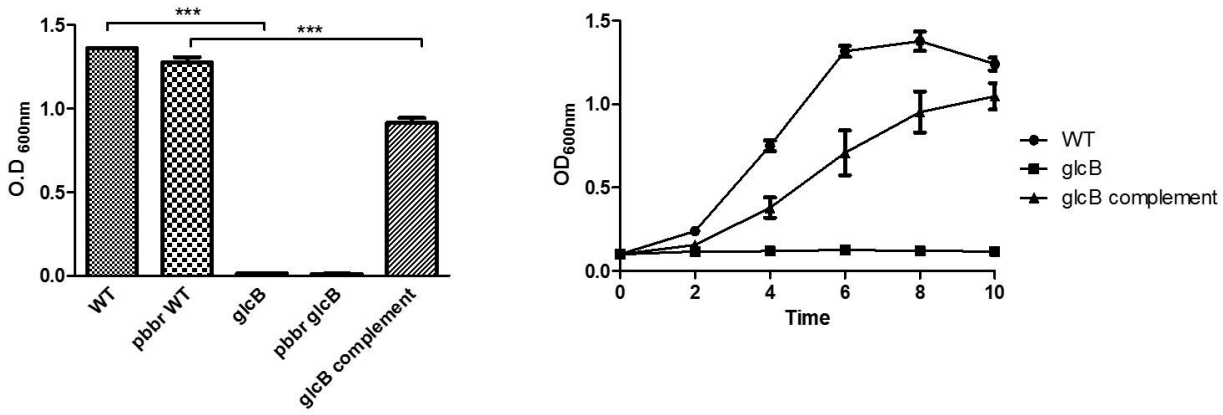

Figure 15. Complementation of the *glcB* mutant in trans restores growth on complex bile with the growth kinetics of the WT strain, the *glcB* mutant and the complemented *glcB* mutant illustrated.

glcB encodes the enzyme malate synthase G, the second enzyme of the glyoxylate shunt, a metabolic pathway that bypasses the later stages of the citric acid cycle allowing for the conversion of isocitrate to malate. The expression of the glyoxylate shunt enzymes has been found to be upregulated in chronic *P. aeruginosa* isolates (105-107) with the *glcB* gene 2.31 fold upregulated in the bile transcriptome. The glyoxylate shunt has been shown to be essential for the utilisation of acetate and fatty acids as carbon sources, with a potential role for this system in the oxidative stress response.

The functional significance for the lack of the glyoxylate shunt system in the presence of bile was investigated with a range if phenotypic assays. The first gene of the glyoxylate pathway was also included in this analysis as it was tested and found to be incapable of utilizing bile as a sole carbon source; *aceA* encoding for the enzyme isocitrate lyase (Appendix Figure 2). This revealed that the phenotypic response to bile is retained in glyoxylate pathway mutants (*aceA* and *glcB).* The induction of biofilm formation and alkyl quinolone signals HHQ and PQS as well as the repression of swarming motility in the presence of bile was comparable to the wild-type in the *aceA* and *glcB*

transposon mutants **(Fig. 16)**. Pyocyanin production was also found to be unaltered in these mutants as in the WT.

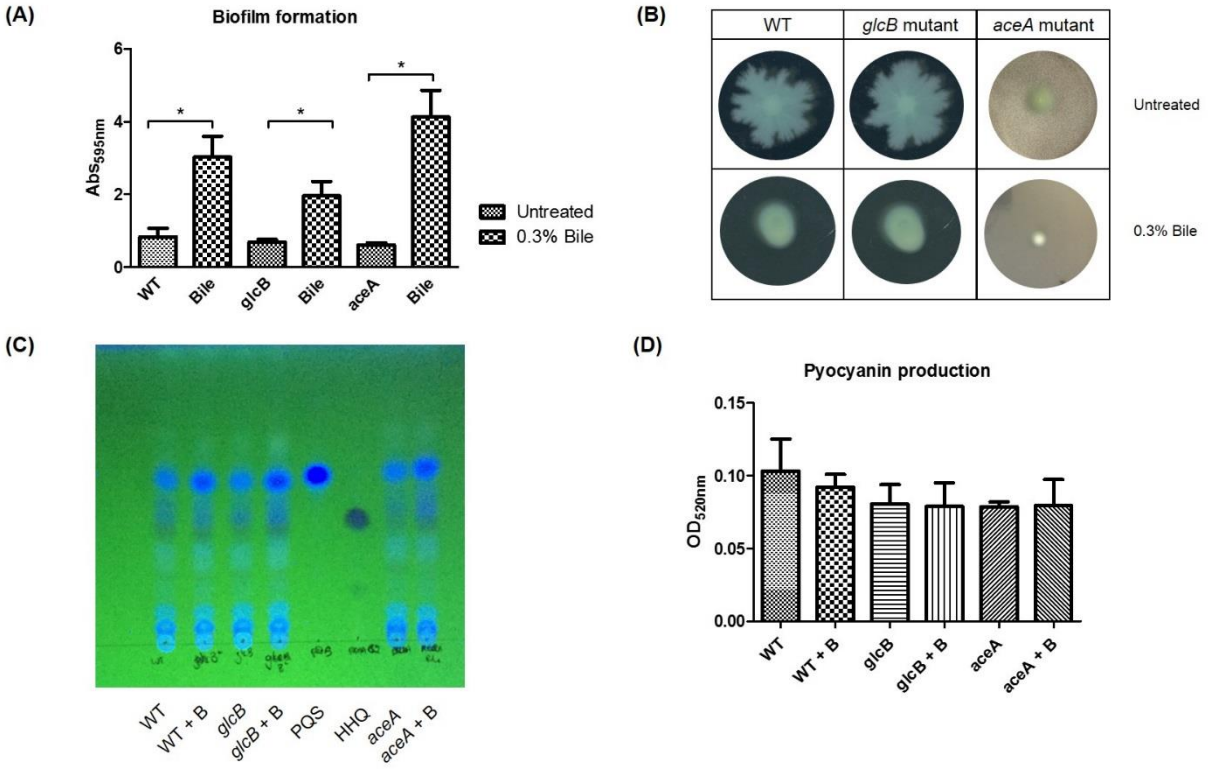

Figure 16. (A) Biofilm formation and (B) swarming motility (C) TLC analysis of AQ production and (D) measurement of pyocyanin production in the WT, *glcB* and *aceA* mutants in the presence and absence of bile. The bile response in the *glcB* and *aceA* mutants are comparable to that of the WT. Data presented is the average of at least three independent biological replicates.

The perturbations of redox potential in the presence of bile was the primary phenotype connecting adaptive metabolism to bile. It was previously hypothesised based on the findings of the bile transcriptome that the reduction of intracellular redox potential was due to the induction of the glyoxylate shunt and the bypassing of the later stages of the citric acid cycle. The TCA is a primary source for the generation of reduced electron carriers such as NADH **(Fig. 9)**. Therefore, the findings that the glyoxylate shunt was essential for utilisation of bile as a carbon source hence

bypassing the later stages of the TCA may underpin this bile altered redox potential. The shift towards reduced redox potential in the presence of bile was not evident in the *glcB* mutant and was found to be restored upon complementation **(Fig. 17)**. The maintenance of the phenotypic response of glyoxylate shunt mutants would suggest that the pathways governing these key transitions occur upstream of flux through the glyoxylate shunt and upstream of the shift in redox potential. These data also indicate that these key phenotypic responses of *P. aeruginosa* to bile are independent of growth on bile as a carbon source though metabolic flux may still play a role.

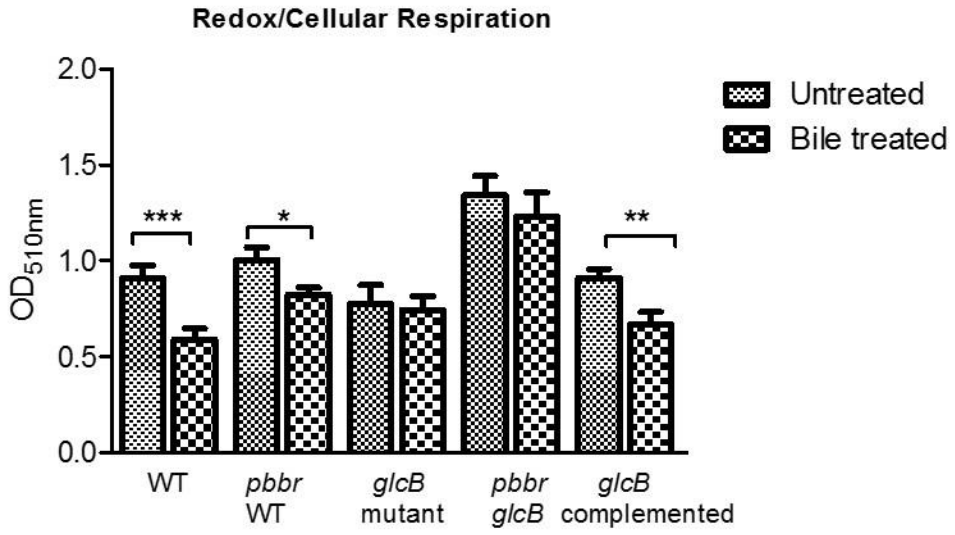

Figure 17. Redox potential is unaffected by bile in a *glcB* mutant, but the repression of redox in the presence of bile is restored in the complemented isogenic strain.

Global screen of the PA14 NR library for loci involved in the biofilm response to bile

As the growth screen identified a single gene which was found to be unaltered in terms of its bile responsive capabilities a global biofilm screen of the PA14 NR mutant library was undertaken to identify other signal transduction components mediating the bile response **(Fig. 14)**. The formation of a biofilm is central to the switch towards chronicity to with bile capable of inducing

this mode of growth. Therefore, the screen aimed at identifying mutants for which there was loss or a reduction in the biofilm response to bile. After a global screen 5800 mutants and a subscreen consisting of 150 sensory and response regulator mutants of in LB treated with 0.3% (w/v) bile, 508 candidates were selected for further testing. These 508 candidates underwent three further independent rounds of testing in LB supplemented with 0.3% (w/v) bile where 246 isolates were selected for a final round of testing. These 246 genes of interest were validated for the bile responsive biofilm phenotype by testing biofilm in both the presence and absence of bile. The final list of gene targets consisted of 30 mutants that exhibited less than a 1.5-fold increase in biofilm formation compared to >3 fold increase in the WT control in the presence of bile **(Table 5)**.

Table 5; Candidates of interest potentially mediating the biofilm response of *P. aeruginosa* to bile.

PA14	**PAO1**	**Gene Name**	**Gene Function**
PA14_05570	PA4292		Phosphate transporter
PA14_07620	PA0584	*cca*	tRNA nucleotidyl transferase
PA14_09470	PA4211	*phzB1*	Phenazine biosynthesis protein
PA14_12260	PA3986		Hypothetical protein
PA14_16430	PA3708	*wspA*	Chemotaxis transducer
PA14_21110	PA3319	*plcN*	Non-hemolytic phospholipase C
PA14_21320	PA3301		Lysophospholipase
PA14_22940	PA3192	*gltR*	Two-component response regulator
PA14_22960	PA3191	*gtrS*	Two-component sensor
PA14_24020	PA3101	*xcpT*	General secretion pathway protein G
PA14_27470	PA2831		Zinc carboxypeptidase
PA14_30840	PA2571		Signal transduction histidine kinase
PA14_32940	PA2449	*gcsR*	Transcriptional regulator
PA14_33220	PA2430		Hypothetical protein
PA14_33430	PA2418		Pirin-related protein
PA14_36100	PA2212	*pdxA*	4-hydroxythreonine-4-phosphate dehydrogenase
PA14_36180	PA2206		LysR type transcriptional regulator
PA14_36220	PA2203		Amino acid permease

PA14_42120	PA1734		Hypothetical protein
PA14_48830	PA1196	*ddaR*	Transcriptional regulator
PA14_49130	PA1183	*dctA*	C4-dicarboxylate transporter
PA14_56700	PA4360		Hypothetical protein
PA14_59990			Hypothetical protein
PA14_61840	PA4674		Antitoxin HigA
PA14_68230	PA5165	*dctB*	Two-component sensor
PA14_68250	PA5166	*dctD*	Two-component response regulator
PA14_70430	PA5335		Hypothetical protein
PA14_72210	PA5471		Hypothetical protein
PA14_72960	PA5530		MFS dicarboxylate transporter

In silico analysis of the finalised list of genes of interest further supported the transcriptome findings that a rewiring of central metabolism plays a role in the *P. aeruginosa* response to bile. The two component system DctB-DctD regulating C4-dicarboxylate transport and the two component system GtrS-GltR regulating glucose transport were identified as bile non-responders with no significant increase in biofilm formation in the presence of bile as in the WT strain **(Fig. 18)**. Interestingly, while *gtrS* and *PA2571* exhibited comparable biofilm levels to the untreated WT, *dctB* trended towards, and *dctD* had, significantly higher levels of biofilm. In addition to the sensory system controlling C4-dicarboxylate transport, the transporter protein DctA itself was also identified as a candidate which was found to be upregulated in the presence of bile in the transcriptome. As well as regulating the DctA transporter, DctB-DctD is also involved in the regulation of the DctPQM transporters with DctA responsible for transport at higher C_4-dicarboxylate concentrations and DctPQM required for transport at lower C_4-dicarboxylate concentrations. In order to test whether the biofilm response to bile was underpinned by these transport systems, these mutants were tested for biofilm formation in the presence of bile. However, while the biofilm response to bile was abolished in *dctA,* the *dctPQM* mutants retained their biofilm response to bile (**Fig. 18)**. These findings suggest that it is unlikely that any single

gene is responsible for signal transduction of the bile response and rather a combination of redundant systems is responsible.

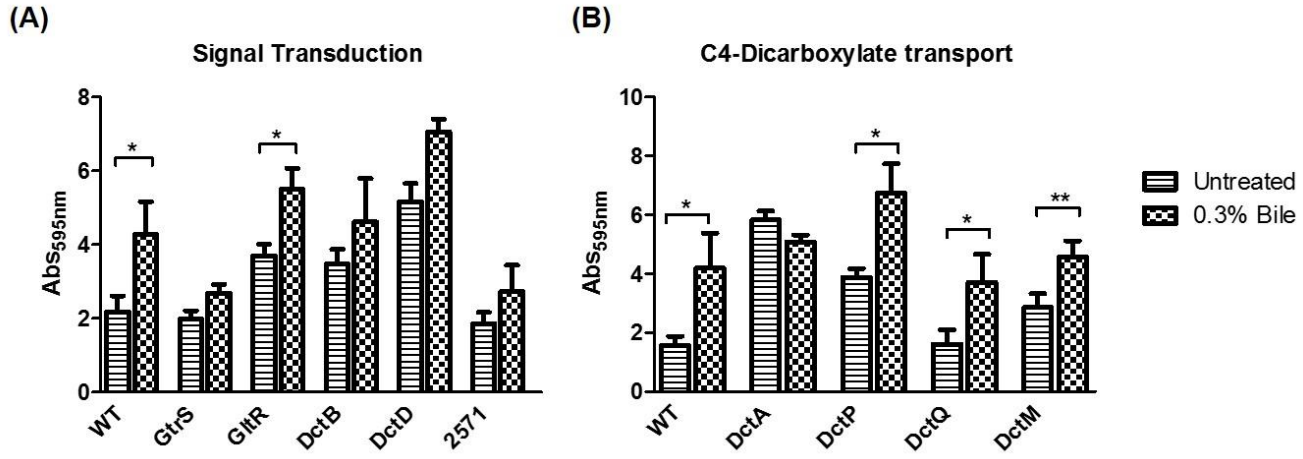

Figure 18. Independent validation of the biofilm screen of the library confirms the results obtained from the global screen of the library, however the *gltR* mutant was found to display a significant increase in biofilm production in the presence of bile. Data presented is the average of at least three independent biological replicates.

Active Component of bile

Bile is a complex mixture consisting primarily of bile salts, fatty acids and cholesterol. With the phenotypic response of *P. aeruginosa* established in the presence of complex bile, investigations into the specific component of bile eliciting these responses was undertaken. This revealed that a complex mixture of bile salts, at clinically significant concentrations of 50 µM and 200 µM, were capable of eliciting an increase in biofilm formation, repression of swarming motility and repression of redox as previously established in complex bile **(Fig. 19)**. However, the increased resistance to the antibiotic polymyxin as measured by growth kinetic analysis was not evident in the presence of bile salts. Additionally, whilst the long chain fatty acid myristic acid was also observed to increase biofilm formation, there was no significant effect on swarming motility and so was not included in further analysis. Therefore, bile acids appear to have a significant impact

on key chronic associated phenotypes and are an active component in the bacteria's response to bile.

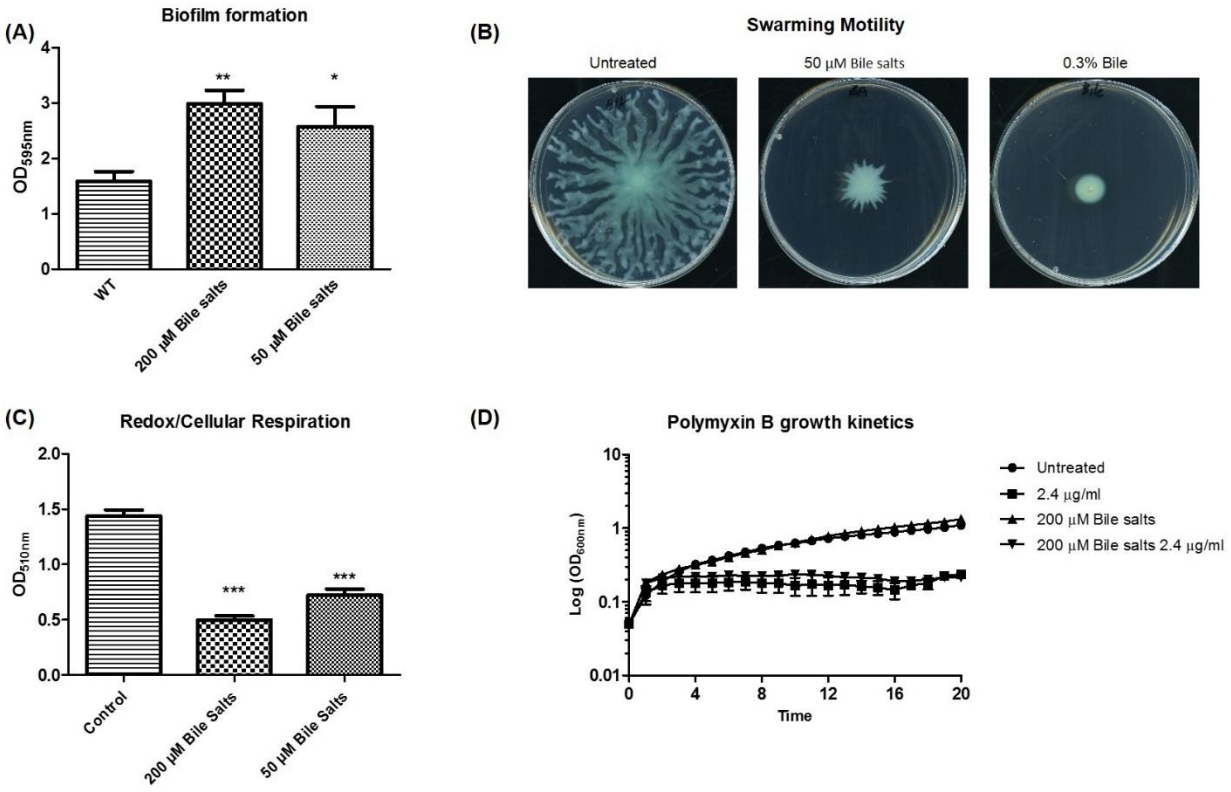

Figure 19. Phenotypic response of *P. aeruginosa* to bile salts; (A) Biofilm formation, (B) Swarming motility, (C) Redox potential and (D) Antibiotic resistance. As observed in the presence of complex bile, bile salts were capable of modulating these key chronic associated phenotypes.

There are twelve abundant bile acids in humans, with evidence that bile acids elicit differential responses within the host depending on their structure. In order to establish whether the different bile acids elicit differential responses in *P. aeruginosa*, twelve individual bile acids were profiled for their impact upon biofilm production and swarming motility. This revealed that though all bile acids tested could reduce swarming motility, only the bile acids CDCA and TLCA significantly increased biofilm formation.

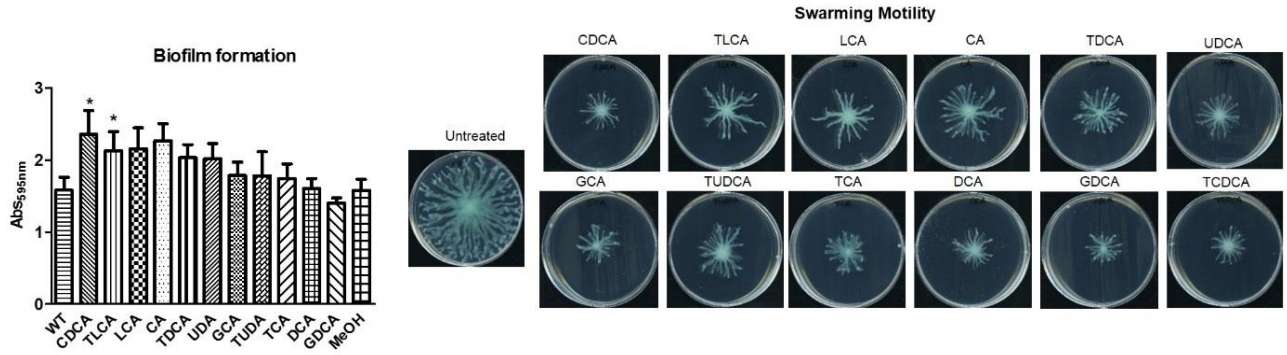

Figure 20. Impact of individual bile acids on two key phenotypes; biofilm formation and swarming motility. The bile acids CDCA and TLCA were capable of increasing biofilm production and repressing swarming motility confirming that not all bile acids display the same biological effects.

Having previously identified key signal transduction components mediating the response to complex bile, it was necessary to establish whether these systems were involved in the bile acids response to bile. This highlighted a loss of the bile acid induced biofilm response to bile in these mutants. This is interesting as *P. aeruginosa* is incapable of utilising bile acids as a sole carbon source and therefore delineating the connection between bile acid signalling and metabolic adaptation should be further investigated.

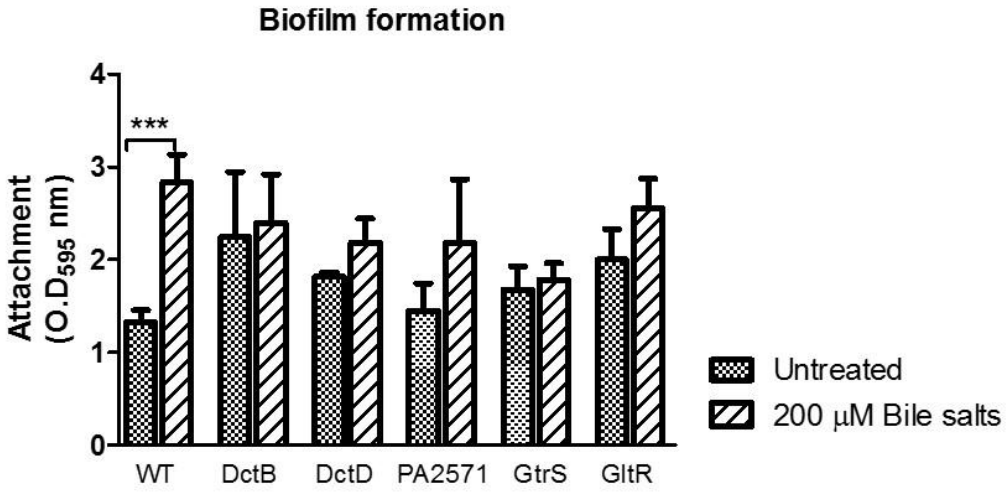

Figure 21. Biofilm formation in response to bile salts in signal transduction mutants highlighting these mutants do not exhibit enhanced biofilm formation in the presence of complex bile salts as in the WT.

Discussion

The emergence of *P. aeruginosa* as the dominant organism within the CF lung microbiota is a hallmark in the pathophysiology of the progression of chronic respiratory disease and the decline in lung function. The transition of this organism towards a biofilm lifestyle presents a major challenge to the clinical management respiratory disease, whereby the organism becomes refractive to antibiotic interventions. Though early intervention strategies have significantly improved patient welfare in patients with CF, *P. aeruginosa* continues to hinder the effective clinical control of this chronic respiratory disease. Furthermore, with the WHO classifying *P. aeruginosa* as a critical pathogen for the identification of novel antimicrobials (108), there is an urgent need for knowledge of the mechanisms through which *P. aeruginosa* exerts its competitive advantage within polymicrobial communities. Deciphering *P. aeruginosa'* emergence and adaptation within the lung environment facilitates the identification of novel drug targets improving early intervention strategies and patient prognosis.

The aspiration of bile into the lungs of respiratory patients has emerged as a major host factor modulating the progression of chronic respiratory disease (5-7, 109), particularly through its impact on the key CF pathogen *P. aeruginosa.* Though investigations into the impact of bile on some of the primary phenotypes present in bile had been conducted, little was known regarding the molecular mechanism mediating these phenotypic responses. In order to gain a deeper insight into the gene expression response potentially governing the phenotypic response, transcriptomics in the presence and absence of bile was undertaken. The bile transcriptome signature was found to be distinct to many *P. aeruginosa* stress response transcriptomes suggesting the transcriptional response to bile was not simply a generalised stress response. The transcriptional response was consistent with the previously reported phenotypic response of *P. aeruginosa* to bile whereby

there was a repression of acute virulence genes such as those involved in type three secretion and pyocyanin production and an up regulation of chronic associated genes such as those associated with antibiotic resistance (*mexAB*) and biofilm formation (*psl*). Furthermore, the increased expression of the diguanylate cyclase *siaD* which is associated with increased levels of cyclic di GMP in the cell is also consistent with an increase in biofilm formation in the presence of bile.

A potential shift in central metabolic flux was evident in the bile transcriptome with a transcriptional induction of the glyoxylate shunt and repression of the later stages of the citric acid cycle. This shift in central metabolism appeared to manifest in an altered redox status in the presence of bile. Furthermore, the identification of *glcB* in the growth screen on bile as a carbon source further reinforces the relative importance of the glyoxylate shunt in *P. aeruginosa,* though the mechanism of uptake of bile into the cell remains unknown. The glyoxylate shunt has been shown to be essential for the utilisation of fatty acids and acetate as carbon sources and allows organisms to bypass the later stages of the TCA in order to conserve carbon for gluconeogenesis (110-114). The regulation of virulence through adaptive metabolism and particularly through the induction of the glyoxylate shunt has been implicated in the pathogenesis of a range of microbes including *Candida albicans, Saccharomyces cerevisiae, Mycobacterium tuberculosis, P. aeruginosa* and will continue to be discerned in the years to come.

In the yeasts *C. albicans* and *S. cerevisiae,* induction of the glyoxylate shunt is observed upon phagocytosis by macrophages (115). In *C. albicans* the glyoxylate enzymes are essential to survival within the phagosome and required for full fungal virulence in a mouse model (115-117). The importance of this system *in vivo* was highlighted in clinical isolates where high activity was observed for the glyoxylate enzymes (118). In the bacterial pathogen *M. tuberculosis,* as observed in *C. albicans,* phagocytosis by macrophage also induced the

glyoxylate genes with mouse models of infection demonstrating increased levels of isocitrate lyase mRNA (119-121). The differential expression in various host cells supports a role for this system in survival and persistence (122, 123). In *Salmonella enterica* serovar *Typhimurium*, the glyoxylate shunt has been shown to be necessary for persistence in chronic infection (124, 125).

In *P. aeruginosa* the link between alterations to central metabolism and virulence is supported by the requirement of the glyoxylate shunt for activation of the T3SS (126, 127). Additionally, the upregulation of glyoxylate enzymes in end stage clinical isolates indicates a role for this system in persistence within the host (106). The glyoxylate shunt has recently been shown to play a role in the mediation of an oxidative stress response in *P. aeruginosa*, and in several other organisms the glyoxylate shunt is induced in response to oxidative stress (128-132).Within the bile transcriptome, there are elements of an oxidative stress response hence the functional significance of the upregulation of the glyoxylate shunt in the presence of bile and the observed enhancement in antibiotic resistance may be in part connected to potential bile induced oxidative stress (133). Although *P. aeruginosa* is capable of utilising bile as a sole carbon source, the ability of glyoxylate shunt mutants to respond to bile for the tested phenotypes; biofilm, swarming motility and AQ production, suggests that the bile response is not solely due to the metabolism of bile as a carbon source. However, metabolic flux and a shift in redox potential appear to be involved in part in the molecular mechanism of the bile response.

Further evidence implicating adaptive metabolism to the bile response came from the biofilm screen of the PA14 mutant library. The production of a biofilm is central to persistence within the host, with bile significantly increasing this key chronicity phenotype. The functional screen identified two two-component systems (TCS), DctBD and GtrS-GltR, and an orphan sensor kinase PA2571 as potentially mediating the signal transduction response to bile. DctBD is

involved in the regulation of C_4-dicarboxylate uptake including succinate, fumarate and malate through control of the C_4-dicarboxylate transporters DctA and DctPQM (134). The orphan sensor kinase PA2571 was previously bioinformatically characterised as a C_4-dicarboxylate regulator so it is possible there is a connection between these two systems. The finding that the later stages of the TCA are transcriptionally repressed, which if reflected at the protein level would result in lower cellular levels of C_4-dicarboxylates, supports the hypothesis of adaptive metabolism mediating the bile response. The GtrS-GltR two component system is involved in the regulation of glucose transport and metabolism (135). This metabolism associated TCS has been linked to the regulation of T3SS and virulence in a mouse model of infection (136). The requirement of two metabolism associated TCSs for bile induced biofilm formation, highlights the complexity of the response. Rather than the classical ligand-receptor systems, the role of these TCSs may be to sense the rewiring of central metabolism and modulate the behaviour of the bacteria accordingly. However, the hyperbiofilm phenotype of some of these mutations may confound the interpretation of the non-responsiveness of these mutants to bile as they already display elevated biofilm prior to the addition of bile.

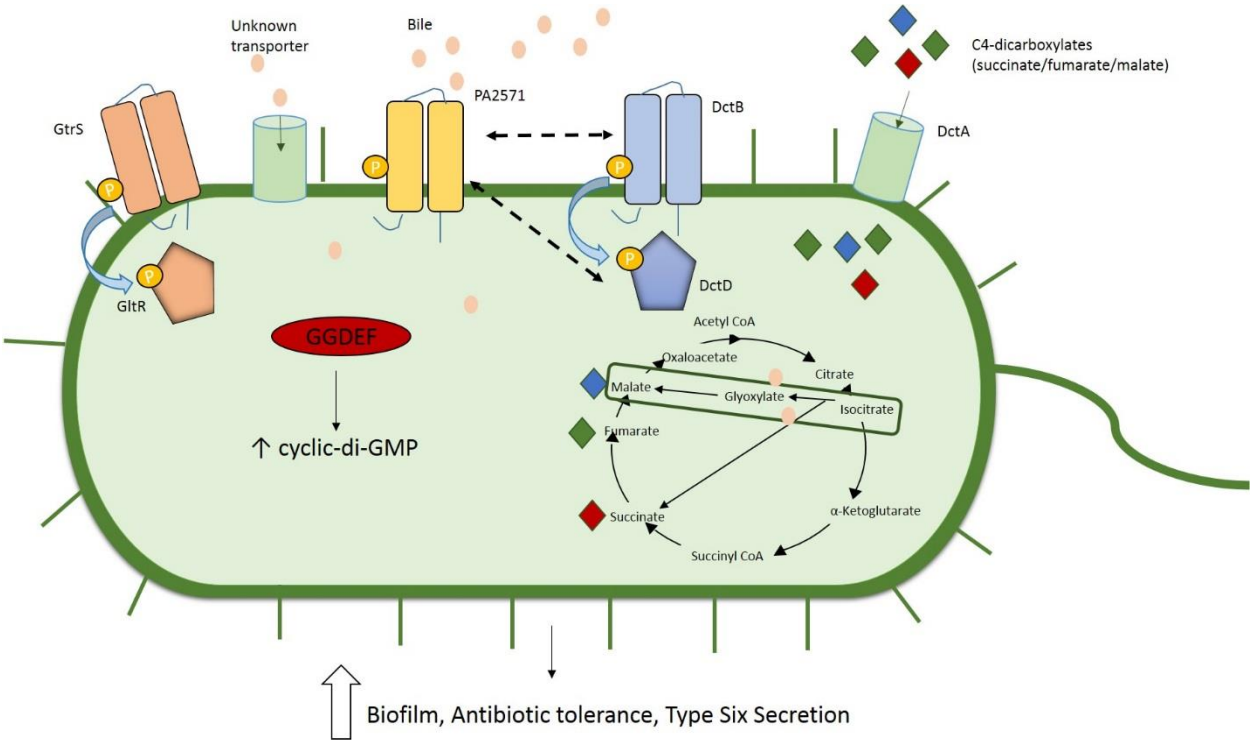

Figure 22. Model of the potential signal transduction systems mediating the response to bile and/or bile acids.

Complex bile is a mixture of a range of components including bile acids, phospholipids, cholesterol and fatty acids. Bile acids are the major constituent of bile constituting roughly 60% of the substance (137). Biofilm, redox and swarming motility were all modulated in response to bile acids in line with the previously observed effects in the presence of bile. *P. aeruginosa* could not use bile acids as a sole carbon source, and yet redox potential was still repressed in the presence of bile acids. This serves to reinforce the view that reprogramming of central metabolism occurs independent of the utilisation of bile as a substrate for growth. Of the 12 bile acids tested, the primary bile acid CDCA was capable of inducing biofilm formation whilst repressing swarming motility. The conjugation of CDCA with either glycine or taurine resulted in the abolishment of these phenotypes. This confirmed previous findings that different bile acids exhibit differential biological effects (138-140). The bile acid conjugation process has been shown to involve residential bacteria in the gut (141, 142). Therefore, the importance of the

composition of the gut microbiota and their contribution to the bile acid profiles present within an individual must be further investigated for their influence on the progression of lung disease **(Fig. 23)**.

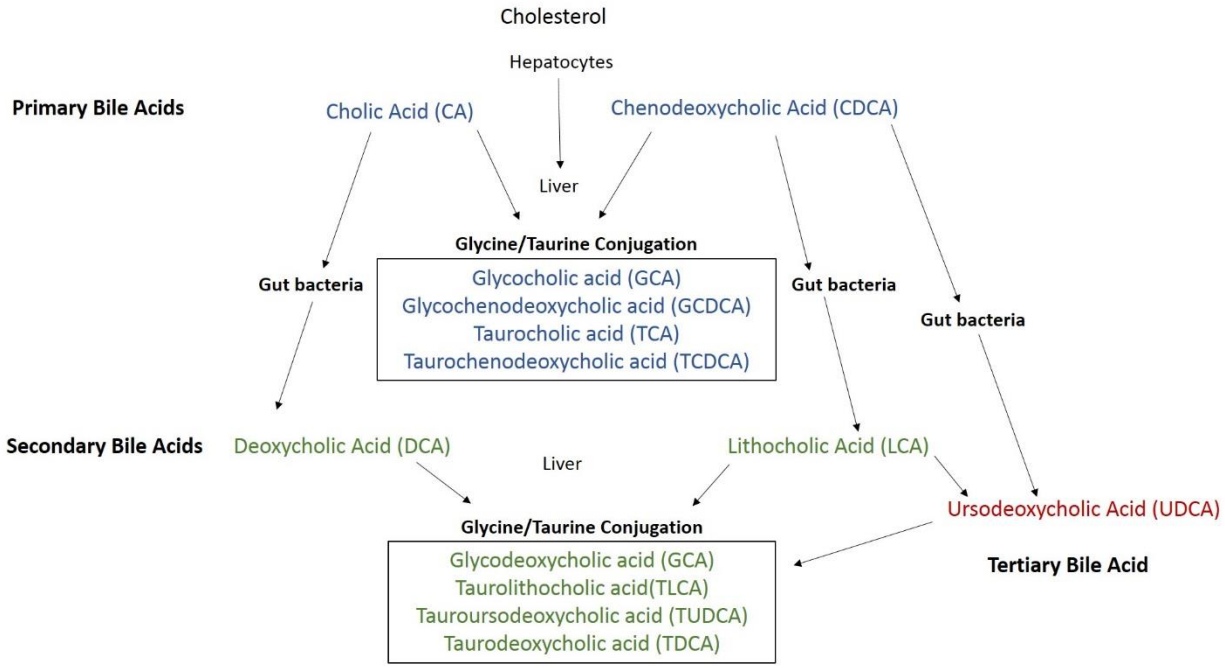

Figure 23. Bile acid synthesis in humans, with three categories of bile acids evident. The formation of primary bile acids (CA, CDCA, GCA, GCDCA, TCA and TCDCA), secondary bile acids (DCA, LCA, GDCA, TLCA, TUDCA and TDCA) and tertiary bile acid (UDCA) is impacted by gut bacteria.

The findings of this research support the previous hypothesis that bile and bile acids are capable of triggering the key respiratory pathogen *P. aeruginosa* to adopt a chronic, antibiotic refractive lifestyle. This behavioural switch appears to be mediated by a combination of adaptive metabolism and altered redox though further investigations will be required to further unravel the complexities of the bile response. The presence of bile acids in the lungs of CF patients is therefore worrying, with this evidence indicating it has a role in the progression of chronic respiratory disease. The design of clinical strategies directed to the

prevention of bile acid transmission into the lungs may be an attractive drug target. The goal of such strategies being the prevention of the onset of chronic bacterial infection ultimately improving patient's quality of life. Surgical intervention in the form of laparoscopic Nissen Fundoplication provides a physical barrier to reflux and is currently the gold standard for the clinical control of reflux and reflux derived bile aspiration (143-145). However, as acquisition and onset of infection can begin early in life, surgery is not always a viable option and can be risky in paediatric patients. Therefore, alternative strategies targeted at clinical control of GOR such as macrolides which display pro-kinetic activity may be of use (146). This option has the added benefit of targeting colonising pathogens. Alternatively, neutralising the biological effects of bile acids through administration of nebulized bile acid sequestrants should also be considered. Clinical interventions may also be based on the prevention of the pathogen's response to bile. The targeting of primary metabolism by narrow spectrum antibiotics may be one such avenue to be explored (147). As we further our understanding of the impact of bile on the pathophysiology of respiratory disease, bile acids may become biological markers for at risk patients. The design of point of care devices that can rapidly detect the presence of bile acids may further improve the effective clinical management of lung disease.

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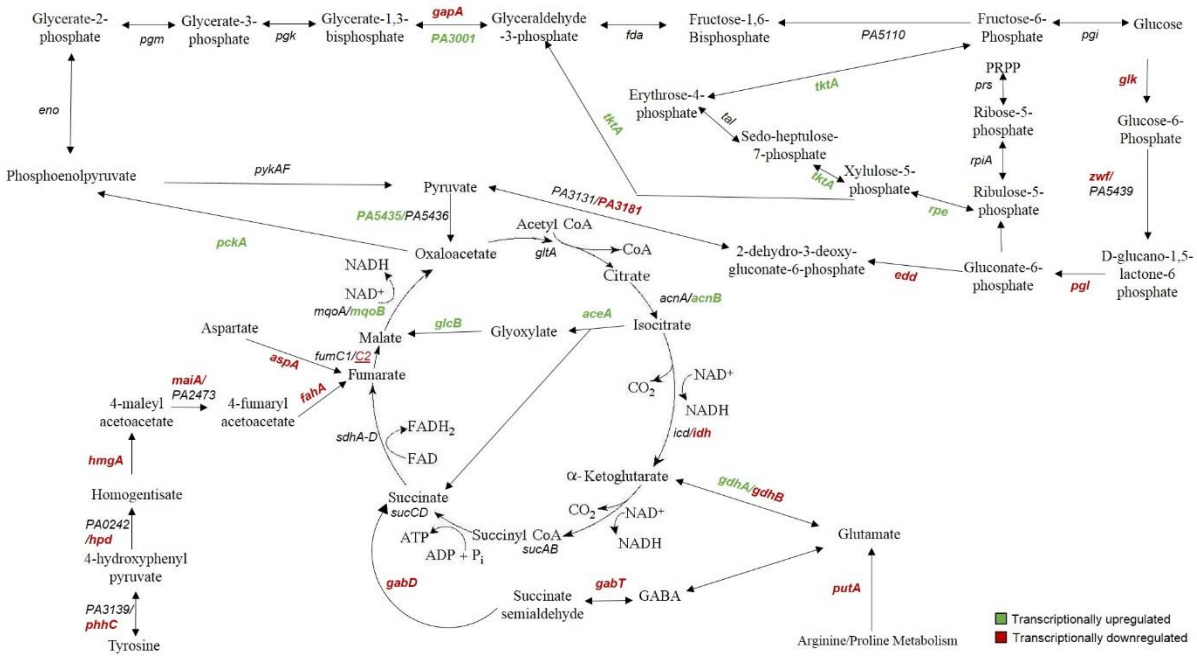
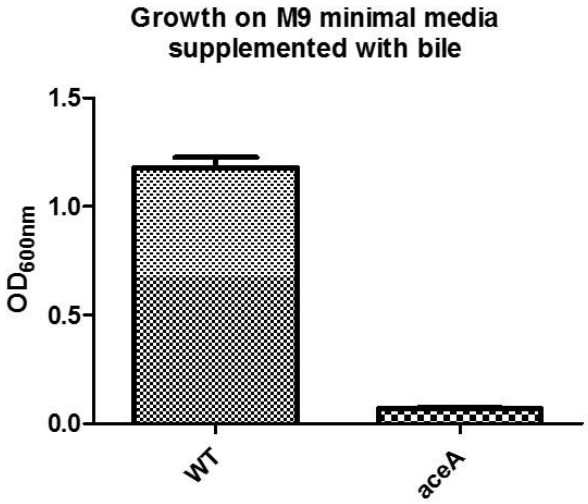
Appendix



Appendix Figure 1. Overview of the impact of bile on central metabolic pathways in *P. aeruginosa;* tricarboxylic acid cycle, Embden-Meyerhof pathway and the Entner-Doudoroff pathway.



Appendix Figure 2. Mutation of the glyoxylate shunt gene *aceA* results in an inability of *P. aeruginosa* to utilise bile as a sole carbon source.

Appendix Table 1; Transcriptome profiling ≥ 1.5 fold change in bile treated vs untreated.

Gene	Name	Fold Change	Gene Description
Upregulated genes			
PA0009	*glyQ*	1.5691702	glycyl-tRNA synthetase alpha chain
PA0013		1.9993705	conserved hypothetical protein
PA0024	*hemF*	1.5071424	coproporphyrinogen III oxidase, aerobic
PA0025	*aroE*	1.6457433	shikimate dehydrogenase
PA0061		1.7197822	hypothetical protein
PA0158	*triC*	1.527921	RND triclosan efflux transporter
PA0165		2.3387754	hypothetical protein
PA0169	*siaD*	2.6718676	diguanylate cyclase (GGDEF) domain
PA0170		2.435536	hypothetical protein
PA0171		2.2755358	hypothetical protein
PA0172	*siaA*	1.9316213	signal transduction protein
PA0195	*pntA*	5.698029	putative NAD(P) transhydrogenase, subunit alpha part 1
PA0196	*pntB*	6.0729065	pyridine nucleotide transhydrogenase, beta subunit
PA0200		1.6779655	hypothetical protein
PA0276		4.9222007	hypothetical protein
PA0293	*aguB*	1.6108009	N-carbamoylputrescine amidohydrolase
PA0305		1.7579664	acylhomoserine lactone acylase B
PA0316	*serA*	1.6069735	D-3-phosphoglycerate dehydrogenase
PA0352		1.7676547	probable transporter
PA0424	*mexR*	2.4193532	multidrug resistance operon repressor
PA0425	*mexA*	1.9427047	RND multidrug efflux membrane fusion precursor
PA0426	*mexB*	1.6745746	RND multidrug efflux transporter
PA0427	*oprM*	1.5616528	multidrug efflux outer membrane prote*in Opr*M precursor
PA0438	*codB*	1.7552981	cytosine permease
PA0482	*glcB*	2.3145316	malate synthase G
PA0506		12.926492	probable acyl-CoA dehydrogenase
PA0507		2.1321254	probable acyl-CoA dehydrogenase
PA0508		41.035236	probable acyl-CoA dehydrogenase
PA0520	*nirQ*	2.6049857	regulatory protein
PA0521		5.02969	probable cytochrome c oxidase subunit
PA0522		2.437193	hypothetical protein
PA0524	*norB*	11.786244	nitric-oxide reductase subunit B
PA0547		1.6460618	probable transcriptional regulator
PA0548	*tktA*	1.877121	transketolase
PA0559		1.875097	conserved hypothetical protein
PA0580	*gcp*	1.6231686	O-sialoglycoprotein endopeptidase
PA0607	*rpe*	1.627546	ribulose-phosphate 3-epimerase
PA0609	*trpE*	1.5326068	anthranilate synthetase component I
PA0642		1.5662348	hypothetical protein
PA0654	*speD*	2.553722	S-adenosylmethionine decarboxylase proenzyme
PA0665		1.741449	conserved hypothetical protein
PA0667		1.9470879	conserved hypothetical protein
PA0715		1.820787	hypothetical protein
PA0729		1.5332638	hypothetical protein
PA0750	*ung*	1.8501233	uracil-DNA glycosylase
PA0775		1.7557627	conserved hypothetical protein
PA0834		2.0055494	conserved hypothetical protein

PA0839		2.3640356	probable transcriptional regulator
PA0840		2.290152	probable oxidoreductase
PA0905	*rsmA*	2.0960267	global regulator protein
PA0916		1.5262712	conserved hypothetical protein
PA0917	*kup*	1.5049281	potassium uptake protein Kup
PA0945	*purM*	1.7995995	phosphoribosylaminoimidazole synthetase
PA0960		1.5693022	hypothetical protein
PA0975		2.0938315	probable radical activating enzyme
PA0976		2.3919008	conserved hypothetical protein
PA0996	*pqsA*	1.610995	AMP-dependent synthetase/ligase
PA1051		6.853995	probable transporter
PA1137		4.652861	probable oxidoreductase
PA1151	*imm2*	1.5625875	pyocin S2 immunity protein
PA1183	*dctA*	5.316506	C4-dicarboxylate transport protein
PA1198		2.0251155	conserved hypothetical protein
PA1228		1.8131458	hypothetical protein
PA1244		1.5826671	hypothetical protein
PA1296		1.5636294	probable 2-hydroxyacid dehydrogenase
PA1299		1.7093493	conserved hypothetical protein
PA1317	*cyoA*	2.316115	cytochrome o ubiquinol oxidase subunit II
PA1318	*cyoB*	2.6973588	cytochrome o ubiquinol oxidase subunit I
PA1319	*cyoC*	2.7420776	cytochrome o ubiquinol oxidase subunit III
PA1320	*cyoD*	3.0416453	cytochrome o ubiquinol oxidase subunit IV
PA1321	*cyoE*	2.38073	cytochrome o ubiquinol oxidase protein
PA1432	*lasI*	1.8146873	autoinducer synthesis protein
PA1554		1.518187	cytochrome c oxidase, cbb3-type, CcoN subunit
PA1596	*htpG*	5.018658	heat shock protein
PA1649		3.7435198	probable short-chain dehydrogenase
PA1687	*speE*	2.2749867	spermidine synthase
PA1736		1.934638	probable acyl-CoA thiolase
PA1748		5.4332643	probable enoyl-CoA hydratase/isomerase
PA1757	*thrH*	1.7832674	homoserine kinase
PA1772		1.8780539	probable methyltransferase
PA1787	*acnB*	1.7395196	aconitate hydratase 2
PA1828		2.5765126	probable short chain dehydrogenase
PA1829		2.7639565	hypothetical protein
PA1830		4.272302	hypothetical protein
PA1831		3.9432259	hypothetical protein
PA1834		1.5483704	hypothetical protein
PA1847	*nfuA*	2.1300695	Fe/S biogenesis protein
PA1959	*bacA*	1.6784254	bacitracin resistance protein
PA1970		2.1638315	hypothetical protein
PA1971	*braZ*	1.7112839	branched chain amino acid transporter protein
PA2063		1.778673	hypothetical protein
PA2120		2.5226676	hypothetical protein
PA2231	*pslA*	1.6181463	Psl exopolysaccharide biosynthesis
PA2232	*pslB*	1.7325034	Psl exopolysaccharide biosynthesis
PA2235	*pslE*	1.666137	Psl exopolysaccharide biosynthesis
PA2237	*pslG*	1.5830181	Psl exopolysaccharide biosynthesis
PA2251		1.5470891	hypothetical protein
PA2260		2.6197445	hypothetical protein

PA2272	*pbpC*	1.9574057	penicillin binding protein 3A
PA2282		1.712276	hypothetical protein
PA2285		3.5870645	hypothetical protein
PA2404		1.6703215	hypothetical protein
PA2453		1.524613	hypothetical protein
PA2524	*czcS*	1.5139198	signal transduction histidine kinase
PA2550		3.6004841	probable acyl-CoA dehydrogenase
PA2619	*infA*	1.5810775	initiation factor
PA2629	*purB*	2.0843713	adenylosuccinate lyase
PA2634	*aceA*	8.60934	isocitrate lyase
PA2653		2.838192	probable transporter
PA2662		6.3886952	conserved hypothetical protein
PA2663	*ppyR*	8.42526	*psl* and pyoverdine operon regulator
PA2705		3.5481186	hypothetical protein
PA2706		2.975919	hypothetical protein
PA2707		2.770848	hypothetical protein
PA2730		1.540463	hypothetical protein
PA2734		1.7171118	hypothetical protein
PA2735		1.8188722	probable restriction modification system protein
PA2740	*pheS*	1.5846324	phenylalanyl-tRNA synthetase alpha-subunit
PA2828		1.9770868	probable amino transferase
PA2849	*ohrR*	2.1531365	Mar-type transcriptional regulator
PA2854		1.6191936	conserved hypothetical protein
PA2876	*pyrF*	1.6983404	orotidine 5'-phosphate decarboxylase
PA2890	*atuE*	1.6387995	putative isohexenylglutaconyl-coA hydratase
PA2901		1.6329596	hypothetical protein
PA2951	*etfA*	2.38636	electron transport flavoprotein alpha subunit
PA2952	*etfB*	2.771388	electron transport flavoprotein beta subunit
PA2953		5.1129184	electron transport flavoprotein-ubiquinone oxidoreductase
PA2956		2.0893328	conserved hypothetical protein
PA2957		2.7422466	probable transcriptional regulator
PA2975	*rluC*	1.5661113	riobosomal large subunit pseudouridine synthase C
PA3001		1.5636102	probable glyceraldehyde-3-phosphate dehydrogenase
PA3006	*psrA*	3.473089	transcriptional regulator
PA3011	*topA*	1.5169479	DNA topoisomerase I
PA3012		3.0956378	hypothetical protein
PA3013	*foaB*	8.261979	fatty-acid oxidation complex beta subunit
PA3014	*faoA*	8.045702	fatty-acid oxidation complex alpha subunit
PA3039		1.6068201	probable transporter
PA3046		1.7461492	conserved hypothetical protein
PA3079		1.5431921	hypothetical protein
PA3080		2.2197092	hypothetical protein
PA3092	*fadH1*	10.941382	2,4-dienoyl-coA reductase
PA3136		4.564559	probable secretion protein
PA3142		1.5115281	integrase
PA3179		2.6249208	conserved hypothetical protein
PA3277		1.5552263	probable short chain dehydrogenase
PA3299	*fadD1*	2.3626924	long-chain-fatty-acid coA ligase
PA3310		1.5319793	conserved hypothetical protein
PA3312		1.6154325	probable 3-hydroxybutyrate dehydrogenase
PA3340		1.8735654	hypothetical protein

PA3397	*fprA*	1.5781447	oxidation-reduction process
PA3430		2.112962	probable aldolase
PA3436		3.7672973	hypothetical protein
PA3441		2.885145	probable molybdopterin-binding protein
PA3454		2.2919114	probable acyl-coA thiolase
PA3530	*bfd*	2.1058738	bacterioferretin-associated ferredoxin
PA3533	*grxD*	1.6208036	cell redox homeostasis
PA3564		1.5504247	conserved hypothetical protein
PA3604	*erdR*	1.8984143	response regulator
PA3608	*potB*	2.6690738	polyamine transport protein
PA3610	*potD*	3.1713614	polyamine transport protein
PA3636	*kdsA*	1.6291608	2-dehydro-3-deoxyphosphooctonate aldolase
PA3648	*opr86*	1.8314524	outer membrane protein
PA3673	*plsB*	1.5458521	glycerol-3-phosphate acyltransferase
PA3713	*spdH*	2.438359	spermidine dehydrogenase
PA3742	*rplS*	1.6403929	50S ribosomal protein L19
PA3810	*hscA*	1.9194839	heat shock protein
PA3815	*iscR*	1.97773	iron-sulfur cluster assembly transcription factor
PA3817		1.9172465	probable methyltransferase
PA3818	*suhB*	2.426292	extragenic suppressor protein
PA3832	*holC*	1.6676788	DNA polymerase III, chi subunit
PA3860		2.786317	probable AMP-binding enzyme
PA3876	*narK2*	2.4774816	nitrite extrusion protein 2
PA3915	*moaB*1	2.683761	molybdopterin biosynthetic protein B1
PA3925		6.362694	probable acyl-coA thiolase
PA3979		2.2068381	hypothetical protein
PA3980		1.5200603	conserved hypothetical protein
PA4053	*ribE*	1.5947592	6,7-dimethyl-8-ribityllumazine synthase
PA4055	*ribC*	2.0001235	rioboflavin synthase alpha chain
PA4139		2.0021958	hypothetical protein
PA4280	*birA*	1.6605364	cellular protein modification process
PA4291		1.5189809	hypothetical protein
PA4292		1.5532227	probable phosphate transporter
PA4385	*groEL*	2.1746135	60 kDa chaperonin
PA4386	*groES*	1.6020151	10 kDa chaperonin
PA4389		2.9126391	probable short chain dehydrogenase
PA4390		2.3305416	hypothetical protein
PA4404		1.5881593	hypothetical protein
PA4432	*rpsI*	2.0233817	30S ribosomal protein S9
PA4435		3.31337	probable acyl-CoA dehydrogenase
PA4519	*speC*	2.5638704	ornithine decarboxylase
PA4524	*nadC*	1.5230906	nicotinate-nucleotide pyrophosphorylase
PA4574		1.8216043	conserved hypothetical protein
PA4588	*gdhA*	4.843752	glutamate dehydrogenase
PA4602	*glyA3*	1.5978534	serine hydroxymethyltransferase
PA4615	*fprB*	1.7907255	ferredoxin reductase-type FAD binding protein
PA4621		1.950093	probable oxidoreductase
PA4625	*cdrA*	2.0494714	cyclic diguanylate-regulated TPS partner A
PA4637		1.6872619	hypothetical protein
PA4640	*mqoB*	1.8581218	malate:quinone oxidoreductase
PA4645		1.7798011	probable purine/pyrimidine phosphoribosyl transferase

PA4646	*upp*	1.5125525	uracil phosphoribosyltransferase
PA4676		1.7414601	probable carbonic anhydrase
PA4729	*panB*	1.6376442	3-methyl-2-oxobutanoate hydroxymethyltransferase
PA4730	*panC*	1.6072731	pantoate-beta-alanine ligase
PA4731	*panD*	2.025211	aspartate 1-decarboxylase precursor
PA4739		1.6100599	conserved hypothetical protein
PA4744	*infB*	1.610814	translation initiation factor IF-2
PA4747	*secG*	2.002634	secretion protein
PA4755	*greA*	1.554067	transcription elongation factor
PA4757		1.7081891	conserved hypothetical protein
PA4758	*carA*	2.2199163	carbamoyl-phosphate synthase small chain
PA4759	*dapB*	4.5988173	dihydrodipicolinate reductase
PA4768	*smpB*	1.660908	ssrA-binding protein
PA4770	*lldP*	6.846915	L-lactate permease
PA4771	*lldD*	6.3667874	L-lactate dehydrogenase
PA4817		1.8832077	hypothetical protein
PA4840		2.423288	conserved hypothetical protein
PA4855	*purD*	1.5000381	phosphoribosylamine -glycine ligase
PA4873		1.8353506	probable heat shock protein
PA4928		2.0750062	conserved hypothetical protein
PA5019		1.8728709	conserved hypothetical protein
PA5020		3.9215884	probable acyl-CoA dehydrogenase
PA5023		2.9872162	conserved hypothetical protein
PA5035	*gltD*	2.323267	glutamate synthase small chain
PA5048		1.5303047	probable nuclease
PA5072		1.859174	probable chemotaxis transducer
PA5076		1.7966675	probable binding protein component of ABC transporter
PA5118	*thiI*	1.7867571	thiazole biosynthesis protein
PA5119	*glnA*	2.0239024	glutamine synthetase
PA5125	*ntrC*	1.7712349	two component response regulator
PA5138		1.514509	hypothetical protein
PA5157		2.4622345	probable transcriptonal regulator
PA5158		2.5326378	probable outer membrane protein precursor
PA5159		3.614299	multdrug resistance protein
PA5192	*pckA*	2.1203341	phosphoenoylpyruvate carboxykinase
PA5215	*gcvT1*	1.6615047	glycine-cleavage system protein T1
PA5275		2.443186	conserved hypothetical protein
PA5296	*rep*	1.955691	ATP-dependent DNA helicase
PA5315	*rpmG*	1.5917709	50S ribosomal protein L33
PA5347		2.2524781	hypothetical protein
PA5407		2.249299	hypothetical protein
PA5430		1.5518764	hypothetical protein
PA5435		2.3119483	probable transcarboxylase subunit
PA5482		2.1968896	hypothetical protein
PA5503		1.51603	probable ATP-binding component of ABC transporter
PA5526		1.5122871	hypothetical protein
PA5530		2.2498746	C5-dicarboxylate transporter
PA5561	*atpI*	1.9720086	ATP-synthase protein I
Pae_tRNA_Ala		2.1793957	
Pae_tRNA_Gln		1.674767	
Pae_tRNA_Gly		1.5203607	

Pae_tRNA_His		1.8042011
Pae_tRNA_Ile		1.868528
Pae_tRNA_Leu		1.9550334
Pae_tRNA_Lys		1.9264927
Pae_tRNA_Thr		3.0495825
Pae_tRNA_Val		1.7678549

Downregulated genes

PA0028		1.5948375	hypothetical protein
PA0039		2.143605	hypothetical protein
PA0048		2.94961	probable transcriptional regulator
PA0050		1.984994	hypothetical protein
PA0078	*tssL1*	2.1075292	Hcp secretion island I (HSI-I) type VI secretion system
PA0079	*tssK1*	1.6049535	Hcp secretion island I (HSI-I) type VI secretion system
PA0081	*fhA1*	1.7814412	Hcp secretion island I (HSI-I) type VI secretion system
PA0082	*tssA1*	2.4476979	Hcp secretion island I (HSI-I) type VI secretion system
PA0083	*tssB1*	2.0212882	Hcp secretion island I (HSI-I) type VI secretion system
PA0084	*tssC1*	2.1767128	Hcp secretion island I (HSI-I) type VI secretion system
PA0085	*hcp1*	1.9435418	Hcp secretion island I (HSI-I) type VI secretion system
PA0086	*tagJ1*	2.6898286	Hcp secretion island I (HSI-I) type VI secretion system
PA0087	*tssE1*	2.6551623	Hcp secretion island I (HSI-I) type VI secretion system
PA0088	*tssF1*	1.9184635	Hcp secretion island I (HSI-I) type VI secretion system
PA0089	*tssG1*	2.39119	Hcp secretion island I (HSI-I) type VI secretion system
PA0093	*tse6*	1.7975425	Hcp secretion island I (HSI-I) type VI secretion system
PA0094		1.6072897	hypothetical protein
PA0095		1.8542596	conserved hypothetical protein
PA0096		2.1944344	hypothetical protein
PA0099		5.3633327	hypothetical protein
PA0100		1.680287	hypothetical protein
PA0112		1.6479193	hypothetical protein
PA0122	*rahU*	3.615853	Hemolysin, aegerolysin type
PA0129	*bauD*	7.6909256	amino acid permease
PA0130	*bauC*	4.95721	oxopropanonate dehydrogenase
PA0131	*bauB*	5.0994606	beta-alanine biosynthetic protein
PA0132	*bauA*	9.548833	beta-alanine:pyruvate transaminase
PA0176	*aer2*	2.1970596	aerotaxis transducer
PA0178		1.7684873	probable two component sensor
PA0179		2.57306	probable two component response regulator
PA0250		1.566561	conserved hypothetical protein
PA0261		1.7094259	hypothetical protein
PA0262	*vgrG2*b	1.8797104	type VI secretion system Vgr family protein
PA0263	*hcpC*	4.5870194	secreted protein
PA0265	*gabD*	1.9571402	succinate-semialdehyde dehydrogenase
PA0266	*gabT*	2.0194545	4-aminobutyrate aminotransferase
PA0296	*spuI*	1.7034112	glutamylpolyamine synthetase
PA0297	*spuA*	2.749863	probable glutamine amidotransferase
PA0298	*spuB*	1.9352928	glutamylpolyamine synthetase
PA0299	*spuC*	1.9077505	Polyamine:pyruvate transaminase
PA0328	*aaaA*	1.7097926	arginine-specific autotransporter
PA0345		1.5220128	hypothetical protein
PA0433		1.5347764	hypothetical protein

PA0459		1.9906839	probable ClpA/B protease ATP binding subunit
PA0492		9.769155	conserved hypothetical protein
PA0493		12.164877	probable biotin-requiring enzyme
PA0494		12.537723	probable acyl-CoA carboxylase subunit
PA0495		14.1487875	hypothetical protein
PA0504	*bioD*	2.1558855	dethiobiotin synthase
PA0527	*dnr*	1.785652	transcriptional regulator
PA0563		2.0404708	conserved hypothetical protein
PA0602		2.9482942	probable binding component of ABC transporter
PA0730		20.264019	probable transferase
PA0744		2.6036005	probable enoyl-coA hydratase/isomerase
PA0745		2.601556	probable enoyl-coA hydratase/isomerase
PA0782	*putA*	2.399258	proline dehydrogenase
PA0783	*putP*	2.7099984	sodium/proline symporter
PA0789		2.135822	probable amino acid permease
PA0796	*prpB*	1.6505334	carboxyphosphonoenolpyruvate phosphonomutase
PA0798	*pmtA*	1.7103677	phospholipid methyltransferase
PA0818		1.538232	hypothetical protein
PA0852	*cbpD*	5.2773623	chitin-binding protein
PA0853		1.5048496	probable oxidoreductase
PA0855		2.013548	hypothetical protein
PA0865	*hpd*	9.271919	4-hydroxyphenylpyruvate dioxygenase
PA0870	*phhC*	3.1358166	aromatic amino acid aminotransferase
PA0871	*phhB*	2.0197833	pterin-4-alpha-carbinolamine dehydratase
PA0872	*phhA*	1.843872	phenylalaine-4-hydroxylase
PA0887	*acsA*	8.322072	acteyl-coenzyme A synthetase
PA1015		1.5982444	probable transcriptional regulator
PA1069		1.9617678	hypothetical protein
PA1073	*braD*	1.8554968	branched chain amino acid transport protein
PA1130	*rhlC*	2.571125	rhamnosyltransferase 2
PA1131		2.6840458	probable major facilitator superfamily transporter
PA1170		3.169312	conserved hypothetical protein
PA1173	*napB*	1.9207349	cytochrome c-type protein precursor
PA1175	*napD*	1.769999	protein of periplasmic nitrate reductase
PA1176	*napF*	2.807975	ferredoxin protein
PA1177	*napE*	2.4080005	periplasmic nitrate reductase protein
PA1293		3.1160045	hypothetical protein
PA1336	*aauS*	1.5600288	signal transduction histidine kinase
PA1338	*ggt*	3.2098217	gamma-glutamyltranspeptidase precursor
PA1377		2.1422617	conserved hypothetical protein
PA1378		2.501052	hypothetical protein
PA1396		2.6544714	probable two component sensor
PA1415		1.5845275	hypothetical protein
PA1418		2.5522227	probable sodium:solute symport protein
PA1469		1.776468	hypothetical protein
PA1494		1.6269441	mucoidy inhibitor gene A
PA1511	*vgrG2*a	2.014915	type VI secretion system Vgr family protein
PA1538		1.5359794	probable flavin-containing monooxygenase
PA1565	*pauB2*	8.70774	FAD-dependent oxidoreductase
PA1591		2.0886438	hypothetical protein
PA1600		1.743144	probable cytochrome c

PA1609	*fabB*	2.2841575	beta-ketoacyl-ACP synthase I
PA1610	*fabA*	2.9927423	beta-hydroxydeacnoyl-ACP dehydrase
PA1611		1.7661884	hybrid sensor kinase
PA1612		1.7176058	hypothetical protein
PA1639		2.3704023	hypothetical protein
PA1647		1.8923525	probable sulfate transporter
PA1656	*hsiA2*	2.6254783	Hcp secretion island I (HSI-II) type VI secretion system
PA1657	*hsiB2*	3.1352513	Hcp secretion island I (HSI-II) type VI secretion system
PA1658	*hsiC2*	3.1408982	Hcp secretion island I (HSI-II) type VI secretion system
PA1659	*hsiF2*	3.9727623	Hcp secretion island I (HSI-II) type VI secretion system
PA1663	*sfa2*	4.714584	Hcp secretion island I (HSI-II) type VI secretion system
PA1664	*orfX*	4.7656765	Hcp secretion island I (HSI-II) type VI secretion system
PA1665	*fha2*	4.4706626	Hcp secretion island I (HSI-II) type VI secretion system
PA1666	*lip2*	2.7888627	Hcp secretion island I (HSI-II) type VI secretion system
PA1667	*hsij2*	3.4455078	Hcp secretion island I (HSI-II) type VI secretion system
PA1668	*dotU2*	3.3361132	Hcp secretion island I (HSI-II) type VI secretion system
PA1669	*icmF2*	3.4615045	Hcp secretion island I (HSI-II) type VI secretion system
PA1670	*stp1*	2.32084	Hcp secretion island I (HSI-II) type VI secretion system
PA1691	*pscT*	1.7019385	translocation protein in type three secretion
PA1692		1.9261502	probable translocation protein in type three secretion
PA1693	*pscR*	1.9027892	translocation protein in type three secretion
PA1694	*pscQ*	2.4248476	translocation protein in type three secretion
PA1698	*popN*	1.8647052	type three secretion outer membrane protein precursor
PA1699	*pcr1*	2.2103293	negative regulator of protein secretion
PA1700	*pcr2*	2.8731177	type three secretion protein
PA1701	*pcr3*	2.8128605	type three secretion protein
PA1703	*pcrD*	2.2703972	type three secretory apparatus protein
PA1705	*pcrG*	2.268675	type three secretion regulator
PA1706	*pcrV*	2.441468	type three secretion protein
PA1707	*pcrH*	2.5031915	regulatory protein
PA1708	*popB*	3.310198	translocator protein
PA1709	*popD*	3.3381078	translocator outer membrane protein precursor
PA1710	*exsC*	3.9618962	exoenzyme S synthesis protein C precursor
PA1711	*exsE*	3.2902606	type three secretion protein
PA1712	*exsB*	3.4639027	exoenzyme S synthesis protein B
PA1713	*exsA*	2.269879	transcriptional regulator
PA1714	*exsD*	2.0926607	negative regulator of protein secretion
PA1715	*pscB*	2.127073	type three export apparatus protein
PA1716	*pscC*	2.4568872	type three secretion outer membrane protein precursor
PA1718	*pscE*	1.9889266	type three secretion export protein
PA1719	*pscF*	2.4587705	type three secretion export protein
PA1720	*pscG*	2.1631007	type three secretion export protein
PA1721	*pscH*	2.7372322	type three secretion export protein
PA1722	*pscI*	2.2609296	type three secretion export protein
PA1725	*pscL*	2.0760052	type three secretion export protein
PA1732		1.9571875	conserved hypothetical protein
PA1759		1.9743236	probable transcriptional regulator
PA1760		3.1952362	probable transcriptional regulator
PA1761		2.0183575	hypothetical protein
PA1762		1.8740104	hypothetical protein
PA1774	*crfX*	2.6833508	hypothetical protein

PA1775	*cmpX*	1.6878084	conserved cytoplasmic membrane protein
PA1797		4.6390796	hypothetical protein
PA1818	*LdcA*	3.1492357	lysine-specific PLP-dependent carboxylase
PA1819		1.9307975	probable amino acid permease
PA1844	*tse1*	2.373152	Hcp secretion island I (HSI-I) type VI secretion system
PA1852		3.399476	hypothetical protein
PA1869		2.6705492	probable acyl-carrier protein
PA1894		2.0885706	hypothetical protein
PA1895		1.9763824	hypothetical protein
PA1897		2.7002308	hypothetical protein
PA1903	*phzE2*	3.8006172	phenazine biosynthesis protein
PA1904	*phzF2*	4.305888	probable phenazine biosynthesis protein
PA1905	*phzG2*	5.7028136	probable pyridoxamine 5'-phosphate oxidase
PA1950	*rbsK*	1.501952	ribokinase
PA1963		2.0812864	hypothetical protein
PA1984	*exaC*	59.43127	NAD+ dependent aldehyde dehydrogenase
PA1986	*pqqB*	2.1094584	pyrroloquinolone quinone biosynthesis protein B
PA1999	*dhcA*	31.309772	dehydrocarnitine coA transferase subunit A
PA2003	*bdhA*	2.8883147	3-hydroxybutyrate dehydrogenase
PA2008	*fahA*	34.142612	fumarylacetoacetase
PA2009	*hmgA*	54.90419	homogentisate 1,2-dioxygenase
PA2010		2.1838396	probable transcriptional regulator
PA2011	*liuE*	3.4714077	3-hydroxy-3-methylglutaryl-CoA lyase
PA2013	*liuC*	12.648429	putative 3-methylglutaconyl-CoA hydratase
PA2014	*liuB*	14.03458	methylcrotonyl-CoA carboxylase, beta subunit
PA2015	*liuA*	10.924191	putative isovaleryl-CoAdehydrogenase
PA2016	*liuR*	7.376461	regulator of *liu* genes
PA2019	*mexX*	1.5939317	RND multi drug efflux membrane fusio*n prote*in precursor
PA2041		2.957639	amino acid permease
PA2044		1.6550866	hypothetical protein
PA2066		3.08255	hypothetical protein
PA2069		5.337103	probable carbamoyl transferase
PA2080	*kynU*	1.9059906	kynureninase
PA2109		13.239002	hypothetical protein
PA2110		9.982603	hypothetical protein
PA2111		11.699324	hypothetical protein
PA2112		10.565109	conserved hypothetical protein
PA2113	*opdO*	15.40829	pyroglutamate porin
PA2114		12.587432	probable major facilitator superfamily transporter
PA2123		1.5930154	probable transcriptional regulator
PA2193	*hcnA*	2.0849488	hydrogen cyanide synthase
PA2194	*hcnB*	2.4813926	hydrogen cyanide synthase
PA2195	*hcnC*	2.7307336	hydrogen cyanide synthase
PA2196		1.603319	tetR family transcriptional regulator
PA2247	*bkdA1*	11.765008	2-oxoisovalerate dehydrogenase alpha subunit
PA2248	*bkdA2*	8.665485	2-oxoisovalerate dehydrogenase beta subunit
PA2249	*bkdB*	8.010507	branched chain alpha keto acid dehydrogenase lipoamide
PA2250	*lpdV*	10.541232	lipoamide dehydrogenase-Val
PA2304	*ambC*	1.5349882	AMB biosynthetic protein
PA2305	*ambB*	1.8090299	AMB biosynthetic protein
PA2358		7.733476	hypothetical protein

PA2378		1.5142914	probable aldehyde dehydrogenase
PA2423		1.9415448	hypothetical protein
PA2464		2.2603655	hypothetical protein
PA2503		1.558646	hypothetical protein
PA2537		1.8237628	probable acyltransferase
PA2538		1.863953	hypothetical protein
PA2539		2.16321	conserved hypothetical protein
PA2540		2.290972	conserved hypothetical protein
PA2541		2.353624	probable CDP-alcohol phosphatidyltransferase
PA2552		10.1244545	probable acyl-CoA dehydrogenase
PA2553		17.720968	probable acyl-CoA thiolase
PA2554		7.5938797	probable short chain dehydrogenase
PA2555		14.5416565	probable AMP-binding enzyme
PA2573		1.7526189	probable chemotaxis transducer
PA2624	*idh*	1.5125592	isocitrate dehydrogenase
PA2637	*nuoA*	1.7138935	NADH dehydrogenase I chain A
PA2682		1.5942562	conserved hypothetical protein
PA2684	*tse5*	2.1057823	protein secretion by the type VI secretion system
PA2685	*vgrG4*	1.5318247	Hcp secretion island I (HSI-I) type VI secretion system
PA2702	*tse2*	1.8557568	protein secretion by the type VI secretion system
PA2703	*tsi2*	2.2335365	Hcp secretion island I (HSI-I) type VI secretion system
PA2725		1.7536601	probable chaperone
PA2726		2.0102458	probable radical activating enzyme
PA2727		1.776595	hypothetical protein
PA2728		1.5782139	hypothetical protein
PA2752		1.720396	conserved hypothetical protein
PA2759		1.9633168	hypothetical protein
PA2761		2.1907022	hypothetical protein
PA2776	*pauB3*	2.2253318	FAD-dependent oxidoreductase
PA2780		2.649256	hypothetical protein
PA2781		1.8431194	hypothetical protein
PA2788		1.7293639	probable chemotaxis transducer
PA2790		1.6003789	hypothetical protein
PA2792		1.6244026	hypothetical protein
PA2867		1.7818938	probable chemotaxis transducer
PA2868		2.5292265	hypothetical protein
PA2883		1.7968694	hypothetical protein
PA2950	*pfm*	2.7392774	proton motive force protein
PA2967	*fabG*	1.761908	3-oxoacyl-(acyl-carrier-protein)reductase
PA2968	*fabD*	2.5239315	malonyl-CoA-(acyl-carrier protein)transacylase
PA3038		4.6687083	probable porin
PA3054		1.6859233	hypothetical protein
PA3068	*gdhB*	3.46976	NAD-dependent glutamate dehydrogenase
PA3089		1.915602	hypothetical protein
PA3111	*folC*	1.9460045	folylpolygultamate synthetase
PA3181		2.4894865	2-keto-3-deoxy-6-phosphogluconate aldolase
PA3182	*pgl*	2.4615443	6-phosphogluconolactonase
PA3183	*zwf*	1.8612951	glucose-6-phosphate 1-dehydrogenase
PA3186	*oprB*	5.547807	glucose/carbohydrate outer membrane protein precursor
PA3187		37.640736	probable ATP binding component of ABC transporter
PA3190		37.495556	probable binding protein component of ABC transporter

PA3191	*gtrS*	2.3700728	glucose transporter sensor
PA3192	*gltR*	2.8294287	two component response regulator
PA3193	*glk*	2.5788982	glucokinase
PA3194	*edd*	2.3691285	phospholgluconate dehydratase
PA3195	*gapA*	11.153055	glyceraldehyde 3-phosphate dehydrogenase
PA3222		1.8008807	hypothetical protein
PA3232		1.7556814	probable nuclease
PA3234		5.911297	probable sodium:solute symporter
PA3266	*capB*	2.062545	cold acclimation protein B
PA3267		2.013526	hypothetical protein
PA3271		2.1071553	probable two component sensor
PA3289		2.1252868	hypothetical protein
PA3294	*vgrG4*a	2.0742862	type VI protein secretion system complex
PA3326	*clpP2*	2.1203053	ATP-dependent Clp protease proteolytic subunit
PA3327		2.6560686	probable non ribosomal peptide synthetase
PA3330		2.478699	probable short chain dehydrogenase
PA3331		2.440465	cytochrome P450
PA3332		2.590828	conserved hypothetical protein
PA3333	*fabH2*	2.5470762	3-oxoacyl-(acyl-carrier-protein)synthase III
PA3335		3.2118356	hypothetical protein
PA3355		2.0893073	hypothetical protein
PA3356	*pauA5*	3.517199	glutamylpolyamine synthetase
PA3361	*lecB*	4.9500566	fucose-binding lectin PA-IIL
PA3362		1.601258	hypothetical protein
PA3363	*amiR*	2.094657	aliphatic amidase regulator
PA3365		2.497356	probable chaperone
PA3366	*amiE*	1.7536775	aliphatic amidase
PA3403		1.6708249	hypothetical protein
PA3471		2.838807	probable malic enzyme
PA3478	*rhlB*	1.5629418	rhamnosyltransferase chain B
PA3483		1.7651366	hypothetical protein
PA3484	*tse3*	2.1280935	Hcp secretion island I (HSI-I) type VI secretion system
PA3485	*tsi3*	2.2205892	Hcp secretion island I (HSI-I) type VI secretion system
PA3487	*tle5*	2.6968684	phospholipase D active protein
PA3488	*tli5*	2.0857	Hcp secretion island I (HSI-I) type VI secretion system
PA3511		1.7608668	probable short chain dehydrogenase
PA3516		8.26889	probable lyase
PA3519		3.9683826	hypothetical protein
PA3535		2.5359788	probable serine protease
PA3560	*fruA*	2.0949857	phosphotransferase system transporter fructose specific IIBC
PA3561	*fruK*	1.8346641	1-phosphofructokinase
PA3562	*fruI*	1.6994203	phosphotransferase system transporter enzyme I
PA3570	*mmsA*	1.8595018	methylmalonate-semialdehyde dehydrogenase
PA3581	*glpF*	3.1833086	glycerol uptake facilitator protein
PA3582	*glpK*	3.2480462	glycerol kinase
PA3583	*glpR*	1.5413748	glycerol-3-phosphate regulon repressor
PA3622	*rpoS*	1.8654066	sigma factor
PA3661		3.144587	hypothetical protein
PA3711		1.5237778	probable transcriptional regulator
PA3716		1.9169841	hypothetical protein
PA3723		1.7067646	probable FMN oxidoreductase

PA3779		1.6678269	hypothetical protein
PA3786		1.5774021	hypothetical protein
PA3791		2.0427399	hypothetical protein
PA3841	*exoS*	2.0348053	exoenzyme S
PA3842	*spcS*	3.8800082	specific Pseudomonas chaperone for ExoS
PA3843		2.883644	hypothetical protein
PA3850		1.5037203	hypothetical protein
PA3858		1.5532614	probable amino acid binding protein
PA3859		1.5182569	carboxylesterase
PA3906		1.6909509	hypothetical protein
PA3924		2.2293808	probable medium chain acyl-CoA ligase
PA3960		1.9433469	hypothetical protein
PA4024	*eutB*	9.765597	ethanolamine ammonia-lyase large subunit
PA4025		3.6506097	ethanolamine ammonia-lyase light chain
PA4040		1.6383013	hypothetical protein
PA4124	*hpcB*	1.984254	homoprotocatechuate 2,3-dioxygenase
PA4126		2.3422012	probable major facilitator superfamily transporter
PA4127	*hpcG*	1.919759	2-oxo-hept-3-ene- 1,7 dioate hydratase
PA4129		2.7885988	hypothetical protein
PA4130		2.168181	probable sulfite or nitrite reductase
PA4131		2.43465	probable iron-sulfur protein
PA4132		2.3007894	conserved hypothetical protein
PA4134		3.8384316	hypothetical protein
PA4198		2.1332781	probable AMP-binding enzyme
PA4211	*phzB1*	8.585621	probable phenazine biosynthesis protein
PA4217	*phzS*	5.672542	flavin-containing monooxygenase
PA4290		3.2010045	probable chemotaxis transducer
PA4296	*pprB*	1.8907439	two component response regulator
PA4312		1.676818	conserved hypothetical protein
PA4316	*sbcB*	1.8292457	exodeoxyribonuclease I
PA4318		1.994757	hypothetical protein
PA4320		1.7371818	hypothetical protein
PA4321		1.8312458	hypothetical protein
PA4348		1.9334596	conserved hypothetical protein
PA4397	*panE*	1.6094146	ketopantoate reductase
PA4441		1.5054107	hypothetical protein
PA4464	*ptsN*	1.5437149	nitrogen regulatory IIA protein
PA4489	*magD*	1.5218936	endopeptidase inhibitor protein
PA4490	*magC*	1.7564965	hypothetical protein
PA4495		1.8454317	hypothetical protein
PA4496		3.494979	probable binding protein component of ABC transporter
PA4497		3.9295037	probable binding protein component of ABC transporter
PA4498	*mdpA*	2.8390453	metallo-dipeptidase aeruginosa
PA4500		3.5027168	probable binding protein component of ABC transporter
PA4502		5.121125	probable binding protein component of ABC transporter
PA4503		3.6120512	probable permease of ABC transporter
PA4504		3.6977658	probable permease of ABC transporter
PA4505		2.5218732	probable ATP binding component of ABC transporter
PA4506		2.7894301	probable ATP binding component of ABC transporter
PA4521	*ampE*	1.6189638	antibiotic response protein
PA4550	*fimU*	1.7110652	type 4 fimbrial biogenesis protein

PA4551	*pilV*	1.9267063	type 4 fimbrial biogenesis protein
PA4583		1.598647	conserved hypothetical protein
PA4591		1.6394689	hypothetical protein
PA4592		1.6008954	probable outer membrane protein precursor
PA4603		1.5413858	hypothetical protein
PA4604		1.9048488	conserved hypothetical protein
PA4605		1.9862462	conserved hypothetical protein
PA4607		1.6575047	hypothetical protein
PA4660	*phr*	1.5931557	deoxyribodipyrimidine photolyase
PA4736		1.645675	hypothetical protein
PA4737		1.5593427	hypothetical protein
PA4846	*aroQ1*	3.026733	3-dehydroquinate dehydratase
PA4847	*accB*	2.2632728	biotin carboxyl carrier protein
PA4848	*accC*	2.665176	biotin carboxylase
PA4890	*desT*	2.196814	negative regulator of fatty acid metabolic process
PA4917		1.6167381	hypothetical protein
PA4978		2.09403	hypothetical protein
PA5015	*aceE*	2.4748633	pyruvate dehydrogenase
PA5016	*aceF*	1.9336244	dihydrolipoamide acetyltransferase
PA5033		1.9714878	hypothetical protein
PA5058	*phaC2*	2.070579	poly(3-hydroxyalkanoic acid) synthase 2
PA5062		1.645185	conserved hypothetical protein
PA5089	*pldB*	1.8433772	phospholipase D active protein
PA5112	*estA*	2.2522578	esterase
PA5113		1.7548673	hypothetical protein
PA5114		1.5116537	hypothetical protein
PA5152		1.6229354	probable ATP binding component of ABC transporter
PA5154		3.0212357	probable permease of ABC transporter
PA5155		1.8666053	amino acid ABC transporter *mem*brane protein
PA5167	*dctP*	2.2897437	C4 dicarboxylate transport
PA5168	*dctQ*	2.9596171	C4 dicarboxylate transport
PA5169	*dctM*	3.794928	C4 dicarboxylate transport
PA5174		10.5041275	probable beta-ketoacyl synthase
PA5184		1.951973	hypothetical protein
PA5208		2.355181	conserved hypothetical protein
PA5212		3.2855444	hypothetical protein
PA5219		1.9625584	hypothetical protein
PA5220		3.035381	hypothetical protein
PA5230		1.5580871	probable permease of ABC transporter
PA5235	*glpT*	1.5808283	glycerol-3-phosphate transporter
PA5266	*vgrG6*	2.6062305	type VI protein secretion system complex
PA5271		1.510594	hypothetical protein
PA5290		1.6901497	conserved hypothetical protein
PA5302	*dadX*	2.7517176	catabolic alanine racemase
PA5303		3.682151	conserved hypothetical protein
PA5304	*dadA*	3.0453987	D-amino acid dehydrogenase,small subunit
PA5312	*pauC*	2.6244156	aldehyde dehydrogenase
PA5313	*gabT2*	1.7187879	transaminase
PA5329		1.5208641	conserved hypothetical protein
PA5348		3.6003451	probable DNA binding protein
PA5355	*glcD*	2.6623282	glycolate oxidase subunit

PA5367	*pstA*	1.555267	membrane protein component of ABC phosphate transporter
PA5374	*betI*	2.1576834	transcriptional regulator
PA5380	*gbdR*	9.957009	putative amidotransferase
PA5396		1.9843612	hypothetical protein
PA5410	*gbcA*	3.55013	putative ring hydroxylating dioxygenase
PA5421	*fdhA*	1.9251707	glutathione-independent formaldhyde dehydrogenase
PA5428		1.5123227	probable transcriptional regulator
PA5429	*aspA*	4.8744445	aspartate ammonia-lyase
PA5458		1.5078423	hypothetical protein
PA5461		1.7103561	hypothetical protein
PA5510		1.8075719	probable transporter
PA5545		2.2842433	conserved hypothetical protein

Chapter 3

Exposure to Bile Leads to the Emergence of Adaptive Signalling Variants in *Pseudomonas aeruginosa*

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Exposure to bile leads to the emergence of adaptive signalling variants in the opportunistic pathogen *Pseudomonas aeruginosa.*

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Abstract

Pseudomonas aeruginosa is the key respiratory pathogen found to dominate the Cystic Fibrosis lung microbiome. This versatile and opportunistic pathogen has been shown to undergo extensive genomic adaptation in order to chronically colonise and persist within the CF lung environment. Rapid evolutionary adaptations of *P. aeruginosa* allows it to both evade the host immune response and outcompete co-colonising residents of the lung microbiota. These adaptations include increased antibiotic resistance, loss of quorum sensing, a switch to a mucoid phenotype and an altered metabolism. However, whilst several studies describe the mutations that frequently arise in clinical isolates of *P. aeruginosa*, it remains unclear as to what the environmental factors governing the emergence of these genetic variants are. Bile aspiration, a consequence of gastro-oesophageal reflux, has recently emerged as a major co-morbidity in CF. This key host factor has been shown to shape the CF microbiota and promote the emergence of dominant proteobacterial pathogens including *P. aeruginosa.* In order to investigate whether bile may act as a selective pressure for genetic adaptation within the CF lung, populations of *P. aeruginosa* were experimentally evolved in Artificial Sputum Medium, a media known to resemble conditions present in the CF lung, in the presence and absence of bile. Pigmented variants of *P. aeruginosa* emerged exclusively in the presence of bile. Whole genome sequencing analysis identified single nucleotide polymorphisms (SNPs) in the pyocyanin (*phzS)* and pyomelanin (*hmgA*) pathways, as well as mutations in a key quorum sensing regulator (*lasR).* Phenotypic characterisation of colonies taken from ASM supplemented with bile, compared with those from untreated ASM, revealed elevated biofilm formation and a selection for the retention of swarming motility. Phenotypic analysis of the pigmented derivatives highlighted an altered bile response profile relative to the ancestral progenitor strain. The

pigmented derivatives exhibited a loss of the biofilm response and redox response to bile with a loss of Pseudomonas Quinolone Signal (PQS) production also observed. The loss of redox repression in the presence of bile could be explained by this defective Alkyl-Quinolone (AQ) production. Together, these findings suggest that the adaptation of *P. aeruginosa* to long term bile exposure is underpinned by the selection of sub-populations of alternative pigment producing cells with a concomitant modulation of pyocyanin production and AQ signalling.

Introduction

Chronic respiratory disease poses a major societal challenge, with the rapid onset of the post-antibiotic era representing a serious threat to the clinical management of infections associated with these conditions (1, 2). As is often the case, the development of chronic microbial infections results in a shift in clinical regimens wherein these infections are no longer treatable using conventional antibiotics. Therefore, understanding the factors that contribute to respiratory pathogen's ability to shift from acute to chronic infections is of paramount importance in order to identify alternative interventions and reduce the global reliance on antibiotics.

Though the prognosis for this life-limiting autosomal recessive disorder Cystic Fibrosis has considerably improved in recent years, challenges remain in the successful implementation of these innovative therapies (3-6). Principal among these is the maintenance of health and lung function in paediatric patients. Lower respiratory infections are reported from as early as the first weeks of life and are strongly associated with the development of pulmonary inflammation and bronchiectasis (7, 8). Early acquisition of respiratory pathogens particularly *P. aeruginosa* is associated with clinical decline and increased mortality with *P. aeruginosa* positive cultures detected in patients as young as 3 months of age (9, 10). Successful early eradication has been associated with reduced prevalence of chronic *P. aeruginosa* infections in later life and hence is of paramount importance in the provision of optimal CF care (11-13).

The pathogen-centric focus to the management of respiratory infections has been challenged somewhat in recent years with the advent of next generation sequencing technologies. Genomics based studies have uncovered richly diverse microbial communities in the lungs of both healthy individuals and hospitalised patients, with perturbations in these populations implicated in many

disease conditions (9, 14). Micro-evolution, the extent of which has been shown to differ between mild and severe cases of disease, of pathogens within these diverse communities has also received considerable attention with a growing appreciation for the extent of phenotypic heterogeneity present within these populations (15-21). The acquisition of highly transmissible strains also contributes to the genetic diversity present in *P. aeruginosa* infection (22)The ability of pathogens such as *P. aeruginosa* to adapt to novel environments, with the presence of multiple phenotypic and genotypic variants reported *in vivo*, facilitates persistence and colonisation in challenging niches such as the lungs of patients with respiratory disease (23-27).

The most common CF pathogen *P. aeruginosa* encounters and must tolerate numerous stressors within the CF lung environment if it is to survive and thrive in the host. These include but are not limited to the toleration of fluctuations in pH and oxygen, nutrient availability, and resisting elimination by residential host immune cells and by-products of inflammation. The emergence of hyper-mutator strains with enhanced rates of spontaneous mutations often due to defective DNA mismatch repair systems (*mutS, mutL, uvrD)* is proposed to be beneficial to the long-term survival of *P. aeruginosa* (28-30). A variety of pathoadaptive mutations have been described in key virulence, regulatory, antibiotic resistance and metabolism genes with the most common mutations identified in *P. aeruginosa* clinical isolates recently outlined in a review by Winstanley and colleagues (31).

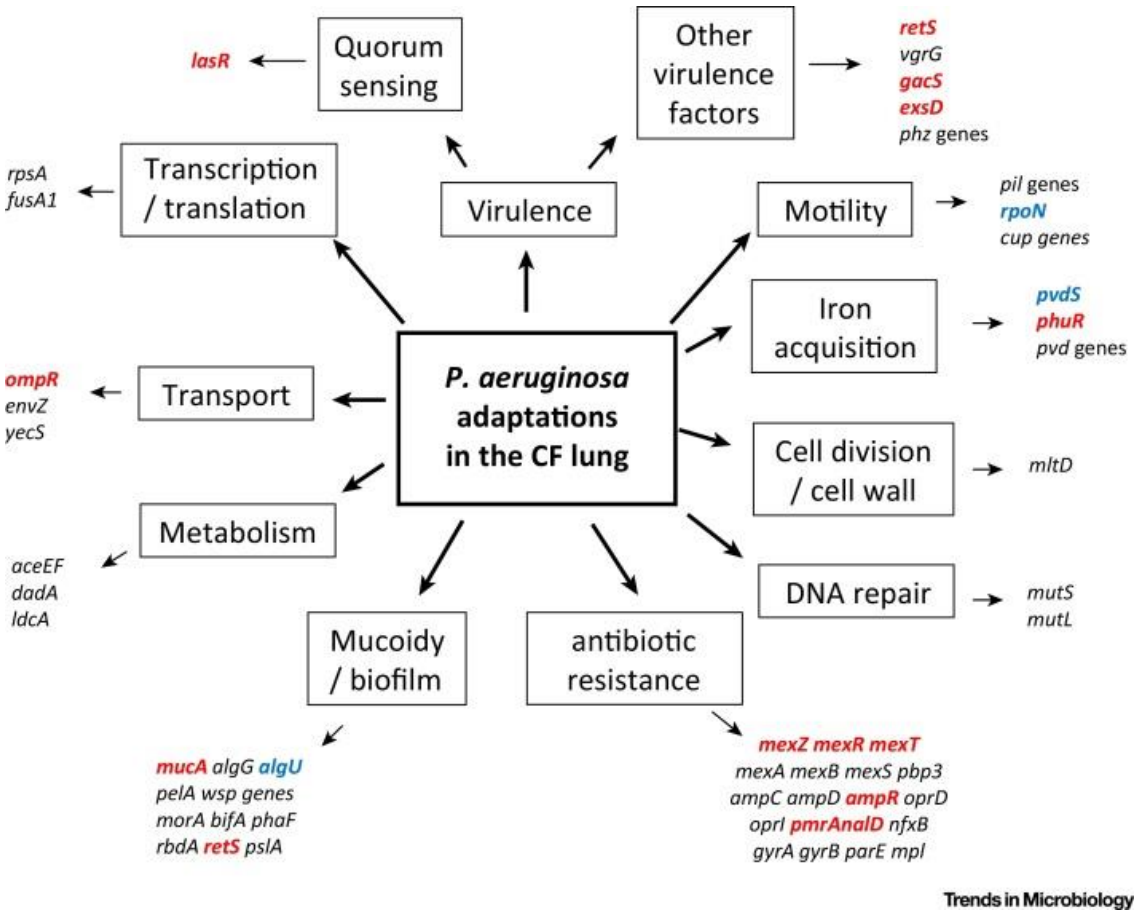


Figure 1. An overview of the most common pathoadaptive mutations by functional category reported to be present in clinical isolates of *P. aeruginosa*. Taken from Winstanley et al., 2016.

Mutations in key signalling pathways e.g. LasR are particularly prevalent among *P. aeruginosa* clinical isolates (32, 33), with pigmented variants also reported in several studies (15, 34). Furthermore, re-wiring of regulatory networks in these clinical isolates has been uncovered, suggesting a complex adaptive response to environmental challenges (33, 35). These changes can lead to growth phase dependent alterations in virulence (36). Though, there are reports of multiple evolutionary trajectories it would appear there is convergent evolution, with mutations in multiple pathways leading to the same beneficial end goal (37, 38). One example of such a phenomenon was described by Marvig et al. whereby individual mutations of genes within the

wsp operon, associated with the regulation of biofilm formation, were capable of yielding the beneficial adaptive phenotype i.e. through multiple different pathways (37). To enhance early treatment strategies of lung disease in CF, improvements in our understanding of the host and environmental factors that contribute to both pathogen acquisition and dysbiosis of the lower respiratory microbiota is essential.

Bile acids, one of the major components of bile, have been detected in the lungs of patients with respiratory disease, and have been shown to correlate directly with increased morbidity and colonisation by *P. aeruginosa* (39, 40). This is further supported by several independent studies that show a correlation between bile reflux and pathogen colonisation in patients with CF (41-43), with evidence of increased inflammation and lung impairment also reported (44). Furthermore, both bile and bile acids have been shown to modulate the behavioural lifestyle of *P. aeruginosa* causing it to adopt a chronic lifestyle (39, 45). This would appear to occur through transcriptional rewiring of the cell, with changes in virulence systems, signalling molecules, and key metabolic pathways including the glyoxylate shunt observed (39). While we have uncovered key insights into the mechanisms through which bile influences the behaviour of *P. aeruginosa*, little is known about how the pathogen responds to long term bile exposure. This would be of particular interest considering the temporal nature of aspiration and its emerging role in shaping the microbiology of the lungs (46).

This study was designed to investigate the response of *P. aeruginosa* to bile in Artificial Sputum Media (ASM) cycled over an extended period. Increased production of pyocyanin (PYO) upon culturing in ASM supplemented with bile was initially observed and confirmed by transcriptional analysis. Over time, pigmented morphologies were observed to emerge exclusively in bile treated cultures. Characterisation of these mutants revealed a loss of AQ signalling and PYO

production, perhaps a consequence of elevated community levels produced in response to bile. Whole genome sequencing and single nucleotide polymorphism analysis revealed key genetic changes in the pigmented variants, including point mutations in the quorum sensing regulator *lasR*. The pigmented mutants were found to be unresponsive to bile with respect to biofilm formation and redox potential suppression. Furthermore, production of the Alkyl-Quinolone PQS was found to be abolished in the red and brown pigmented variants. Defective AQ production was found to underpin the abolished redox repression in the presence of bile. These findings implicate a role for AQs in the bile induced shift in redox potential with additional unidentified factors likely to be involved in the altered biofilm response to bile on the pigmented mutants.

Materials and Methods

Bacterial culture

Strains and plasmids utilised in this study are outlined in Table 1 below. All cultures of *P. aeruginosa* were routinely grown in Tryptic Soy Broth (TSB) media or Luria-Bertani broth (LB) with shaking at 180 rpm at 37˚C. Strains were maintained on Tryptic Soy Agar (TSA) or Luria-Bertani agar (LBA). For the purpose of the antibiotic resistance assays Mueller-Hinton (MH) agar or broth was used. Bovine bile at a concentration of 0.3% (w/v) was added to media either prior to autoclaving at 105˚C for 30 min or after autoclaving by filter sterilisation with a 0.2 µM filter.

Table 1; Description of strains and plasmids utilised in the study.

Strain/plasmid	Description	Source
PA14	Wild type	(47)
PA14::*mutS*	PA14 harboring mariner Tn7 transposon insertion in PA14_17500 (*mutS);Gm^R*	(47)
PA14::*phzM*	PA14 harboring mariner Tn7 transposon insertion in PA14_09490 (*phzM);Gm^R*	(47)
PA14::*phzS*	PA14 harboring mariner Tn7 transposon insertion in PA14_09400 (*phzS);Gm^R*	(47)
PA14::*hmgA*	PA14 harboring mariner Tn7 transposon insertion in PA14_38510 (*hmgA);Gm^R*	(47)
PA14::*pqsA*	PA14 harboring mariner Tn7 transposon insertion in PA14_51430 (*pqsA);Gm^R*	(47)
PA14::*pqsC*	PA14 harboring mariner Tn7 transposon insertion in PA14_51410 (*pqsC);Gm^R*	(47)
PA14::*pqsE*	PA14 harboring mariner Tn7 transposon insertion in PA14_51380 (*pqsE);Gm^R*	(47)
PA14::*pqsH*	PA14 harboring mariner Tn7 transposon insertion in PA14_30630 (*pqsH);Gm^R*	(47)

Table 2; Primers utilised in this study.

Primer Name	Sequence 5'-3'	Reference
PA14_46660 F	AGCCAGCGAAGACCTGTTCG	This study
PA14_46660 R	GGCGAACATGGTCGACCAGT	This study
aer F	CGCTGATCGCGCAGATGTAC	This study
aer R	TCGGCGATGCCCTTGATCAC	This study
sppR F	AACCAGCGCCTGCAACTGGT	This study
sppR R	AAGTCCGGCGAGGTGAAGGA	This study
fha1 F	TTCGCCGAACTGCTCGGCAA	This study
fha1 R	CGGGTTGTTCTGCACAGGCT	This study
cupB5 F	ATGTTCGGCCACGTCTCCGA	This study
cupB5 R	CGCTGGCATCCACGTTGAAC	This study
PA14_15200 F	CACGACCTGGCGTTCCTCAA	This study
PA14_15200 R	GCCAGCATTACTGCGCCGAT	This study
PA14_70580 F	TCAAGGTCGCCGACCTGATG	This study
PA14_70580 R	AGGTCCAGCAACTGGCTCTG	This study
PA14_25030 F	TCGAGTACCGCATCACGCTG	This study
PA14_25030 R	CATCAGGTACAGCGGCACCA	This study
gnd F	CGGCGAAGCTGGTGATCATC	This study
gnd R	GGTCGAGGTCGAGGTAGTTG	This study
PA14_00970 F	CGAAGCGCAGATGGAAGCCA	This study
PA14_00970 R	TCAGGAACGCCGTAGTCGAG	This study
PA14_57610 F	CCGAGAACCCTGTTCAGCTC	This study
PA14_57610 R	CGTACTCGTCGCCATCCAGT	This study
pchF F	ACGCCACGGTGATCCACGAT	This study
pchF R	GTGGAGCCGGAGGTGTAGAT	This study
PA14_32015 F	TCGACCAGAGCTTCATCGCC	This study
PA14_32015 R	AGGCTACGTCGGATGCGCTT	This study
hmgA F	GGAAGCAGGTCTCGTTGAGC	This study
hmgA R	AGTCGAGGTTCATCTGAGGC	This study
phzM F	TTCCGCAACGAGATCCAG	This study
phzM R	CGTTCGTCAACGTCATCG	This study
phzS F	CGGTGGATAACCGAATGCGG	This study
phzS R	TGGCGTCTTCGTTCCTGGTC	This study
lasR F	GATATCGGGTGCCGAATC	This study
lasR R	TAGAAGGGCAAATTACCG	This study
Taq-proC F	CTTCGAAGCACTGGGTGGAG	This study
Taq-proC R	TTATTGGCCAAGCTGTTCG	This study
Taq-*phzS* F	CTGGGCTGGTTCGACATC	This study
Taq-*phzS* R	CGGGTACTGCAGGATCAACT	This study
Taq-*phzM* F	ACCTGCTGAGGGATGTCG	This study
Taq-*phzM* R	GAACTCCTCGCCGTAGAACA	This study
Taq-*phzH* F	GGTGTTCGGCATCGTTTC	This study
Taq-*phzH* R	GTACCCCTCCGGATGCTC	This study

Pyocyanin assay in ASM

Overnight cultures of *P. aeruginosa* were adjusted to an OD_{600nm} 0.05 in ASM with and without 3%, 0.3% and 0.03% bovine bile (w/v) and incubated at 37˚C with shaking at 180 rpm. After 24 h and 96 h, test cultures (10 mL) were centrifuged at 5000 rpm for 15 min to obtain a cell free supernatant. Chloroform (3 mL) was added to the cell free supernatant, vortexed and centrifuged for 5 min at 5000 rpm. The bottom blue phase was transferred to a tube containing 2 ml of 0.2 M hydrochloric acid, vortexed and centrifuged at 5000 rpm for 5 min. The absorbance of the top pink phase was read at $OD_{520nm.}$

Pyocyanin and Alkyl quinolone extraction

Overnight cultures of *P. aeruginosa* were adjusted to an OD_{600nm} 0.02 in 20 mL TSB with or without 0.3% (w/v) bovine bile and incubated at 37˚C with shaking at 180 rpm for 8 h. Culture was centrifuged at 5000 rpm for 15 min to obtain a cell free supernatant with resulting supernatant split into two 10 mL tubes. Pyocyanin was extracted as described in the pyocyanin assay conducted for ASM. Alkyl quinolones were extracted by addition of 10 mL of acidified ethyl acetate followed by vortexing and centrifugation for 5 min at 5000 rpm. The top clear phase was transferred to a fresh tube and stored at -20˚C overnight. Rotary evaporation was completed to remove the solvent with extracts re-suspended in 1 mL of methanol for analysis by thin layer chromatography.

Thin layer chromatography

A normal phase silica TLC plate was prepared by immersing in a solution of potassium phosphate monobasic for half an hour. The TLC plate was activated in a hybridisation oven for 1 h at 100˚C. 20 µL of extract was spotted onto the prepared TLC plate with synthetic PQS/HHQ

loaded as controls. The plate was developed in 95:5 dichloromethane:methanol and visualised under UV light.

Cycled culture of *P. aeruginosa* in ASM

ASM cultures were initiated by inoculation of 2 x 10^6 cells from an overnight culture of a PA14 *mutS* transposon mutant into 20 mL ASM in the presence and absence of 3% (w/v) bovine bile. The *mutS* transposon mutant was selected due to the mutation in its DNA repair system resulting in enhanced mutation frequency therefore speeding up the rate of mutation and experimental outcome (48). Cultures were incubated at 37˚C static for 96 h with transfer (1:100) into fresh ASM media after mechanical homogenisation. Transfers were repeated for 180 days (encompassing 45 transfers and approximately 315 bacterial generations).

Colony morphology assay

3 µL of overnight TSB cultures were spotted on to TSA with or without 0.3% (w/v) bovine bile. The spot was allowed to dry at room temperature before incubation at 37˚C overnight. Plates were transferred back to room temperature, with colony morphology monitored and recorded over a period of 7 days.

Pigment extraction

Strains of interest were inoculated at an O.D of 0.05 in 50 mL of MH in a 250 ml conical flask for 48 hrs with visual indication of pigment production. Pigments were successfully extracted by centrifugation of 40 mL of culture at 5000 rpm for 10 min. 10 mL of ethanol was added to 2 mL of the cell free culture supernatant. The supernatant/ethanol mixture was centrifuged at max speed for a further 10 min. The resulting supernatant was rotary evaporated with compounds collected using methanol. A preparative TLC was undertaken.

Biofilm assay

Overnight cultures were adjusted to an $O.D_{600nm}$ 0.05 in TSB in the presence and absence of bile. 1 mL was transferred in to 24-well plates and incubated static at 37˚C overnight. Biofilm formation was measured by removing spent culture by pipetting. Wells were washed with water by gentle pipetting to remove any unattached biofilm. Attached biofilm was measured by staining for 30 min with 1 mL of 0.1% (w/v) crystal violet. 100% (v/v) ethanol was used to solubilise the crystal violet followed by a measurement of the absorbance at a wavelength of 595nm.

Antibiotic resistance assay

Overnight cultures of *P. aeruginosa* were adjusted to 0.5 MacFarland units in MH broth. MH agar plates supplemented with or without 0.3% (w/v) bile were uniformly swabbed with culture. E-strips (Thermo Scientific) were placed on to the surface of the agar manually. Plates were left to incubate at 37˚C overnight after which the diameter of the zone of inhibition was recorded.

Swarming motility assay

Swarming motility was measured on LB 0.8% (w/v) agar or 0.6% (w/v) Eiken Agar in the presence and absence of 0.3% (w/v) bile. Sterile toothpicks were used to gently inoculate a single colony onto the surface of the Eiken Agar with minimal pressure. Plates were incubated overnight for 1-2 days with degree of motility visualised and recorded.

Redox assay

Overnight cultures of *P. aeruginosa* were adjusted to $O.D_{600nm}$ 2.0 with 200 µL of adjusted cultured added to 25 mL of TSB supplemented with 0.01 mg/ml of tetrazolium violet (Sigma

Aldrich). Cultures were incubated at 37˚C shaking at 180 rpm for 24 h. Formazan production was measured by centrifuging 5 mL of culture at 5,000 g for 5 min. The supernatant was discarded with the pelleted cells re-suspended in 1.2 mL of dimethyl sulfoxide and centrifuging again at 5,000 g for 5 min. The $O.D_{510nm}$ was recorded of the cell free supernatant.

Whole genome sequencing

Genomic DNA was extracted from bacterial strains of interest using a Gentra PureGene DNA Extraction Kit (QIAGEN) with isolated DNA re-suspended in sterile water. Paired end sequencing was conducted by Eurofins Genomics using Illumina MiSeq V3 with 2x300bp reads. Reads were mapped to the *P. aeruginosa* UCBPP-PA14 NC_008463.1 reference genome and delivered as BAM and BAI files. Further sequence analysis for SNP identification was conducted using the Integrative Genome Viewer software platform using the reference strain UCBPP-PA14 and the more recent PA14 Or genome sequence (NZ_LT608330.1).

Statistical Analysis

Data presented is the average of at least three independent biological replicates. Statistical analysis was performed by student's t-test (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$).

Results

Pyocyanin production is elevated in ASM supplemented with bile

Bile has emerged as a central host factor capable of modulating behavioural changes in the key opportunistic respiratory pathogen *P. aeruginosa* (39, 45). Transcriptomic analysis conducted in the presence of bile has previously revealed changes in the expression of an array of quorum sensing and PYO related genes (39). Furthermore, the shift in redox towards that of decreased reducing potential in the cell observed in that study was notable considering the inherent relationship between PYO (and other phenazine pigments) and the maintenance of redox homeostasis within the cell. Therefore, we investigated the impact of bile on PYO production in *P. aeruginosa* PA14 grown in ASM, which is known to mimic conditions found within the CF lung environment.

Bile, at various sub-inhibitory concentrations was shown to significantly increase production of PYO in *P. aeruginosa* in ASM when compared to untreated ASM cultures (**Fig. 2A**). Induction was evident at 24 h incubation and was found to be most pronounced at 96 h in the presence of 3% (w/v) bile. Gene expression analysis as measured by qRT-PCR (primers outlined in Table 2) analysis of three branch point PYO biosynthetic genes (*phzH, phzM, phzS*) revealed a significant increase in the expression of *phzS* in bile treated ASM cultures (**Fig. 2B**). A trend towards increased expression of *phzH* and *phzM* was also observed although it did not reach statistical significance. PhzS, encoding a flavin-containing monooxygenase, is the final gene of the PYO biosynthetic pathway and is responsible for the conversion of 5-methylphenazine-1-carboxylic acid betaine to PYO. The increased expression of this gene may therefore underpin the overproduction of PYO following *P. aeruginosa* exposure to bile.

Growth curve analysis was undertaken to investigate the impact that the accumulation of PYO within the community may have on growth rate. An upper range concentration of 50 μg/ml, representative of the elevated levels observed in bile treated cultures, was selected. A recent study by Meirelles and co-workers has shown that elevated levels of PYO can have both beneficial and detrimental effects on *P. aeruginosa* populations (49). Consistent with these findings we found that a concentration of 50 μg/ml of PYO reduced the growth rate and resulting biomass accumulation of *P. aeruginosa* (**Fig. 2C**). To investigate the effects of longer-term bile exposure on PYO production, *P. aeruginosa* was serially transferred into fresh ASM at 96 h intervals. Subsequent PYO analysis following three rounds of 96 h growth confirmed that PYO levels remain elevated following the prolonged exposure to bile (**Fig. 2D**). Therefore, it was essential to understand the mechanism(s) by which *P. aeruginosa* might adapt to these elevated, toxic levels of PYO which would potentially represent a selective pressure for the evolution of *P. aeruginosa* within a bile positive lung environment.

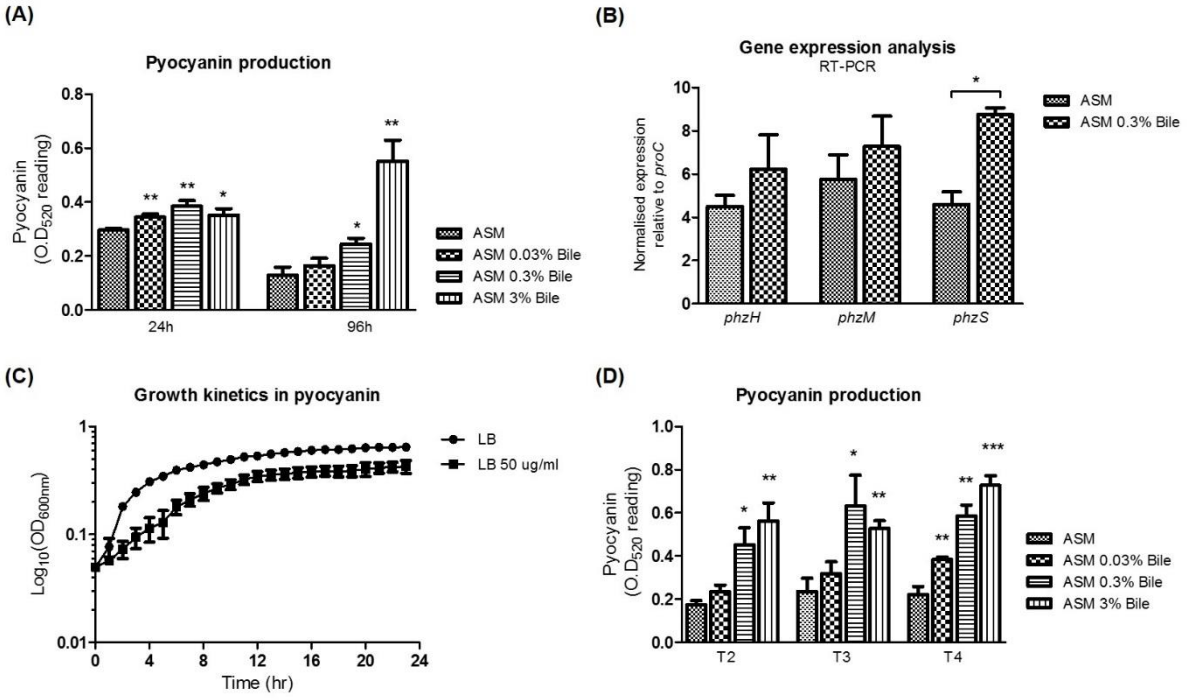


Figure 2. (A) The phenazine PYO is elevated in ASM supplemented with a range of bile concentrations (B) Enhanced PYO production is underpinned by increased *phzS* expression, the final step of the PYO biosynthetic pathway, as measured by RT-PCR analysis. No significant change in gene expression was observed for *phzH* or *phzM*, the two other central genes in PYO biosynthesis. (C) PYO, at a concentration of 50 µg/ml reduces the growth rate and biomass of *P. aeruginosa.* (D) Serially cycling *P. aeruginosa* in ASM supplemented with bile confirms the consistent up-regulation of PYO production over time.

Pigmented variants of *Pseudomonas aeruginosa* emerge exclusively in the presence of bile

Upon confirmation that PYO levels remained elevated upon sequential transfer of *P. aeruginosa* in ASM supplemented with bile, serial transfers were performed for a duration of 6 months encompassing approximately 45 transfers outlined in Figure 3 below (n=3). Regular sampling was undertaken in order to monitor the emergence of phenotypic variants and to assess whether these variants are stably retained in the population. As described in studies by Wassermann et al, a *mutS* transposon mutant was selected for the analysis as disruption of this mismatch repair

protein is known to increase the spontaneous mutation frequency of the strain hence facilitating rapid *in vitro* evolution experimentation (48, 50-53). Furthermore, the high rate of *mutS* mutation observed in clinical isolates of *P. aeruginosa* isolated from the CF lung environment further supports the use of this mutant *in vitro* (54-56).

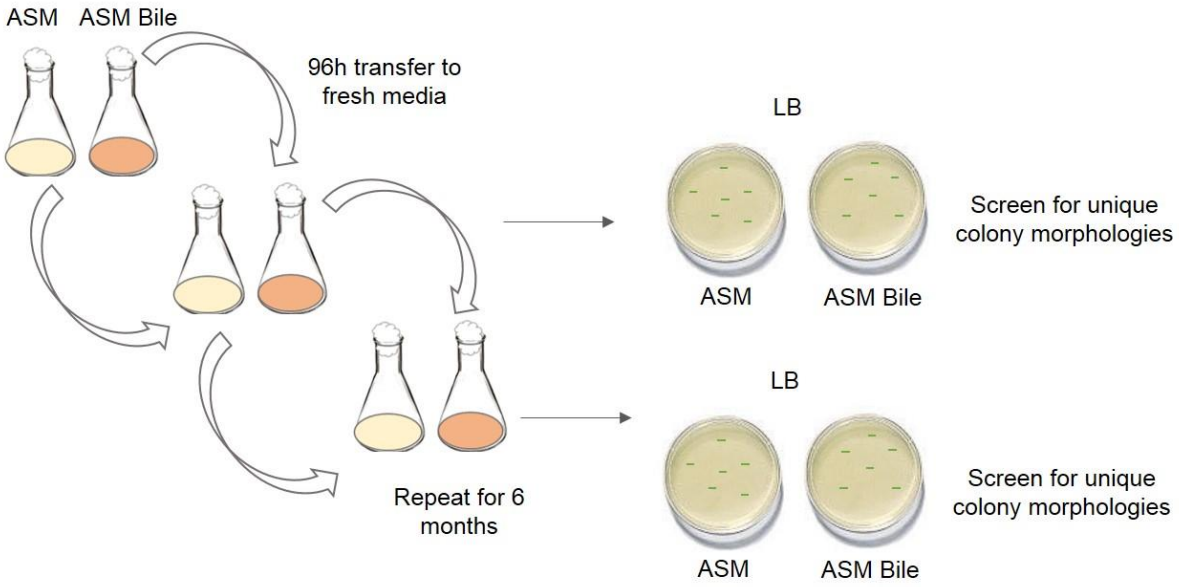


Figure 3. Outline of experimental design assessing the impact of bile and subsequent enhanced pyocyanin production on the evolution of *P. aeruginosa.* Serial transfer of culture was conducted every 96 h for the duration of 6 months with sampling of cultures at regular intervals to monitor phenotypic diversification in the presence of bile. Phenotypic analysis was conducted on end time point isolates.

Biofilm analysis of 9 randomly selected isolates from untreated ASM and 9 randomly selected isolates from bile treated ASM obtained from 3 independent biological replicates revealed that bile adapted ASM isolates displayed elevated biofilm levels relative to untreated ASM isolates (**Fig. 4**). Consistent with the findings of Winstanley and colleagues, swarming motility appeared to be dramatically reduced in the ASM colonies, with an apparent selection for the maintenance

of swarming motility in several of the bile treated ASM colonies. These findings were further confirmed by a global analysis of isolates taken from the final replicate of the adaptive experiment encompassing 48 colonies from ASM treated bile and 48 colonies from untreated ASM. The same pattern of enhanced biofilm and retention of swarming motility was observed (**Appendix Figure 1 & 2**). This suggests that the presence of bile in the environment selects for isolates adapted towards enhanced biofilm formation. This analysis was considered to be representative of all three experimental replicates for which the emergence of pigmented variants were observed.

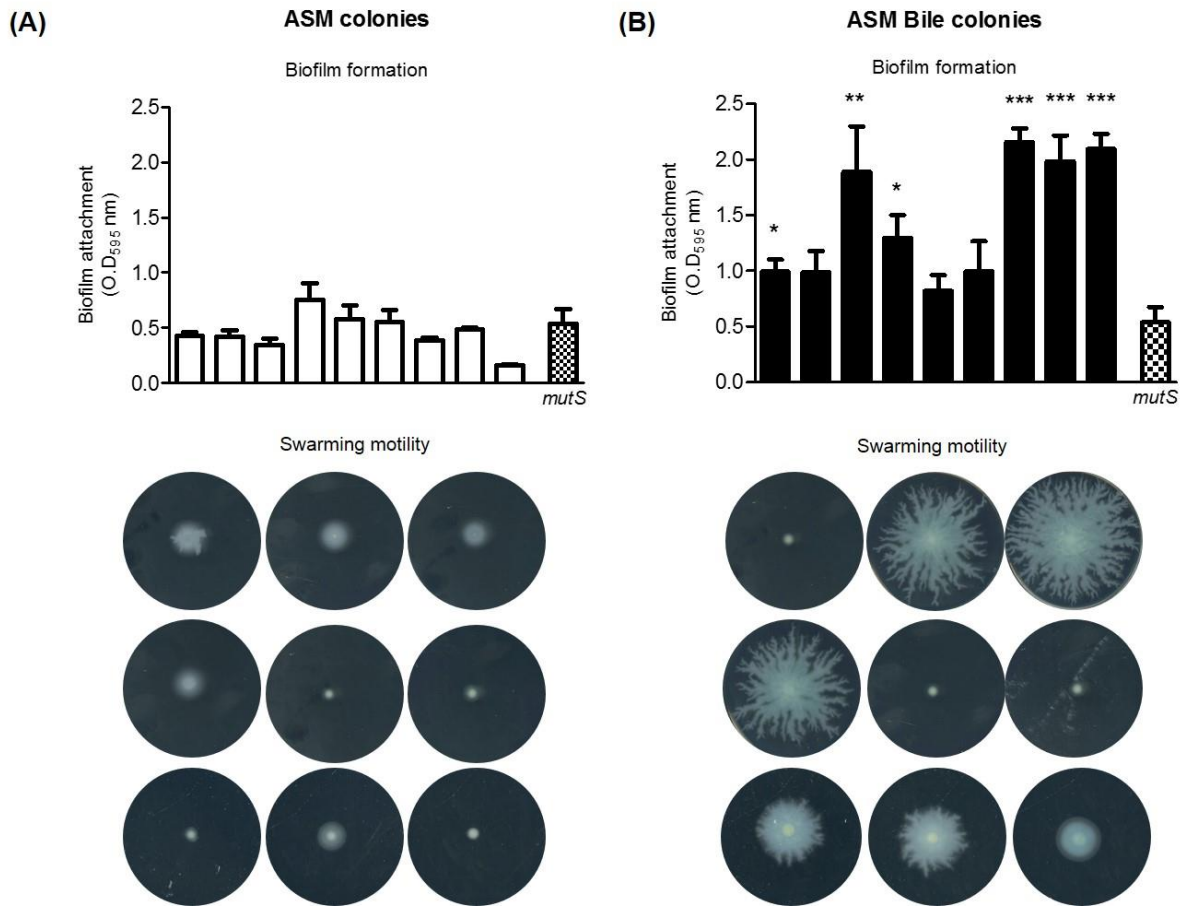


Figure 4. Biofilm formation and swarming motility in 9 colonies isolated from (A) untreated ASM and (B) bile treated ASM. Colonies recovered from untreated ASM display biofilm levels comparable to that of the ancestral progenitor strain with a loss of the swarming motility phenotype. In contrast, colonies recovered from bile treated ASM exhibit enhanced biofilm formation relative to the ancestral progenitor strain with a retention of swarming motility.

The bile responsiveness of the strains from the global analysis was also investigated. We have previously established that *P. aeruginosa* exhibits increased biofilm formation in the presence of bile therefore it would be expected that UT isolates which have had no previous exposure to bile would behave in this way. The majority of the UT colonies (80%) displayed a significant increase in biofilm formation in the presence of bile (**Fig. 5**). Therefore, the biofilm response to bile of these isolates has not been affected during its adaptation within ASM.

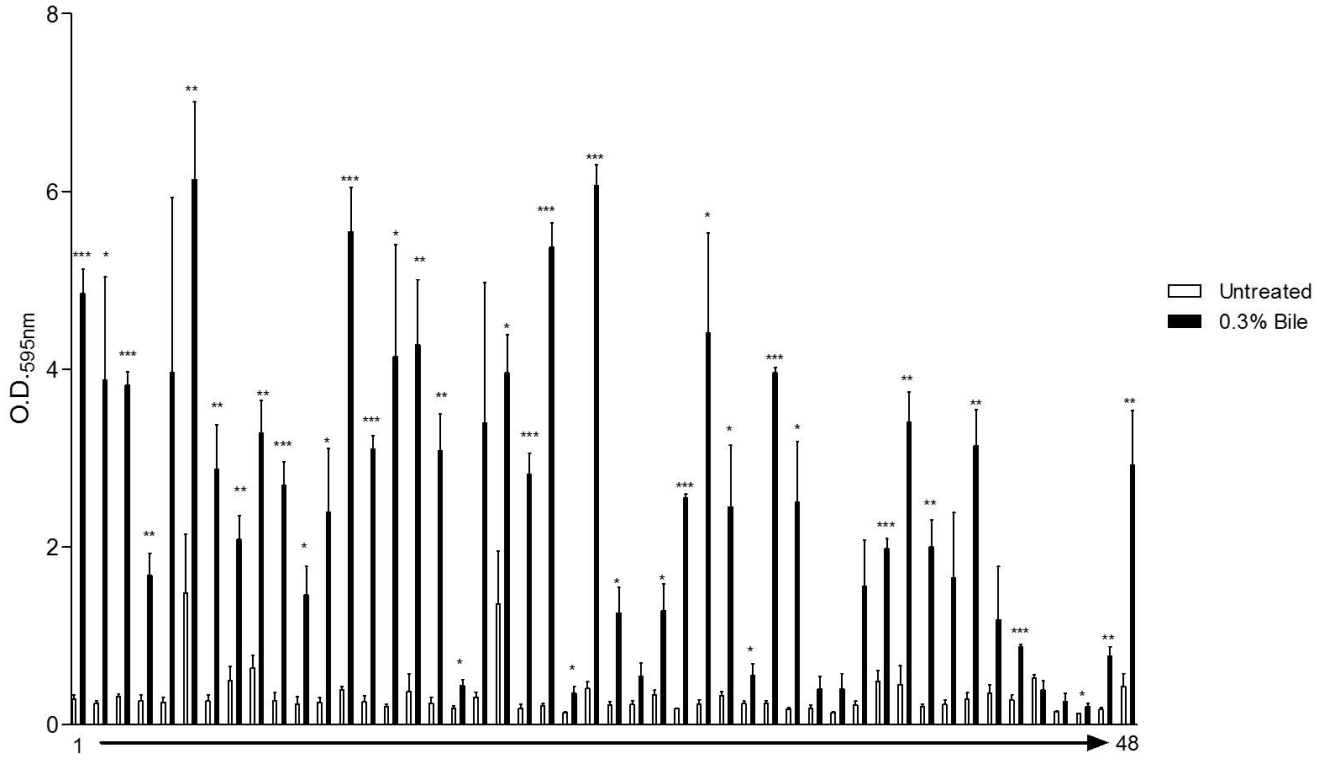


Figure 5. Biofilm formation of untreated ASM isolates in the presence and absence of 0.3% (w/v) bile highlighting that the majority of colonies recovered from untreated ASM retain their responsiveness to bile.

Regarding the BT isolates, as the screen showed that many of the isolates display a hyper-biofilm phenotype, it was important to establish whether biofilm formation could be further induced or has a bile adaptive mutation locked these isolates in to a hyper-biofilm active state. In contrast to UT isolates, the majority of BT colonies (71%) biofilm formation was not significantly increased in the presence of bile (**Fig. 6**). As mentioned above, this is possibly due to the already elevated biofilm in the untreated strains. Taken together this analysis highlights that the process of adaptation in an ASM bile environment results in the selection of isolates that favour biofilm overproduction.

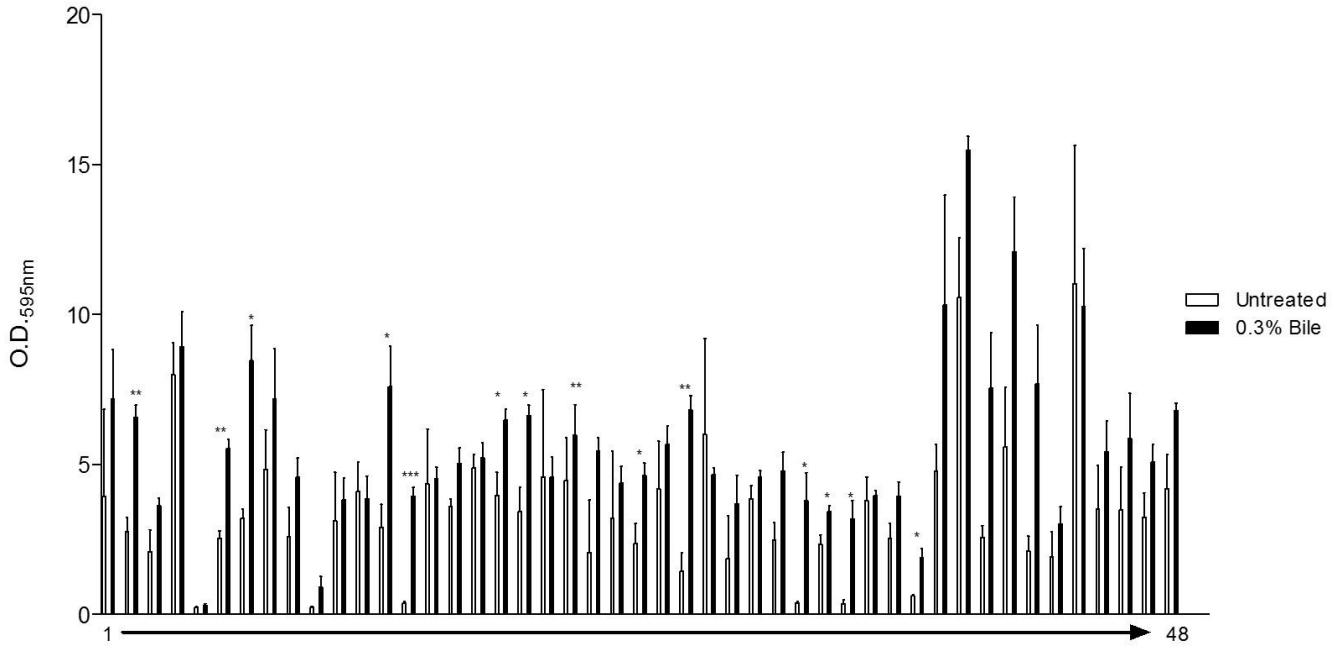


Figure 6. Biofilm formation of colonies recovered from bile treated ASM isolates in the presence and absence of 0.3% (w/v) bile. The majority of these colonies do not exhibit a statistically significant increase in biofilm formation in the presence of bile which may be attributed to the prior adaptation to bile.

Swarming motility in the UT and BT isolates was also investigated, due to its relationship with biofilm formation. Global screen analysis was conducted on LB 0.8% (w/v) agar (**Fig. 7**) with validation carried out on Eiken agar which is the media more routinely used for the testing of swarming motility in *P. aeruginosa.* For the majority of UT colonies there is an apparent loss of swarming motility on LB 0.8% (w/v) agar i.e. strains appear to be non-motile (**Fig. 7**). Interestingly, even those isolates which appeared to be swarming on LB 0.8% (w/v) agar, upon validation on Eiken agar the resulting motility appeared to resemble a more swimming like motility rather than that of the classical swarming motility which is characterised by the

formation of tendrils. Furthermore, unlike in the ancestral strain where bile represses swarming motility there is little to no repression of motility in the UT colonies which relates back to the apparent loss of swarming motility even in the absence of bile (Appendix Figure 2). The majority of BT colonies retained their ability to swarm on LB 0.8% (w/v) agar (**Fig. 7)** with a repression of this motility observed in the presence of bile (Appendix Figure 2). It is noteworthy that although the BT colonies exhibit increased biofilm production, there also appears to be a strong selection for the retention of swarming motility in ASM supplemented with bile. This differs from reports of the inverse nature of the relationship between biofilm formation and swarming motility and should be further investigated.

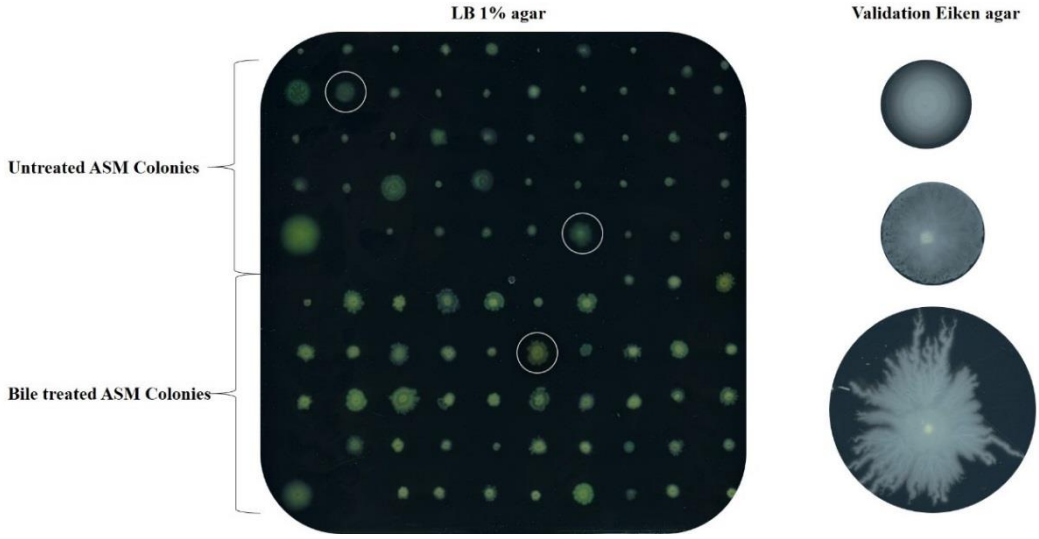


Figure 7. Swarming Motility of 48 strains isolated from untreated ASM and 48 strains isolated from ASM supplemented with bile on LB 1% (w/v) agar with a representative validation of colonies on Eiken agar. Colonies isolated from ASM supplemented with bile retained their ability swarm while those isolated from untreated ASM appeared to become swarming deficient.

Colony morphology analysis revealed the emergence of uniquely pigmented variants of *P. aeruginosa* derived from ASM in the presence of bile, with yellow, red and brown pigmented derivatives observed (**Fig. 8**). The yellow pigmented variant was the first to emerge, followed by the brown and red pigmented variants, respectively. The yellow pigmented variant emerged after approximately 40 generations, the brown pigmented variant emerged after approximately 120 generations, followed by the red pigmented variant which appeared after approximately 144 generations.

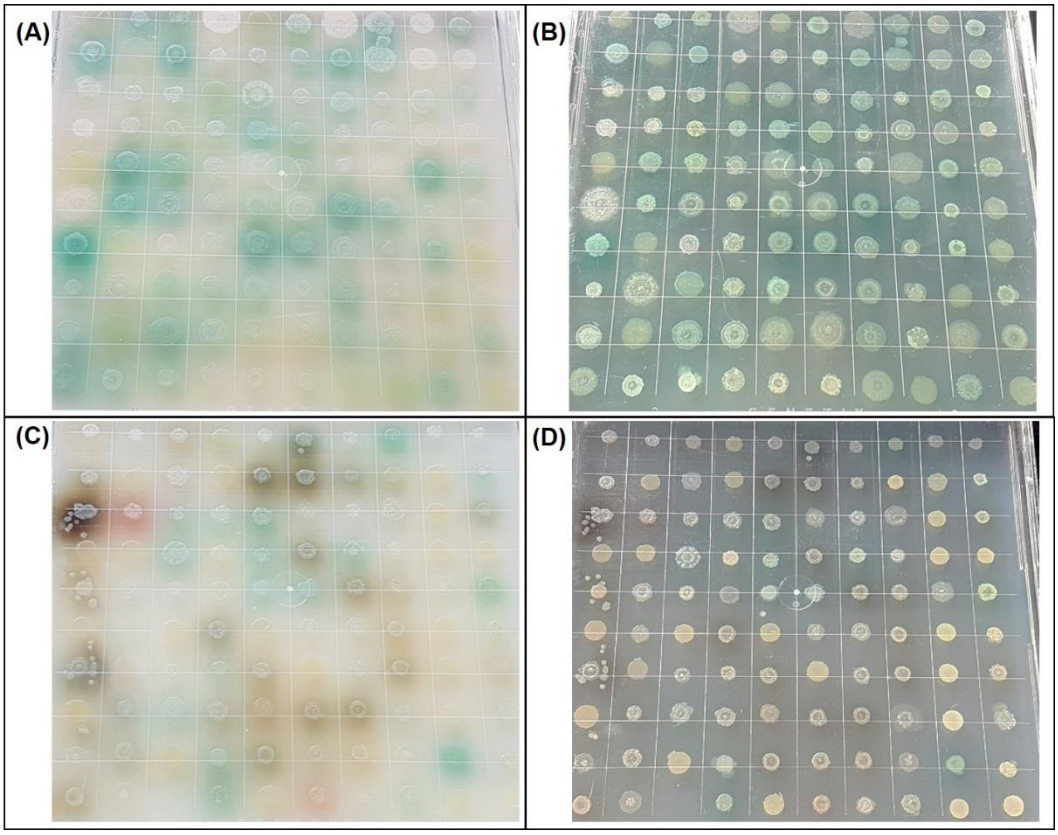


Figure 8. Colony morphology analysis on LB agar of (A) UT colonies top view (B) UT colonies bottom view (C) BT colonies top view and (D) BT colonies bottom view. By comparing both the top and bottom view images the colony pigment status can be designated.

The yellow pigmented variant became the most dominant member of the community representing approximately 60% of the population with the brown and the red variants comprising approximately 10% and 5% of the population respectively upon completion of the experiment. Interestingly, though the brown and red derivatives were maintained in the community, they did not significantly increase in abundance suggesting that though their presence is an advantage there may be some fitness cost preventing them from outcompeting within the population. The apparent overexpression of a green pigment potentially the phenazine pyocyanin in the presence of bile may contribute to the appearance of alternative pigmented variants of *P. aeruginosa.*

Colony morphology analysis revealed the unique pigment production of BT colonies. In contrast to the bile treated ASM cultures, the majority of colonies obtained from untreated ASM appear to either retain green pigmentation, most likely a result of intact phenazine production, or lose pigmentation completely. Furthermore, colony morphologies of the untreated ASM cells appeared larger and more rugose than their bile treated counterparts, which were smaller and more smooth (**Fig. 8**).

Genotypic profiling of the mutants to characterise the genetic changes underpinning the pigmented variants

As the pigmented derivatives emerged exclusively in the presence of bile, red pigmented variants (n=3), brown pigmented variants (n=3) and yellow pigmented variants (n=3) were selected for further analysis in order to elucidate their functional importance in the presence of bile. Colony morphology analysis confirmed the stable production of the brown/red/yellow pigment which appears to be extruded from the colony (**Fig. 9A**). However, in the presence of bile there appears to be a further increase in the production of these pigments. Therefore, though a mutational event

likely underpins the production of these alternative pigments, the regulatory pathway governing the regulation of pigment production appears to still be responsive to bile (**Fig. 9B**).

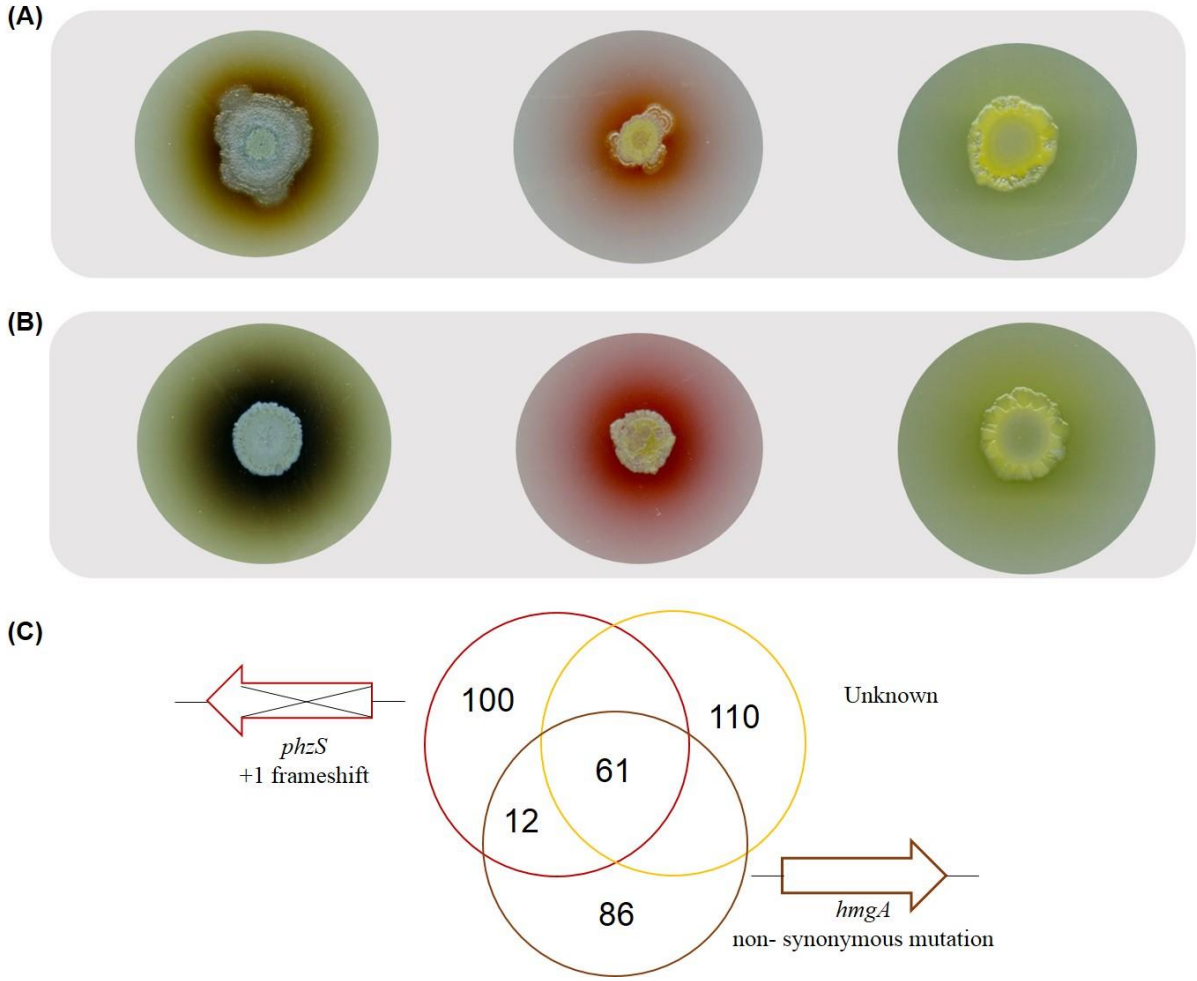


Figure 9. (A) Representative colony morphology analysis of the brown, red and yellow pigmented variants on TSB agar and (B) TSB agar supplemented with 0.3% (w/v) bile highlighting pigment production with further induction in the presence of bile. (C) Whole genome sequence analysis with strain PA14 Or as the reference strain.

Whole genome sequencing analysis was conducted on a representative red, brown and yellow pigmented isolate. Paired end sequencing was conducted by Eurofins Genomics using Illumina MiSeq V3 with 2x300bp reads. Reads were mapped to the *P. aeruginosa* UCBPP-PA14 NC_008463.1 reference genome and delivered as BAM and BAI files. Further sequence analysis

for SNP identification was conducted using the Integrative Genome Viewer software platform using the reference strain UCBPP-PA14 and the more recent PA14 Or genome sequence (NZ_LT608330.1). Sequence validation following PCR amplification using gene-specific primers outlined in Table 2 was carried out on selected target genes in the *mutS* ancestral strain. While all three isolates were found to have 294 SNP's in common when using the UCBPP-PA14 reference strain, this number was reduced down to 61 SNP's when using PA14 Or as a reference strain (**Fig. 9C**). This finding is important when considering the use of these reference genomes for SNP analysis of *P. aeruginosa* clinical isolates and highlights the potential importance of using multiple reference genomes in clinical genome analysis. An overview of the genetic events within each of the individual isolates is described in Table 3 with further information available in Appendix Table 1-5. The presence of mutations in common between the brown and red isolate suggest they derived from the same strain before diverging.

Table 3; Genetic events present in all three pigmented variants, the brown pigmented variant alone, the red pigmented variant alone, the yellow pigmented variant alone and in both the brown and red isolates.

	Coding Regions			**Intergenic**	
	Indels	**Nucleotide substitutions**		**Indels**	**Nucleotide substitutions**
		Synonymous	**Non-synonymous**		
Common	11	8	32	2	8
Brown	14	9	50	5	8
Red	7	23	52	4	15
Yellow	10	21	70	3	6
Brown/Red	0	3	7	2	0

Nucleotide substitutions outlined in Table 3 may be considered as synonymous or non-synonymous. Synonymous mutations do not cause a change to the encoded amino acid due to the redundancy of the genetic code and therefore will not affect the functionality of the protein and hence are considered silent mutations. Non-synonymous mutations cause a change to the encoded amino acid which have the potential to alter protein functionality depending on the location of the amino acid within the protein. All of the SNP's (mutation events) identified were mapped across the total PA14 genome (kb) to identify potential hot spots of mutation **(Fig. 10)**.

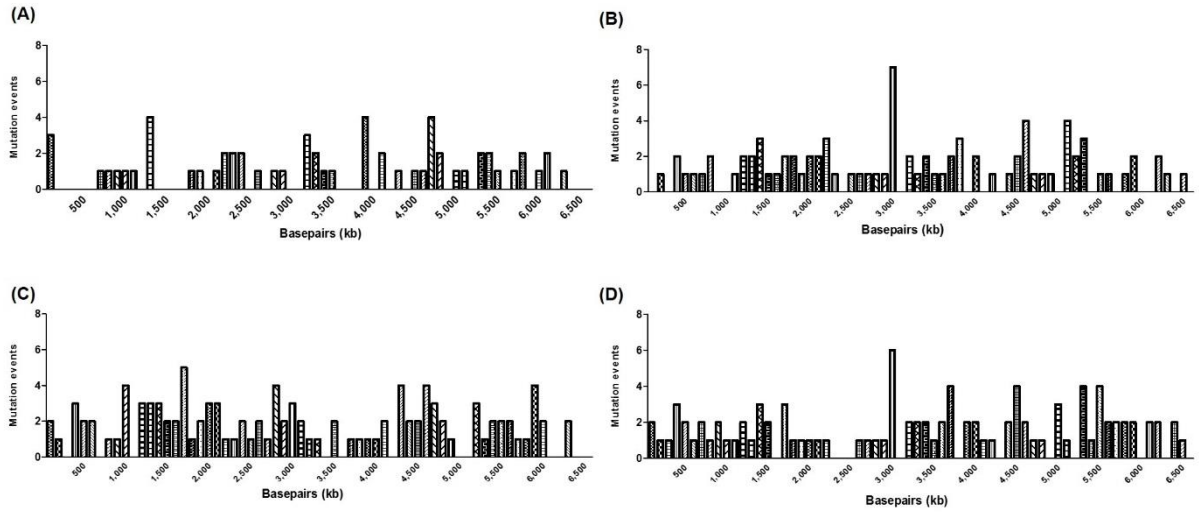
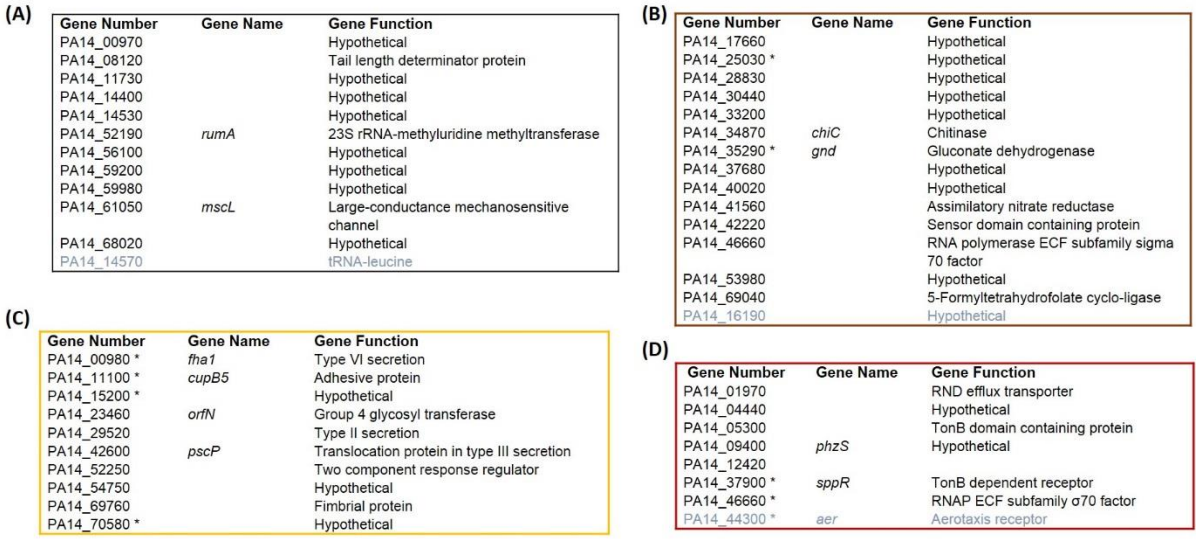


Figure 10. Mutation events across the PA14 genome in (A) All three pigmented derivatives (B) Brown pigmented derivative (C) Yellow pigmented derivative and (D) Red pigmented derivative.

Indels in coding regions represent the most important category as these will definitively alter the protein are outlined in Figure 11.



(A)

Gene Number	Gene Name	Gene Function
PA14_00970		Hypothetical
PA14_08120		Tail length determinator protein
PA14_11730		Hypothetical
PA14_14400		Hypothetical
PA14_14530		Hypothetical
PA14_52190	*rumA*	23S rRNA-methyluridine methyltransferase
PA14_56100		Hypothetical
PA14_59200		Hypothetical
PA14_59980		Hypothetical
PA14_61050	*mscL*	Large-conductance mechanosensitive channel
PA14_68020		Hypothetical
PA14_14570		tRNA-leucine

(B)

Gene Number	Gene Name	Gene Function
PA14_17660		Hypothetical
PA14_25030 *		Hypothetical
PA14_28830		Hypothetical
PA14_30440		Hypothetical
PA14_33200		Hypothetical
PA14_34870	*chiC*	Chitinase
PA14_35290 *	*gnd*	Gluconate dehydrogenase
PA14_37680		Hypothetical
PA14_40020		Hypothetical
PA14_41560		Assimilatory nitrate reductase
PA14_42220		Sensor domain containing protein
PA14_46660		RNA polymerase ECF subfamily sigma 70 factor
PA14_53980		Hypothetical
PA14_69040		5-Formyltetrahydrofolate cyclo-ligase
PA14_16190		Hypothetical

(C)

Gene Number	Gene Name	Gene Function
PA14_00980 *	*fha1*	Type VI secretion
PA14_11100 *	*cupB5*	Adhesive protein
PA14_15200 *		Hypothetical
PA14_23460	*orfN*	Group 4 glycosyl transferase
PA14_29520		Type II secretion
PA14_42600	*pscP*	Translocation protein in type III secretion
PA14_52250		Two component response regulator
PA14_54750		Hypothetical
PA14_69760		Fimbrial protein
PA14_70580 *		Hypothetical

(D)

Gene Number	Gene Name	Gene Function
PA14_01970		RND efflux transporter
PA14_04440		Hypothetical
PA14_05300		TonB domain containing protein
PA14_09400	*phzS*	Hypothetical
PA14_12420		
PA14_37900 *	*sppR*	TonB dependent receptor
PA14_46660 *		RNAP ECF subfamily σ70 factor
PA14_44300 *	*aer*	Aerotaxis receptor

Figure 11. Insertion/Deletions and premature stop codons (grey) in coding regions (A) All three pigmented derivatives (B) Brown pigmented derivative (C) Yellow pigmented derivative and (D) Red pigmented derivative.

Within the genome of the brown pigmented variant a single base pair substitution at nucleotide 984 of a T to a C was identified in the *hmgA* gene. This non-synonymous substitution results in a change of the amino acid phenylalanine (TTC) to leucine (CTC). This single base pair mutation was also located in the *hmgA* gene of the two other brown pigmented variants tested (**Fig. 12**).

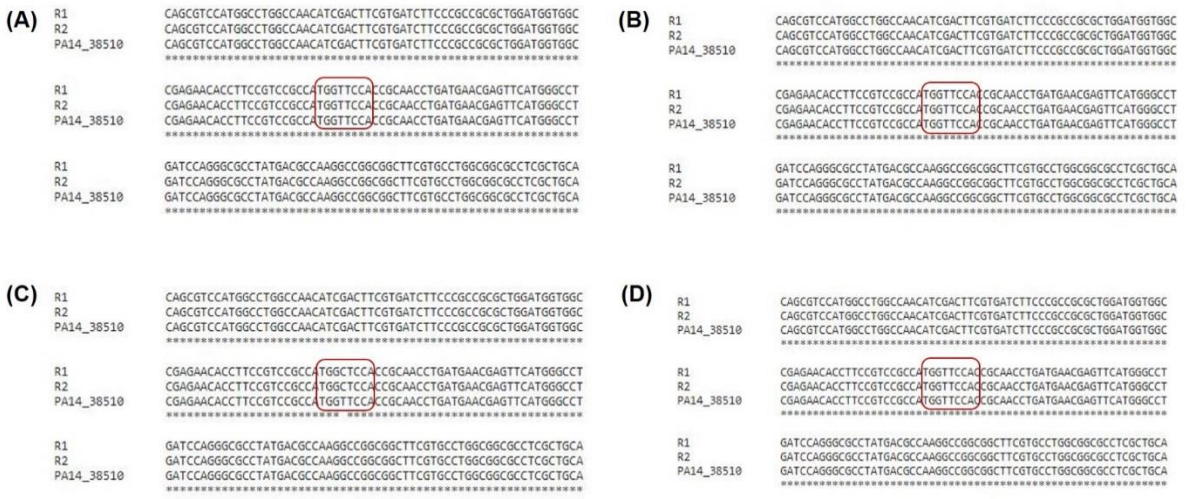


Figure 12. *hmgA* sequence anaylsis in (A) *mutS* original strain (B) red pigmented variant (C) brown pigmented variant and (D) yellow pigmented variant depicting the SNP exclusively present in the brown pigmented derivative.

Loss of function mutations in *hmgA* have previously been shown to result in production of the brown polymeric pigment pyomelanin by *P. aeruginosa* **(Fig. 14)** (57). Though there is no crystal structure available for *hmgA* in *P. aeruginosa*, a crystal structure has been described in *Pseudomonas putida.* Phenylalanine 328 is located in the binding pocket of the protein, with substitution of this amino acid to leucine likely to impact protein function. However, both phenylalanine and leucine are non-polar hydrophobic amino acids therefore definitively attributing production of the brown pigment to the SNP identified in this study would require protein structure analysis to determine whether the change amino acid would have a significant impact on the protein conformation and functionality.

Within the genome of the red pigmented variant sequence analysis revealed the insertion of a cytosine nucleotide at position 125 of the *phzS* encoded protein. The production of a red pigment in *P. aeruginosa* has previously been reported for *phzS* mutants, suggesting that this insertion underpins the pigmentation the red variant (58). The insertion of a cytosine at position 125 resulted in a +1 frameshift mutation which was confirmed by PCR sequencing (**Fig. 13**) and extended to all other red variants tested (data not shown). The emergence of a *phzS* mutation which would prevent the production and accumulation of pyocyanin could therefore be in response to the overexpression of this phenazine (**Fig. 14**).

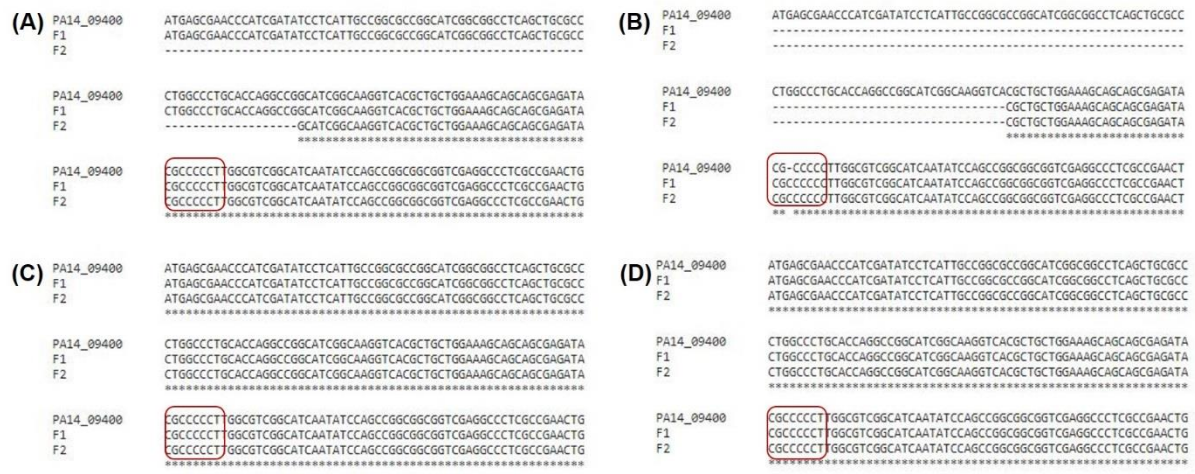


Figure 13. *phzS* sequence anaylsis in (A) *mutS* original strain (B) red pigmented variant (C) brown pigmented variant and (D) yellow pigmented variant highlighting the insertion of a C nucleotide exclusively in the red pigmented derivative.

Interestingly, subsequent plating of red pigmented variants consistently led to loss of pigmentation in a subset of colonies. Sequencing of these colonies revealed reversion back to the original ancestral sequence. This is consistent with previous studies describing a high frequency of reversion for +1 frameshift mutations in a *mutS* mutant (59). This finding validated that insertion of the single C nucleotide into the *phzS* sequence was responsible for production of the

red pigment in these mutants. Additionally, pigment extraction and preparative TLC analysis from a *phzS* transposon mutant and the red derivative further reinforced these findings with an RF value of 0.92 obtained for both the *phzS* mutant and the red pigmented isolate. LC mass spectrometry analysis would ideally be applied for definitive confirmation that these are the same pigment.

While the potential causative mutations could be identified for the red and brown pigmented variants, no likely mutation could be located that could underpin production of the yellow pigment, with *phzM,* which has previously been proposed to result in production of a yellow pigment, remaining unchanged in all strains.

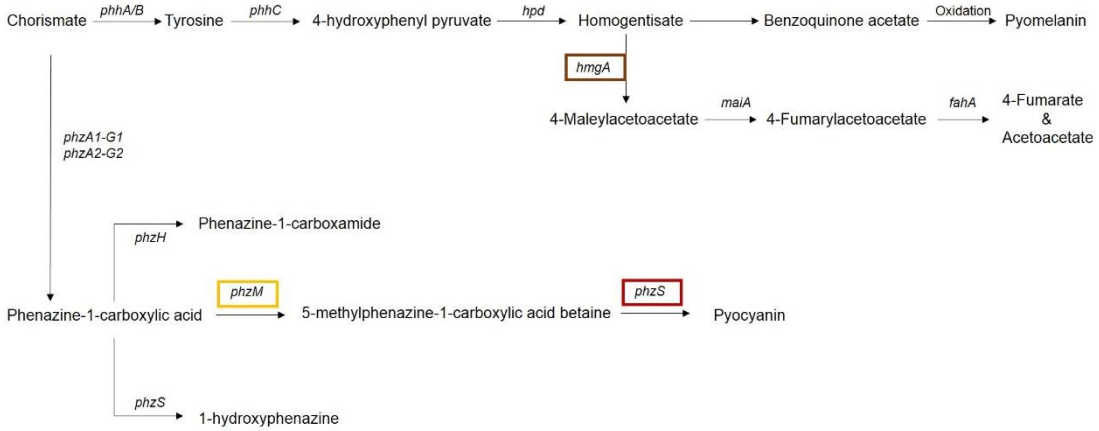


Figure 14. Genetic basis of the pyomelanin and pyocyanin biosynthetic pathway. All of the SNP's (mutation events) identified were mapped across the total PA14 genome (kb) to identify potential hot spots of mutation **(Fig. 10)**.

Phenotypic analysis of the pigmented variants

In order to further elucidate the importance of the emergence of the pigmented variants in the presence of bile red pigmented variants (n=3), brown pigmented variants (n=3) and yellow pigmented variants (n=3) were isolated for further phenotypic investigation. Phenotypic analysis

including, biofilm formation, swarming motility, antibiotic resistance, growth and redox was conducted in order to further understand the potential functionality of these isolates within the total community.

Biofilm formation

Biofilm formation was assessed in a crystal violet assay **(Fig. 15)**. The brown pigmented variants displayed a significantly higher biofilm formation relative to the *mutS* mutant strain in untreated media. However, in contrast to *mutS* there was no significant increase in biofilm observed in the presence of bile. This finding was also evident in the red pigmented variant, however only one red isolate displayed enhanced biofilm formation relative to the original strain. The behaviour of the yellow pigmented strains more closely resembled that of the control strain with enhanced biofilm formation in the presence of bile observed for two of the three isolates tested. Interestingly, even in the absence of bile these mutants were found to be hyper-biofilm formers, exhibiting enhanced biofilm formation relative to the ancestral strain, as measured by the crystal violet assay. Hence, supporting a transition towards a biofilm lifestyle in these isolates. However, there is a degree of phenotypic heterogeneity present amongst these isolates consistent with reports from other evolutionary adaptation experiments as well as strains recovered from the CF lung environment.

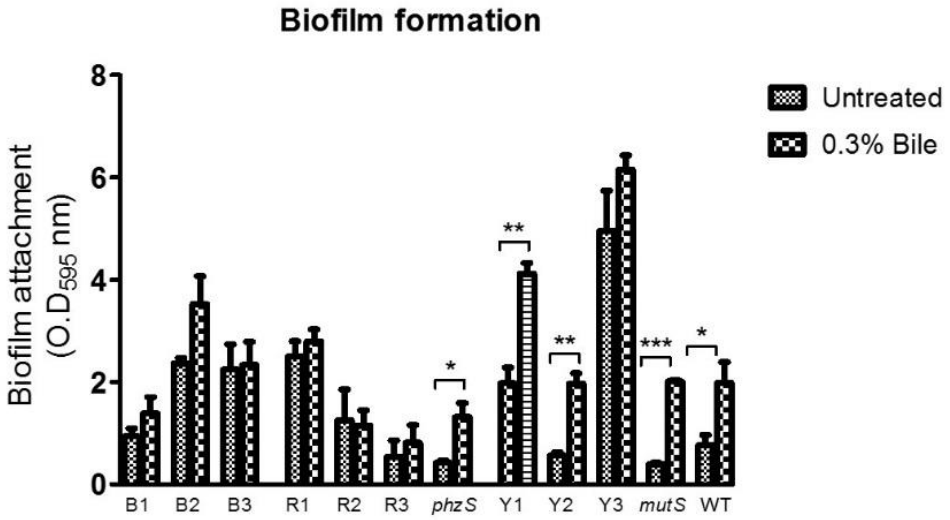


Figure 15. Biofilm formation in TSB in the presence and absence of bile in the pigmented derivatives. The red and bown pigmented derivatives displayed no significant increase to bile in the presence or bile while two of three yellow variants tested did.

Swarming Motility

The swarming motility of the respective pigmented variants was tested on Eiken agar and Eiken agar supplemented with bile **(Fig. 16)**. The three brown mutants displayed a form of motility that would not be strictly characterised as true swarming motility as there wasn't the formation of the classical swarming tendrils. Repression of motility in the presence of bile was also observed. Two out of three of the red pigmented variants were swarming mutants whilst one displayed reduced swarming capability. The yellow pigmented variants retained their capacity to swarm and displayed a marked reduction in swarming in the presence of bile as is observed in the *mutS* mutant ancestral strain.

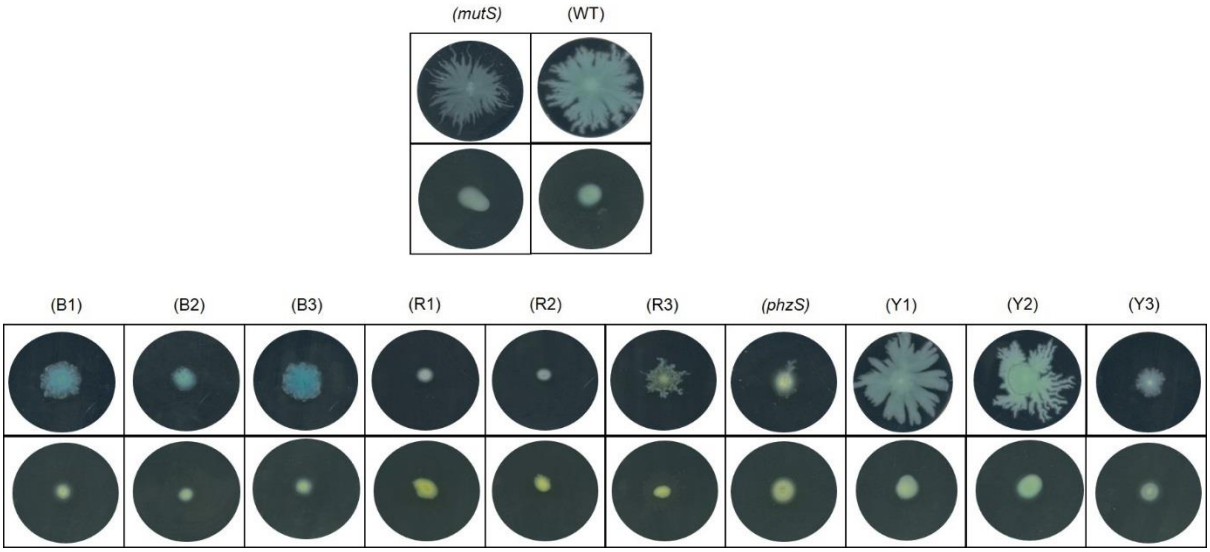


Figure 16. Swarming motility in the brown (B), red (R) and yellow (Y) mutants and the *MutS* transposon mutant. The brown pigmented derivatives did not display a classical swarming motility phenotype but there was a repression of this motility in the presence of bile. The red pigmented derivative was found to be a swarming mutants whilst the yellow pigmented derivative retained its ability to swarm with a repression of motility in the presence of bile.

Antibiotic resistance

P. aeruginosa isolates from the CF lung have been shown to display altered antibiotic susceptibility profiles. This is potentially mediated by exposure to antibiotics within the lung environment resulting in adaptation and increased resistance. We therefore wanted to investigate whether exposure to bile influenced antibiotic resistance profiles in our pigmented mutant derivatives. Three clinically relevant antibiotics, previously investigated in chapter 2 were examined; erythromycin, colistin and polymyxin B **(Fig. 17)**. Resistance to erythromycin in the untreated experiment was comparable in all pigmented derivatives with the increase in resistance in the presence of bile retained in all strains except R2 and Y2. The brown pigmented isolates were shown to have an even greater increase in resistance to erythromycin in response to bile relative to the *mutS* mutant suggesting that bile has selected for a genetic variant linked to

erythromycin antibiotic resistance. The brown and red pigmented variants retained the same antibiotic profile to the *mutS* mutant. The yellow mutants however displayed a reduction in their resistance to colistin and polymyxin in the presence of bile.

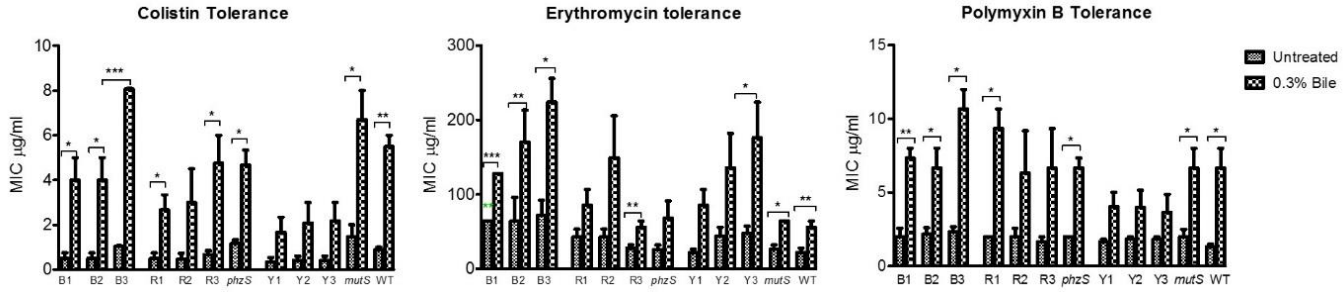


Figure 17. Erythromycin, colistin and polymyxin B resistance in the presence and absence of bile. The bile responsive profile of the red and brown pigmented derivatives was retained whilst the yellow pigmented derivative appeared to have lost its increase in resistance to the antibiotics tested in the presence of bile.

Redox

In elucidating the molecular mechanism underpinning the response of *P. aeruginosa* to bile, alterations in redox status appear intricately connected to the organism's ability to thrive in bile. Furthermore, the functional role of pigments in *P. aeruginosa* is linked to the maintenance of redox homeostasis within the cell. Therefore, it was imperative to investigate by a tetrazolium violet reduction assay whether the redox status of the cell was altered in comparison to the original ancestral strain. The perturbations of redox homeostasis appear intricately connected to the ability of *P. aeruginosa* to thrive in bile. Transcriptional profiling has previously shown changes in the expression of genes encoding metabolic pathways, while redox was shown to be repressed in the presence of bile (39). Furthermore, the functional role of pigments within *P. aeruginosa* is linked to the maintenance of redox homeostasis within the cell (60, 61). Therefore,

the redox status of the pigmented variants was investigated and compared to the original ancestral isolate. The *phzS* transposon mutant exhibited a similar response to the WT and *mutS* mutant, with a repression of redox in the presence of bile (**Fig. 18**). However, while redox potential in the pigmented variants was comparable to wild-type, *mutS*, and *phzS* mutants in the absence of bile, no reduction in potential was evident upon challenge with bile. This indicates that redox potential is 'locked in' in these variants and the metabolic changes underpinning its repression are not manifesting in these strains. Furthermore, similar to the antibiotic resistance and biofilm phenotypes, the modulation of chronic associated phenotypes in the red pigmented variants therefore cannot solely be attributed to its pigment production capabilities (**Fig. 18**).

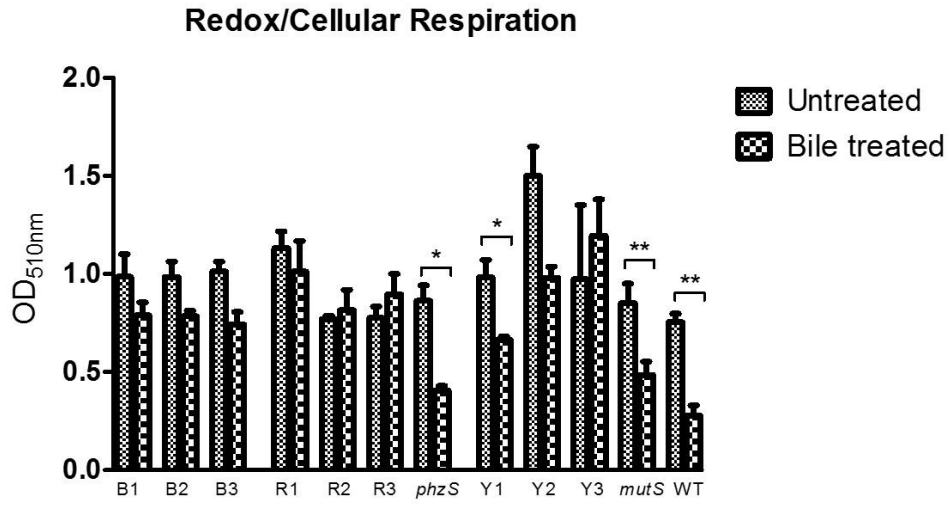


Figure 18. Redox assay of the brown, red and yellow pigmented variants. The brown and red pigmented variants no longer display a suppression of redox potential in the presence of bile with a varied response in the tested yellow variants.

Akyl quinolone signalling is central to an effective bile response in *P. aeruginosa*

The observation that pigmented variants no longer exhibited increased biofilm formation or reduced redox potential in the presence of bile led us to further investigate the possible mechanisms underlying this. The finding that a *phzS* mutant still retained an intact biofilm response to bile suggested that neither pigment production alone, nor loss of PYO production through mutation of *phzS*, were solely responsible. This raised the intriguing possibility that another signalling system was involved, potentially upstream of PYO, with the AHL-PQS axis considered a likely target. Evidence supporting this hypothesis was provided through the SNP analysis whereby LasR was found to exhibit a single amino acid change in both the red and brown mutants from independent replicates of the serial cycling experiment (Appendix Table 1-5). Loss of LasR functionality has been previously shown to result in decreased levels of PQS within the cell, with *lasR* mutations described in clinical isolates of *P. aeruginosa* isolated from the lungs of CF patients (32). Interestingly, the yellow pigmented mutants in which redox is still suppressed in the presence of bile, did not show any change in *lasR* sequence.

Analysis of PYO production revealed PYO production was abolished in the red and brown pigmented variants **(Fig. 19A)**. This is unsurprising in the red pigmented mutant given the loss of *phzS* activity. Analysis of AQ production by Thin Layer Chromatography of the red pigmented variant for which the underlying mutation has been identified revealed altered AQ profiles, consistent with their genotypic profile as putative *lasR* mutants. PQS production was abolished in these strains, while its biological precursor HHQ was retained, comparable to the profile of a *pqsH* mutant (**Fig. 19B**). Interestingly, increased HHQ production was observed in the presence of bile in these strains, indicating that autoinduction by PQS is not required to elicit this

response. It was also notable that both PQS and HHQ production was retained in the *phzS* mutant. (**Fig. 19B**).

From the community perspective, loss of PQS signalling might be expected to result in a less competitive sub-population of *P. aeruginosa*, considering its central role in virulence regulation and its emerging role as an inter-kingdom effector (62, 63). Though loss of PQS would result in reduced PYO levels within the cell, thus potentially addressing any toxicity issues arising from the induction of this pigment in ASM supplemented with bile, this would already be achieved by mutation of the *phzS* gene. Therefore, the role of PQS in response to bile may be more central to adaptation of the population, within what would appear to be an enhanced biofilm community.

To investigate this, several mutants affected for AQ signalling were tested for their biofilm and redox response to bile. Loss of the PQS biosynthetic genes did not result in the loss of bile induced biofilm formation. This suggested that HHQ and other AQs produced by the *pqsA-E* operon were not required for a functional bile-induced biofilm response (**Fig. 19C**). Interestingly, the loss of the PQS biosynthetic genes resulted in a loss of redox repression in the presence of bile with redox potential considerably higher than the WT counterpart in these mutants (**Fig. 19D)**. This finding would strongly implicate a role for alkyl-quinolones in the perturbations of redox potential observed in the presence of bile with the red and brown pigmented derivatives representing bile-adapted redox isolates.

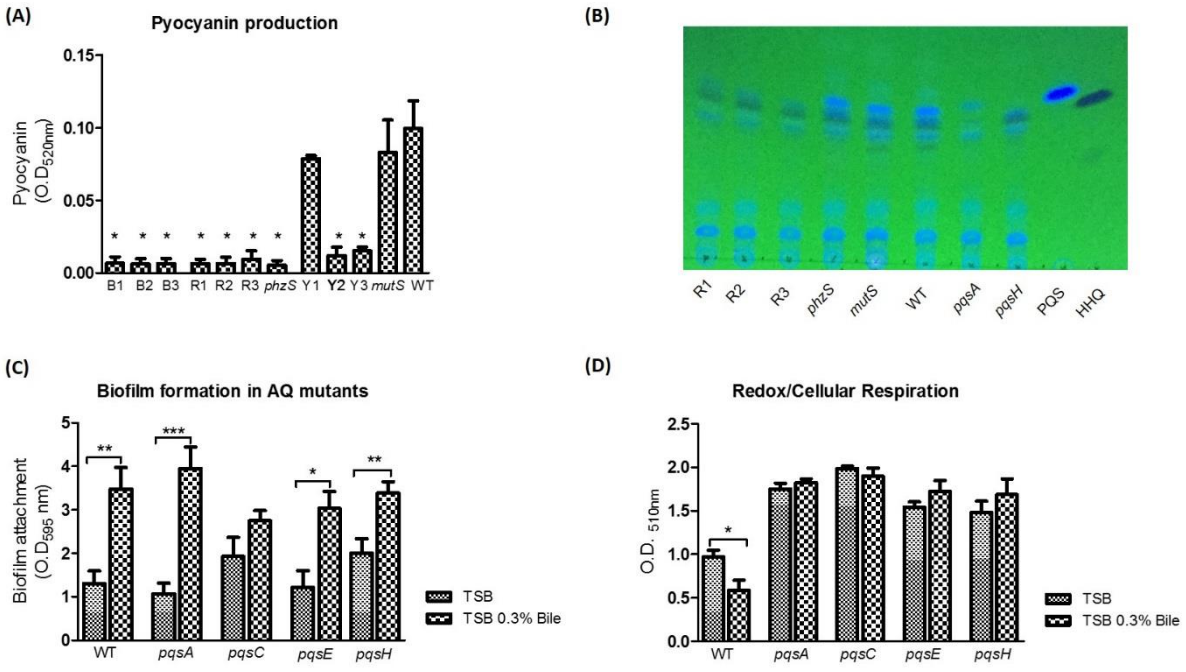


Figure 19. (A) Loss of PYO production in the red and brown pigmented variants and *phzS* mutant. (B) TLC analysis indicates loss of PQS production in the red pigmented derivative, consistent with the possible loss of *lasR* identified in the SNP comparisons. A *phzS* mutant retains PQS production, indicating loss of PQS occurs independent of pigment production. (C) Mutations of the PQS biosynthetic genes did not significantly affect the ability of *P. aeruginosa* to increase biofilm formation in response to bile. (D) Mutations of the PQS biosynthetic genes resulted in a loss of redox repression in the presence of bile.

Discussion

The clinical treatment of respiratory disease is faced with several challenges, not least the rapid decline in novel antibiotic discovery (64). Innovative approaches to pathogen control have been the focus of intensive research efforts, yet resistance continues to increase globally in spite of improved stewardship and hygiene control (65). Studies into host factors capable of modulating bacterial behaviour are important in facilitating the development of these innovative intervention strategies and will be key to their successful implementation. There is increasing evidence implicating a role for gastro-oesophageal reflux and subsequent pulmonary aspiration in the progression of CF and other chronic respiratory diseases (39, 40, 42-44, 46). To date, experimental analysis investigating bile/bacterial interactions has focused on phenotypic responses linked to the acute-chronic switch in *P. aeruginosa* and pathogenesis in general in gastrointestinal pathogens. However, little is known about the consequences for longer term exposure to bile in these organisms and the nature of the adaptation that might occur. Therefore, in this study we performed a cycling experiment with *P. aeruginosa* cultured in ASM, a synthetic media designed to more closely mimic conditions encountered within the sputum rich CF lung. Adaptation through enhanced biofilm formation, *lasR* mutation and pigmentation was observed in bile treated samples. The emergence of non-synonymous mutation in the brown pigmented variant in the *hmgA* gene, a member of the tyrosine catabolic operon, encoding the enzyme homogentisate-1,2-dioxygenase is interesting in light of the 55 fold downregulation of this gene in the bile transcriptome. Mutation of this gene results in the overproduction of the red/brown pigment pyomelanin. Pyomelanogenic *P. aeruginosa* clinical isolates are frequently recovered from the lungs of CF patients with 5% of *P. aeruginosa* strains isolated from the lungs found to overproduce pyomelanin (57, 66-69). The clinical relevance of HmgA has been

investigated and revealed that inactivation of *hmgA* confers enhanced resistance to oxidative stress, reduced bacterial clearance and increased persistence in a chronic infection model, phenotypes potentially connected to the production of pyomelanin (57, 70). The degradation of chorismate is a branch point which can be directed either to the production of pyomelanin or to the production of the AQ PQS, therefore the repression or mutation of *hmgA* in the repression of bile may be mechanistically favourable allowing for the enhanced production of AQ (71, 72). The persistent Australian Epidemic Strain-1 was shown to down regulate the transcription of *hmgA* though this repression did not result in overproduction of pyocyanin. The downregulation rather than mutational inactivation may be employed by clinical isolates providing more flexibility for adaptation to changing conditions in the lung (73). Adaptive evolution experiments in the presence of the antibiotics piperacillin and ciprofloxacin resulted in the emergence of pyomelanin hyper producers (74). Reconciling the link between the genotype and phenotype in these studies is essential to determining the functionality of *hmgA* mutation.

The expression of the quorum sensing signalling systems by *P. aeruginosa* has been shown to be accompanied by global cellular metabolic changes, with significant changes in observed in TCA, therefore the global metabolic changes observed in the presence of bile may be connected to the enhanced expression of QS (75). Recent studies have described the evolution of the quorum sensing signalling in chronic infection, providing a plausible explanation for the emergence of LasR mutants in clinical isolates through a combination of social cheating and reliance on an independent RhllR system (33, 76, 77). LasR mutants were demonstrated to induce a pro-inflammatory response resulting in the recruitment of neutrophils and the production of pro-inflammatory cytokines (78). Hence, quorum sensing may still play a central role in the establishment of chronic infection despite the loss of the LasR signalling system.

Phenazine production has been shown to enhance anaerobic survival of *P. aeruginosa* (79), while phenazines have also been shown to influence the physiology of host cells and competing organisms. Production of the phenazine pigment PYO was significantly increased in response to bile in ASM cultures after 24 h and 96 h. The increased levels of PYO in the cultures were found to be toxic to the growth of *P. aeruginosa*. This key virulence factor contributes to the pathogenicity of *P. aeruginosa* with significant quantities of this redox-active molecule both recovered from the lungs of infected CF patients and produced by clinical isolates obtained from the lungs (80, 81). The production of PYO is known to be under the regulatory control of the quorum sensing signalling systems with its biosynthesis linked to two distantly encoded operons (*phzA-G*), as well as the additional modification genes *phzH/M/S.* Several studies have reported a connection between PYO production and redox and virulence potential within *P. aeruginosa* (49, 60, 61). From the host perspective, PYO is capable of causing direct damage to human host cells, inhibiting cellular respiration, inducing neutrophil apoptosis and enhancing binding to airway epithelial cells (82-85). The importance of PYO to the *in vivo* success of *P. aeruginosa* was demonstrated in both acute and chronic mouse models, where PYO mutants were less competitive than their wild-type counterparts. However, recent evidence has shown that reduced autophagy by bronchial epithelial cells is associated with a reduction in pyocyanin production *in vivo* facilitating long term persistence within the host (86).

From the bacterial perspective, PYO has been demonstrated to play an important role in the establishment of *P. aeruginosa* biofilms, both through its modulation of signalling of the second messenger c-di-GMP and through its contribution to extracellular DNA release as a result of cellular auto-poisoning and subsequent cell death and through it (87). In contrast, PYO is also capable of promoting cellular survival within the hypoxic regions of a biofilm due to its ability to

serve as an alternative electron acceptor (49). A recent study by Meirelles et al. has further confirmed that PYO production may have both toxic and beneficial consequences to *P. aeruginosa* (49), analogous in some ways to the role proposed for PQS by Haussler and Becker (88). Sub-populations of PYO-tolerant cells were shown to emerge from within nutrient-limited biofilms, with the PYO-susceptible population sacrificed for eDNA release and structural support in a 'net-benefit' arrangement for the population. It is possible therefore that the emergence of pigmented variants that no longer produce PYO following long term exposure to bile is an adaptation within the population to the elevated 'toxic' levels encountered in the artificial sputum environment. Elevated toxic levels of PYO may select for the emergence of a sub-population of PYO defective mutants within the ASM bile microenvironment. Alternatively, the emergence of PYO defective mutants could be another form of social cheating akin to that of siderophore cheating (89). This would allow the PYO non producers to invade the population of producing cells. This cooperation and coexistence could potentially allow the population to mitigate the stress of toxic levels of PYO (81, 90, 91). The enhanced production of pyocyanin present within a bile positive CF lung should therefore be of consideration regarding the evolutionary trajectory of residing *P. aeruginosa* populations.

The occurrence of pigmented variants within the lungs of patients with respiratory disease has been reported for decades (92, 93). Distinction between red and brown pigmented clinical isolates was complicated further by the observation of some isolates that turned from 'yellowish to red' (94). Advances in sequencing and high throughput screening technologies have reinforced the finding that *P. aeruginosa* populations within the lungs of patients with CF are not uniform, but rather display a remarkable level of genotypic and phenotypic heterogeneity (95). The rationale underpinning the function of these pigmented mutants within lung populations

remains to be understood. In our study, while maintained within the cycled populations in bile treated ASM, neither the brown nor the red pigmented mutants ever exceeded more than 10% of the population. This would suggest that their function may be crucial in maintaining a PYO positive population of *P. aeruginosa*. Reversion of some colonies following subsequent culturing of red pigmented variants would support this hypothesis. Indeed, the frequency of red pigmented variants in clinical samples is low, ranging from 3.5-6% (96). Brown pyomelanin pigmented variants have been reported at higher frequencies of up to 13% in chronically infected CF patients (97). The selective pressure underpinning the emergence of these pigmented variants in the lungs, urine, bile and wounds of patients' remains to be determined. Increased persistence through maintenance of redox-balanced populations within biofilms (49), intraspecific competition through production of pyocins (98), and tolerance to oxidative stress (57), may all contribute to the necessity for these pigmented sub-populations.

The quorum sensing AQ signalling system is an effective strategy utilised by *P. aeruginosa* allowing a coordinated gene expression response at the population level. This level of regulation serves as an additional global mechanism of adaptation to external stimuli and has been demonstrated to play a role in both virulence factor production and biofilm formation (99, 100). The Pseudomonas Quinolone Signal (PQS) has been shown to exert differential effects on individual members of communities of *P. aeruginosa* capable of both sensitising cells to external stresses and inducing effective stress response (88). The presence of high levels of PQS has been shown to induce cellular autolysis whilst remaining unaffected cells are triggered to transition to a PQS tolerant reduced metabolic state. The synergy between this selective impact on a population and the recent findings related to the action of PYO on *P. aeruginosa* populations is intriguing and points to a concerted control of populations. As with PYO, PQS represents a

central factor in the modulation of population structure and adaptation. The red pigmented derivative was shown to be defective in the production of PQS but not its precursor HHQ, possibly as a consequence of changes in *lasR* sequence. As with the loss of PYO production in a subpopulation of cells, the global reduction of the PQS signalling molecule may represent a successful strategy offsetting any negative effects resulting from over production in a sputum rich environment.

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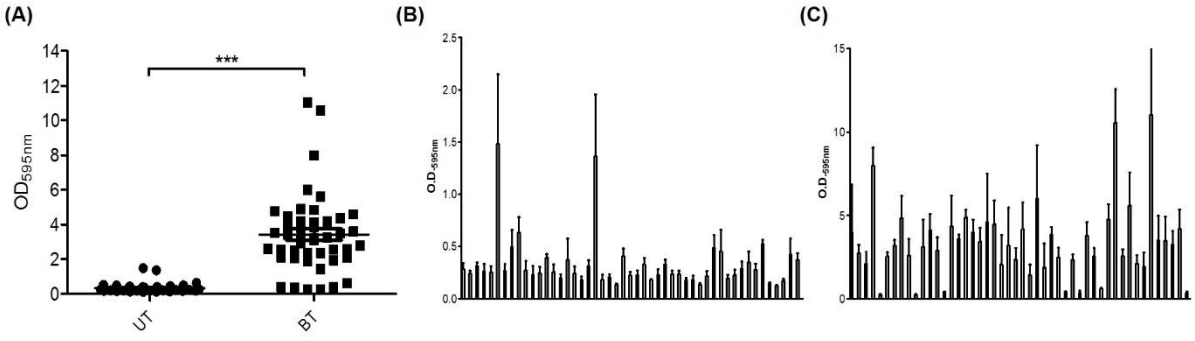
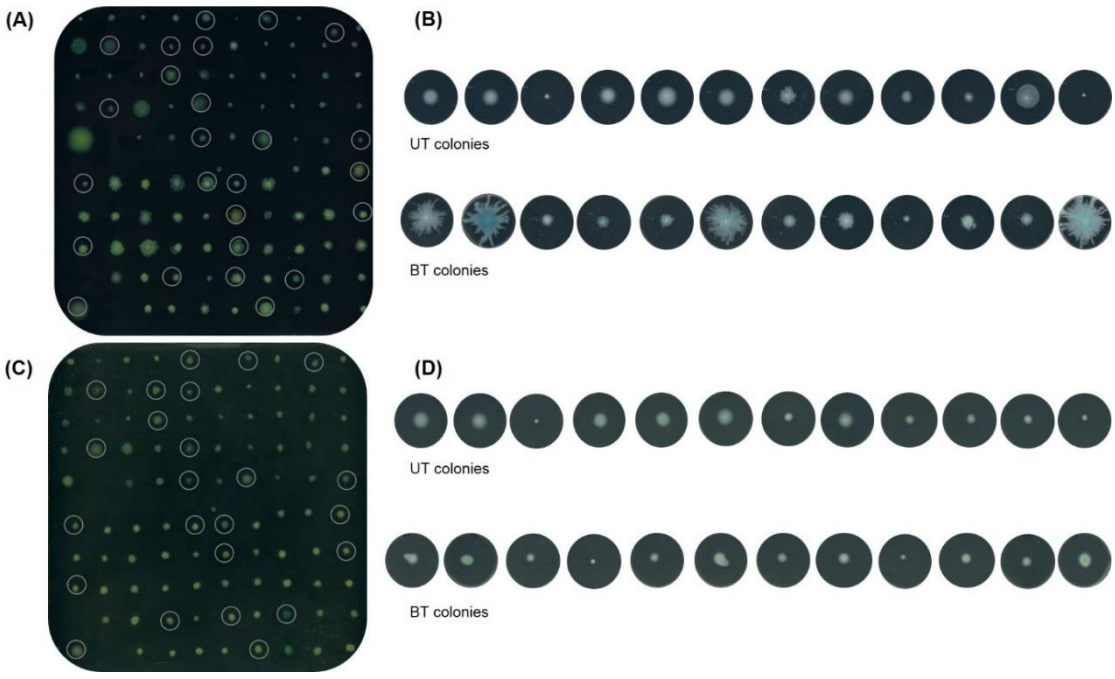
Appendix



Appendix Figure 1. (A) Biofilm formation in 48 strains isolates from untreated ASM versus 48 strains isolates from ASM supplemented with bile. Biofilm is significantly higher in strains isolates from ASM supplemented with bile. (B) Biofilm formation in the aforementioned 48 strains isolated from ASM and (C) 48 strains isolates from ASM supplemented with bile. Data is the mean of at least three independent biological replicates. Statistical analysis was performed by Student's t-test (***$p \leq 0.001$).



Appendix Figure 2. Swarming motility of colonies recovered from untreated ASM and colonies recovered ASM supplemented with bile on LB 0.8% (w/v) agar in the presence and absence of bile with validation on Eiken agar.

Appendix Table 1; Mutations present in all three pigmented isolates.

Location	Sequence Change	Codon change	Amino acid change	Gene Number PA14	Gene Number PAO1	Gene I.D	Product Annotation
72,440	G/C	GAG/CAG	Glutamic acid/Glutamine	PA14_00740	PA0062		Lipoprotein
84,664	C/A	CCG/CAG	Proline/Glutamine	PA14_00875	PA0074	*ppkA*	Serine/threonine protein kinase
96,452	Insertion of A	Frameshift		PA14_00970			Hypothetical
698,265	Insertion of G*	Frameshift		PA14_08120	PA0625		Tail length determinator protein
747,764	G/A	GGC/GAC	Glycine/Aspartic acid	PA14_08760	PA4270	*rpoB*	DNA-directed RNA polymerase subunit beta
801,696	C/T	CCG/CTG	Proline/Leucine	PA14_09340	PA4221	*fptA*	Fe(III)-pyochelin outer membrane receptor
934,050	G/A	GCG/GCA	Alanine	PA14_10800	PA4109	*ampR*	Transcriptional regulator
1,016,097	Insertion of G	Frameshift		PA14_11730			Protein kinase
1,228,955	Deletion of G	Frameshift		PA14_14400			Hypothetical
1,243,658	Insertion of G*	Frameshift		PA14_14530	PA3825		Hypothetical
1,244,748	G/T	GAG/TAG	Glutamic acid/STOP	PA14_14570			t-RNA-leucine
1,269,267	C/T	CCG/CTG	Proline/Leucine	PA14_14940	PA3798		Aminotransferase
1,744,494	C/T	Intergenic					
1,880,872	C/G	CGC/AGC	Arginine/Serine	PA14_21690	PA3272		ATP-dependent DNA helicase
2,040,973	G/A	GCT/ACT	Alanine/Threonine	PA14_23460	PA3145	*orfN*	Group 4 glycosyl transferase
2,105,091	A/G	AGC/GGC	Serine/Glycine	PA14_24260			Hypothetical
2,149,425	A/C	CAA/CAC	Glutamine/Histidine	PA14_24600	PA3054		Carboxypeptidase
2,209,726	A/G	AAG/AGG	Lysine/Arginine	PA14_25250	PA3001		Glyceraldehyde-3-phosphate dehydrogenase
2,277,057	G/A	Intergenic					
2,339,963	Insertion of G	Intergenic					
2,354,159	Deletion of G	Intergenic					
2,589,402	C/T	Intergenic					
2,790,309	A/G	ACG/GCG	Threonine/Alanine	PA14_32060	PA2519	*xylS*	Transcriptional regulator
2,864,042	A/G	ACG/GCG	Threonine/Alanine	PA14_32790	PA2462		Hypothetical
3,107,103	A/G	GAC/GGC	Aspartic acid/Glycine	PA14_34900	PA2298		Oxidoreductase
3,162,561	T/G	GTT/GGT	Valine/Glycine	PA14_35590	PA2243	*pslM*	FAD-binding dehydrogenase
3,166,109	T/G	CTG/CGG	Leucine/Arginine	PA14_35620	PA2241	*pslK*	Hypothetical
3,217,257	C/A	GGC/GGA	Glycine	PA14_36100	PA2212	*pdxA*	4-hydroxythreonine-4-phosphate dehydrogenase
3,267,665	T/C	TGC/CGC	Cysteine/Arginine	PA14_36690	PA2155		Cardiolipin synthase 2

3,390,498	A/C	TAG/TCG	STOP/Serine	PA14_38000			Hypothetical
3,453,679	T/C	CTG/CCG	Leucine/Proline	PA14_38730	PA1993		Major facilitator superfamily transporter
3,832,407	T/C	Intergenic					
3,832,408	C/G	Intergenic					
3,874,888	C/A	ACG/AAG	Threonine/Lysine	PA14_43510	PA1625		Hypothetical
3,879,553	C/G	CTC/GTC	Leucine/Valine	PA14_43570	PA5341		Hypothetical
4,030,027	G/A	GTG/GTA	Valine	PA14_45180	PA1488		Oxidoreductase
4,092,528	C/A	CGC/AGC	Argine/Serine	PA14_46010	PA1425		ABC transporter ATP-binding protein
4,265,772	G/A	GGC/GAC	Glycine/Aspartic acid	PA14_47920	PA1260		ABC transporter substrate-binding protein
4,486,152	A/C	CAC/CCC	Histidine/Proline	PA14_50500	PA2753		Hypothetical
4,572,692	G/T	CCG/CCT	Proline	PA14_51450	PA0994	*cupC3*	Usher cupC3
4,607,562	G/A	Intergenic					
4,629,533	Insertion of G	Frameshift		PA14_52190	PA0933	*rumA*	23S rRNA-methyluridine methyltransferase
4,659,805	A/G	Intergenic					
4,659,822	A/G	Intergenic					
4,704,034	C/A	CCG/ACG	Proline/Threonine	PA14_53050		*aroP2*	Aromatic amino acid transport protein
4,787,386	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_53980	PA0793		Hypothetical
4,939,410	C/A	GCC/GCA	Alanine	PA14_55480	PA0682	*hxcX*	HxcX
5,010,234	Insertion of C**	Frameshift		PA14_56100			Hypothetical
5,269,324	T/C	GTG/GCG	Valine/Alanine	PA14_59180			Topoisomerase I-like protein
5,270,796	Insertion of C	Frameshift		PA14_59200			Hypothetical
5,284,844	T/C	CGT/CGC	Arginine	PA14_59320		*pilS2*	Type IV B pilus protein
5,341,373	Insertion of C	Frameshift		PA14_59980			Hypothetical
5,390,088	G/A	GCG/GCA	Alanine	PA14_60470	PA4569	*ispB*	Octaprenyl-diphosphate synthase
5,444,993	Deletion of G	Frameshift		PA14_61050	PA4614	*mscL*	Large-conductance mechanosensitive channel
5,698,849	T/G	CTC/CGC	Leucine/Arginine	PA14_63960	PA4837		Outer membrane protein
5,716,479	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_64140	PA4850	*prmA*	50S ribosomal protein L11 methyltransferase
5,716,484	G/A	CCG/CCA	Proline	PA14_64140	PA4850	*prmA*	50S ribosomal protein L11 methyltransferase
5,939,960	A/C	GAC/GCC	Aspartic acid/Alanine	PA14_66570	PA5036	*gltB*	Gluatamate synthase subunit alpha
6,070,078	T/C	TGA/CGA	STOP/Arginine	PA14_68010			
6,070,105	Deletion of A	Frameshift		PA14_68020			

6,238,553	G/A	GCC/ACC	Alanine/Threonine	PA14_69925	PA5297	*poxB*	Pyruvate dehydrogenase (cytochrome)

Appendix Table 2. Mutations present in the red pigmented isolate.

Location	**Sequence Change**	**Codon change**	**Amino acid change**	**Gene Number PA14**	**Gene Number PAO1**	**Gene I.D**	**Product Annotation**
32,841	G/T	GGC/GTC	Glycine/Valine	PA14_00360	PA0030		Hypothetical
61,992	G/T	GGC/GTC	Glycine/Valine	PA14_00620			Hypothetical
178,759	Insertion of C	Frameshift		PA14_01970	PA0158		RND efflux transporter
221,070	C/T	GCC/GCT	Alanine	PA14_02450	PA0195		NAD(P) transhydrogenase subunit alpha part 1
349,077	C/T	CCC/CCT	Proline	PA14_03870	PA0297	*spuA*	Glutamine amidotransferase
360,459	A/C	CAG/CCG	Glutamine/Proline	PA14_03980	PA0305		Hypothetical
397,464	Deletion of C	Frameshift		PA14_04440	PA0340		Permease
434,354	C/T	ACC/ACT	Threonine	PA14_04920	PA0375	*ftsX*	Cell division protein
463,063	Insertion of G	Frameshift		PA14_05300	PA0406		TonB domain containing protein
519,061	T/G	GTG/GGG	Valine/Glycine	PA14_05870	PA0450		Phosphate transporter
632,707	C/T	CTG/TTG	Leucine	PA14_07370	PA0567		Hypothetical
653,104	C/T	GAC/GAT	Aspartic acid	PA14_07570	PA0580	*gcp*	DNA binding/iron metalloprotein/AP endonuclease
783,257	G/T	GGC/TGC	Glycine/Cysteine	PA14_09240	PA4228	*pchD*	Pyochelin biosynthesis protein
806,392	Insertion of G	Frameshift		PA14_09400	PA4217	*phzS*	Hypothetical
814,995	T/G	TAC/GAC	Tyrosine/Aspartic acid	PA14_09500	PA4208	*opmD*	Outer membrane protein
989,594	C/T	Intergenic					
1,070,507	Deletion of GC	Frameshift		PA14_12420			
1,100,285	A/G	ACC/GCC	Threonine/Alanine	PA14_12820	PA4112		Two component sensor
1,131,231	A/G	CAG/CGG	Glutamine/Arginine	PA14_13150	PA3921		Transcriptional regulator
1,238,559	G/A	GCG/ACG	Alanine/Threonine	PA14_14490	PA3829		Hydrolase
1,327,770	C/T	Intergenic					
1,336,935	A/C	TAC/TCC	Tyrosine/Serine	PA14_15720	PA3764		Transglycosylase
1,346,934	G/T	GGG/GTG	Glycine/Valine	PA14_15790	PA3760		Phosphoenolpyruvate-protein phosphotransferase
1,465,300	A/G	TGT/GTG	Methionine/Valine	PA14_17080	PA3654	*pyrH*	Uridylate kinase

1,475,661	A/G	AGC/GGC	Serine/Glycine	PA14_17210	PA3644	*lpxA*	UDP-N-acetylglucosamine acyltransferase
1,611,301	C/T	TCG/TTG	Serine/Leucine	PA14_18720	PA3526		OmpA family membrane protein
1,642,140	T/G	TTC/GTC	Phenylalanine/Valine	PA14_19020	PA3484	*tse3*	
1,676,663	T/G	CTG/CGG	Leucine/Arginine	PA14_19390	PA3457		Hypothetical
1,709,809	C/T	Intergenic					
1,863,419	A/G	CCA/CCG	Proline	PA14_21490	PA3290	*tle1*	Type IV secretion lipase effector
1,910,808	C/T	Intergenic					
2,082,340	G/T	GAC/TAC	Aspartic acid/Tyrosine	PA14_23970	PA3105	*xcpQ*	General secretion pathway protein D
2,108,371	C/T	AAC/AAT	Asparagine	PA14_24290	PA3082	*gbt*	Glycine betaine transmethylase
2,576,533	T/G	CTG/CGG	Leucine/Arginine	PA14_29760	PA4633		Chemotaxis transducer
2,629,940	C/A	CGG/AGG	Arginine	PA14_30350	PA2610		Hypothetical
2,772,303	C/T	ACC/ACT	Threonine	PA14_31870	PA2528		RND efflux membrane fusion protein
2,886,521	C/T	ACC/ATC	Threonine/Isoleucine	PA14_33000	PA2445	*gcvP2*	Glycine dehydrogenase
2,948,483	G/A	GCG/GCA	Alanine	PA14_33500	PA2413	*pcvdH*	Diaminobutyrate-2-oxoglutarate amino transferase
2,983,709	A/T	GGA/GGT	Glycine	PA14_33650	PA2399	*pvdD*	Pyoverdine synthetase D
2,983,721	C/T	GAC/GAT	Aspartic acid	PA14_33650	PA2399	*pvdD*	Pyoverdine synthetase D
2,983,898	G/A	ACG/ACA	Threonine	PA14_33650	PA2399	*pvdD*	Pyoverdine synthetase D
2,984,162	T/G	CTT/CTG	Leucine	PA14_33650	PA2399	*pvdD*	Pyoverdine synthetase D
2,993,193	G/T	TGG/TGT	Tryptophan/Cystein	PA14_33720	PA2394	*pvdN*	
3,119,426	T/G	CTC/CGC	Leucine/Arginine	PA14_35030	PA2285		Hypothetical
3,194,938	C/T	GCC/GCT	Alanine	PA14_35890			Diamino-2-oxogluturate aminotransferase
3,208,076	T/G	TTG/GTG	Leucine/Valine	PA14_36020			Paraquat-inducible protein B
3,265,868	A/G	GAC/GGC	Aspartic acid/Glycine	PA14_36660	PA2119		Alcohol dehydrogenase
3,382,210	Insertion of C	Frameshift		PA14_37900	PA2057	*sppR*	TonB dependent receptor
3,392,069	C/T	Intergenic					
3,481,455	G/A	GTG/GTA	Valine	PA14_39050	PA1971	*braZ*	Branched chain amino acid transport carrier
3,521,696	C/A	CCG/ACG	Proline/Threonine	PA14_39560	PA1930		Chemotaxis transducer
3,539,750	G/A	CCG/CCA	Proline	PA14_39720	PA1918		Amino acid oxidase
3,657,223	C/A	CCG/CAG	Proline/Glutamine	PA14_41010	PA1819		Amino acid permease
3,628,465	G/T	GGC/GTC	Glycine/Valine	PA14_46040	PA1846	*cti*	Cis/trans isomerase
3,631,015	Insertion of C	Intergenic					

3,645,621	Deletion of T	Intergenic					
3,657,223	C/A	CGG/AGG	Arginine	PA14_41070	PA1814		Hypothetical
3,813,048	C/A	AGC/AGA	Serine/Arginine	PA14_42900	PA1669	*icmF2*	
3,842,568	A/C	ACC/CCC	Threonine/Proline	PA14_43130	PA1654		Aminotransferase
3,917,378	A/C	ACC/CCC	Threonine/Proline	PA14_44010	PA1585	*sucA*	2-oxoglutarate dehydrogenase E1
3,944,395	C/T	CAG/TAG	Glutamine/STOP	PA14_44300	PA1561	*aer*	Aerotaxis receptor
4,049,785	A/C	ACC/CCC	Threonine/Proline	PA14_44500	PA1464		Purine binding chemotaxis protein
4,159,861	Insertion of two Cs	Frameshift		PA14_46660	PA1363		RNA polymerase ECF subfamily sigma 70 factor
4,355,575	T/C	Intergenic					
4,371,981	C/A	ATC/ATA	Isoleucine	PA14_49200	PA1178	*oprH*	Outer membrane protein
4,437,782	G/A	GGC/AGC	Glycine/Serine	PA14_49910	PA1118		Hypothetical
4,454,838	T/G	CTG/CGG	Leucine/Arginine	PA14_50130	PA1102	*fliG*	Flagellar motor switch protein
4,460,921	T/G	ATG/AGG	Methionine/Arginine	PA14_50220	PA1097	*fleQ*	Transcriptional regulator
4,487,961	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_50520	PA1074	*braZ*	Branched chain amino acid transport protein
4,521,022	G/A	CTG/CTA	Leucine	PA14_50850	PA1044		Hypothetical
4,530,389	G/A	Intergenic					
4,685,183	G/T	GGC/GTC	Glycine/Valine	PA14_52850	PA0883		Acyl coA lyase subunit beta
4,717,281	G/A	GGC/GAC	Glycine/Aspartic acid	PA14_53210	PA0855		Hypothetical
4,941,683	T/C	Intergenic					
4,976,703	A/C	GAA/GAC	Glutamic acid/Aspartic acid	PA14_55750	PA4290		Chemotaxis transducer
4,993,902	A/C	AAC/ACC	Asparagine/Threonine	PA14_55930	PA4305		Pilus assembly protein
5,083,361	G/A	Intergenic					
5,235,518	G/A	GGG/AGG	Glycine/Arginine	PA14_58750	PA4526	*pilB*	Type 4 fimbrial biogenesis protein
5,278,478	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_59240		*pilL2*	Type IV B pilus protein
5,278,574	Insertion of C	Intergenic					
5,291,762	A/G	Intergenic					
5,539,405	A/C	ACA/CCA	Threonine/Proline	PA14_60150			tRNA-lysine
5,376,673	T/C	Intergenic					
5,404,653	C/T	ACC/ACT	Threonine	PA14_60630	PA4582		Hypothetical
5,441,128	G/A	Intergenic					
5,445,689	C/A	CTC/CTA	Leucine	PA14_61060	PA4615		Oxidoreductase
5,447,589	G/A	GTC/ATC	Valine/Isoleucine	PA14_61090	PA4617		Hypothetical
5,526,548	G/A	GTG/GTA	Valine	PA14_61980	PA4685		Hypothetical

5,670,468	A/C	GAA/GAC	Glutamic acid/Aspartic acid	PA14_63605		*fdnG*	Formate dehydrogenase-O major subunit
5,676,686	C/T	Intergenic					
5,703,909	A/C	AGC/CGC	Serine/Arginine	PA14_63990	PA4839	*speA*	Arginine decarboxylase
5,742,997	C/T	Intergenic					
5,834,228	T/C	TCC/CCC	Serine/Proline	PA14_65500	PA4957	*psd*	Phosphatidylserine decarboxylase
5,889,326	C/A	CCG/ACG	Proline/Threonine	PA14_66110	PA1390		Glycosyl transferase family protein
6,003,335	A/C	TAC/TCC	Tyrosine/Serine	PA14_67230	PA5090	*vgrG5*	
6,098,091	Deletion of C	Intergenic					
6,119,371	T/C	TGG/CGG	Tryptophan/Arginine	PA14_68620	PA5194		Hypothetical
6,188,351	A/G	Intergenic					
6,310,168	T/G	TAC/GAC	Tyrosine/Aspartic acid	PA14_70850	PA5368	*pstC*	Membrane protein component of ABC phosphate transporter
6,355,746	T/C	CTG/CCG	Leucine/Proline	PA14_71320	PA5402		Hypothetical
6,437,415	A/G	TAC/TGC	Tyrosine/Cysteine	PA14_72250	PA5474		Hypothetical

Appendix Table 3. Mutations present in the yellow pigmented isolate.

Location	**Sequence Change**	**Codon change**	**Amino acid change**	**Gene Number PA14**	**Gene Number PAO1**	**Gene I.D**	**Product Annotation**
79,546	C/T	CCG/TCG	Proline/Serine	PA14_00810	PA0069		DNA repair photolyase
96,549	Deletion of ACCGAC	Frameshift		PA14_00980	PA0081	*fha1*	Fha1
110,810	G/T	GCG/GCT	Alanine	PA14_01120	PA0092	*tsi6*	Tsi6
317,111	C/T	CGG/TGG	Arginine/Tryptophan	PA14_03470	PA0267		Hypothetical
356,687	C/T	CTG/TTG	Leucine	PA14_03950	PA0303	*spuG*	Polyamine transport protein
358,353	A/G	Intergenic					
482,414	T/G	TGC/GGC	Cysteine/Glycine	PA14_05460	PA0420	*bioA*	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase
484,343	C/T	CCG/TCG	Proline/Serine	PA14_05480	PA0421		Hypothetical
588,284	G/T	GGC/TGC	Glycine/Cysteine	PA14_06740	PA0518	*nirM*	Cytochrome c-551
588,730	T/G	TTC/GTC	Phenylalanine/Valine	PA14_06750	PA0519	*nirS*	Nitrite reductase
727,931	G/C	GCC/CCC	Alanine/Proline	PA14_08500	PA0664		Hypothetical
841,422	Insertion of three Cs	Intergenic					
936,585	T/G	TTC/GTC	Phenylalanine/Valine	PA14_10820	PA4108		HDIG domain containing protein

939,122	T/G	GTC/GGC	Valine/Glycine	PA14_10850	PA4100		Dehydrogenase
964,247	Insertion of G	Frameshift		PA14_11100	PA4082	*cupB5*	Adhesive protein
974,418	C/T	CCG/CTG	Proline/Leucine	PA14_11190	PA4073		Aldehyde dehydrogenase
1,126,953	T/C	GTG/GCG	Valine/Alanine	PA14_13130	PA3422		Hypothetical
1,145,198	T/C	GAT/GAC	Aspartic acid	PA14_13330	PA3910		Hypothetical
1,193,464	A/C	AGC/CGC	Serine/Arginine	PA14_13890	PA3868		Integrase
1,202,660	G/T	GCC/TCC	Alanine/Serine	PA14_14020	PA4908		Hypothetical
1,264,653	C/T	GCC/GCT	Alanine	PA14_14890	PA3802	*hisS*	Histidyl-Trna synthetase
1,287,749	Insertion of G	Frameshift		PA14_15200	PA5167		Hypothetical
1,338,118	G/T	GCC/TCC	Alanine/Serine	PA14_15720	PA3764		Transglycosylase
1,346,872	G/T	GCC/TCC	Alanine/Serine	PA14_15790	PA3760		Phosphoenolpyruvate-protein phosphotransferase
1,356,413	G/T	GGC/TGC	Glycine/Cysteine	PA14_15920	PA3749		Major facilitator transporter
1,400,568	A/G	GAG/GGG	Glutamic acid/Glycine	PA14_16370	PA3712		Hypothetical
1,413,327	C/T	GCG/GTG	Alanine/Valine	PA14_16500	PA3702	*wspR*	Two component response regulator
1,574,661	G/T	GGC/GTC	Glycine/Valine	PA14_18350	PA3554		Bifunctional UDP-glucoronic acid decarboxylase/UDP-4-amino-4deoxy-L-arabinose formyltransferase
1,593,148	T/G	TGG/GGG	Tryptophan/Glycine	PA14_18565	PA3541	*alg8*	Alginate biosynthesis protein
1,633,780	G/T	CGG/CTG	Arginine/Leucine	PA14_18950	PA3489		Na(+)-translocating NADH-quinone reductase subunit E
1,651,176	A/G	ACC/GCC	Threonine/Alanine	PA14_19120	PA3477	*rhlR*	Transcriptional regulator
1,675,303	G/A	GGC/AGC	Glycine/Serine	PA14_19380	PA3458		Transcriptional regulator
1,682,069	A/G	AGG/GGG	Arginine/Glycine	PA14_19450	PA3453		Hypothetical
1,690,445	C/T	CCC/TCC	Proline/Serine	PA14_19560	PA2356	*ssuD*	Alkanesulfonate monooxygenase
1,775,138	C/T	GCC/GCT	Alanine	PA14_20620	PA3360		HlyD family secretion protein
1,832,860	C/A	GCC/GAC	Alanine/Aspartic acid	PA14_21190	PA3311		Hypothetical
1,887,084	T/G	TGG/GGG	Tryptophan/Glycine	PA14_21700	PA3271		Two component sensor
1,965,600	A/G	ATC/GTC	Isoleucine/Valine	PA14_22590	PA3219		Hypothetical
1,967,248	G/T	GTG/TTG	Valine/Leucine	PA14_22620	PA3217	*cyaB*	Hypothetical
1,993,384	A/G	AAG/AGG	Lysine/Arginine	PA14_22980	PA3190		Sugar ABC transporter substrate binding protein
2,040,287	Deletion of G	Frameshift		PA14_23460		*orfN*	Group 4 glycosyl transferase
2,047,335	C/T	ACC/ACT	Threonine	PA14_23510	PA3138	*uvrB*	Exinuclease ABC subunit B

2,089,517	A/G	CAC/CGC	Histidine/Arginine	PA14_24060	PA3098	*xcpW*	General secretion pathway protein J
2,111,816	C/T	Intergenic					
2,265,266	G/A	GGC/AGC	Glycine/Serine	PA14_25970	PA2944	*cobN*	Cobaltchelatase subunit
2,309,068	C/T	GGC/GGT	Glycine	PA14_26485	PA2906		Oxidoreductase
2,334,848	C/T	GCC/GCT	Alanine	PA14_26810	PA2882		Two component sensor
2,439,069	T/C	GTG/GCG	Valine/Alanine	PA14_28170	PA2777		Formate/nitrate transporter
2,560,944	Deletion of C	Frameshift		PA14_29520	PA2674		Type II secretion system protein
2,573,823	C/T	CGC/CGT	Arginine	PA14_29720	PA2658		Hypothetical
2,658,395	C/T	ACC/ACT	Threonine	PA14_30670	PA2584	*pgsA*	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyl transferase
2,716,172	T/G	TTT/GTT	Phenylalanine/Valine	PA14_31240			NrbE like protein
2,723,025	T/C	GGT/GGC	Glycine	PA14_31310	PA2568		Hypothetical
2,723,330	A/G	TAC/TGC	Tyrosine/Cysteine	PA14_31310	PA2568		Hypothetical
2,734,321	T/C	Intergenic					
2,821,688	C/A	CCC/CAC	Proline/Histidine	PA14_32450	PA2917		AraC family transcriptional regulator
2,822,329	A/G	Intergenic					
2,937,204	A/G	GAA/GAG	Glutamic acid	PA14_33360			Hypothetical
2,950,156	G/T	GAC/TAC	Aspartic acid/Tyrosine	PA14_33530	PA2410		Hypothetical
2,969,825	A/G	CAC/CGC	Histidine/Arginine	PA14_33610	PA2402		Peptide synthase
3,021,989	C/A	GCC/GCA	Alanine	PA14_33990	PA2371	*clpV3*	
3,086,570	A/G	CAG/CGG	Glutamine/Arginine	PA14_34750	PA3935		Taurine catabolism dioxygenase
3,142,806	T/G	TCC/GCC	Serine/Alanine	PA14_35340	PA2261		2-ketogluconate kinase
3,299,356	A/C	ATC/CTC	Isoleucine/Leucine	PA14_37030	PA2130	*cupA3*	Usher
3,406,931	A/C	ACA/ACC	Threonine	PA14_38190	PA2036		Hypothetical
3,423,559	T/C	GTC/GCC	Valine/Alanine	PA14_38410	PA2018	*amrB*	Multidrug efflux protein
3,624,692	T/C	ATG/ACG	Methionine/Threonine	PA14_40600	PA3782		Transcriptional regulator
3,789,839	Insertion of CAACGC	Frameshift		PA14_42600	PA1695	*pscP*	Translocation protein in type III secretion
3,886,462	G/A	GGC/AGC	Glycine/Serine	PA14_43650	PA1613		Hypothetical
3,904,801	G/T	GGC/GTC	Glycine/Valine	PA14_43850	PA1596	*htpG*	Heat shock protein 90
4,050,445	C/A	CGC/AGC	Arginine/Serine	PA14_45110	PA1463		CheW-domain containing protein
4,083,113	A/G	CAG/CGG	Glutamine/Arginine	PA14_45930	PA1433		Hypothetical
4,250,055	G/T	GTC/TTC	Valine/Phenylalanine	PA14_47760	PA1273	*cobB*	Cobyrinic acid a,c-diamide synthase

4,279,422	G/T	ACG/ACT	Threonine	PA14_48090	PA1248	*aprF*	Alkaline protease secretion outer membrane protein precursor
4,282,181	C/A	GCC/GCA	Alanine	PA14_48115	PA1246	*aprD*	Alkaline protease secretion protein
4,290,384	C/A	CGC/AGC	Arginine/Serine	PA14_48170	PA1242		Hypothetical
4,334,674	C/A	ATC/ATA	Isoleucine	PA14_48750	PA1203		Hypothetical
4,385,780	Insertion of G	Intergenic					
4,412,053	T/C	CTG/CCG	Leucine/Proline	PA14_45980	PA1991		Iron containing alcohol dehydrogenase
4,469,500	T/G	CTG/CGG	Leucine/Arginine	PA14_50300	PA1390		Hypothetical
4,532,529	Insertion of C	Intergenic					
4,533,567	G/T	GGG/TGG	Glycine/Tryptophan	PA14_51020	PA1029		Hypothetical
4,452,943	T/G	CTG/CGG	Leucine/Arginine	PA14_51120	PA1020		Acyl coA dehydrogenase
4,573,748	C/A	TCC/TCA	Serine	PA14_51450	PA0994	*cupC 3*	Usher cupC3
4,635,225	Insertion of C	Frameshift		PA14_52250	PA0929		Two component response regulator
4,675,304	C/A	GGC/GGA	Glycine	PA14_52750	PA0892	*aotP*	Arginine/ornithine transport protein
4,678,446	A/C	ATC/CTC	Isoleucine/Leucine	PA14_52780	PA0889	*aotQ*	Arginine/ornithine transport protein
4,725,064	T/C	CTG/CCG	Leucine/Proline	PA14_53310	PA0847		Hypothetical
4,786,878	G/A	GGC/AGC	Glycine/Serine	PA14_53970	PA0794		Aconitate hydratase
4,853,488	Insertion of C	Frameshift		PA14_54750			Hypothetical
5,155,582	T/C	TCC/CCC	Serine/Proline	PA14_57930	PA4461		ABC transporter ATP-binding protein
5,163,385	C/A	CCC/CCA	Proline	PA14_58030	PA4470	*fumC*	Fumarate hydratase
5,187,968	A/C	AAG/ACG	Lysine/Threonine	PA14_58250	PA4489		Hypothetical
5,235,921	T/C	CTG/CCG	Leucine/Proline	PA14_58750	PA4526	*pilB*	Type 4 fimbrial biogenesis protein
5,314,049	G/A	GTG/ATG	Valine/Methionine	PA14_59710		*cupD 1*	Fimbrial protein
5,331,047	T/C	GGT/GGC	Glycine	PA14_59840			Hypothetical
5,432,474	A/G	GAG/GGG	Glutamic acid/Glycine	PA14_60870	PA4601	*morA*	Motility regulator
5,496,057	C/A	AGC/AGA	Serine/Arginine	PA14_61590	PA4656		Hypothetical
5,501,873	C/A	CAC/CAA	Histidine/Glutamine	PA14_61650	PA4661	*pagL*	Lipid A 3-O-deacylase
5,597,574	G/A	Intergenic					
5,633,276	A/G	Intergenic					
5,762,000	C/A	CCG/ACG	Proline/Threonine	PA14_64690	PA4895		Transmembrane sensor
5,800,486	T/G	TTC/GTC	Phenylalanine/Valine	PA14_65080	PA4928		Hypothetical

5,819,003	C/A	CGT/AGT	Arginine/Serine	PA14_65300	PA4943	*hflX*	GTP-binding protein
5,827,207	G/A	GGC/GAC	Glycine/Aspartic acid	PA14_65400	PA4950		Iron sulfur cluster binding protein
5,894,399	C/A	CCT/ACT	Proline/Threonine	PA14_66160	PA1391		Glycosyl transferase family protein
5,947,261	C/A	GCT/GAT	Alanine/Aspartic acid	PA14_66620	PA5040	*pilQ*	Type 4 fimbrial biogenesis outer membrane protein precursor
5,847,448	A/C	ACG/CCG	Threonine/Proline	PA14_66620	PA5040	*pilQ*	Type 4 fimbrial biogenesis outer membrane protein precursor
6,222,145	Insertion of two Cs	Frameshift		PA14_69760	PA5284		Fimbrial protein
6,288,100	Insertion of C	Frameshift		PA14_70580	PA5346		Hypothetical

Appendix Table 4. Mutations present in the brown pigmented isolate.

Location	**Sequence Change**	**Codon change**	**Amino acid change**	**Gene Number PA14**	**Gene Number PAO1**	**Gene I.D**	**Product Annotation**
154,905	C/T	AGC/AGT	Serine	PA14_01680	PA0137		ABC transporter permease
304,728	T/G	CTG/CGG	Leucine/Arginine	PA14_03340			Hypothetical
375,119	G/T	GGC/TGC	Glycine/Cysteine	PA14_04190	PA0321		Acetylpolyamine aminohydrolase
400,739	C/A	GCG/GAG	Alanine/Glutamic acid	PA14_04510	PA0344		Hypothetical
559,260	T/G	GTC/GGC	Valine/Glycine	PA14_06320	PA0485		Hypothetical
676,385	C/T	ACC/ATC	Threonine/Isoleucine	PA14_07850	PA0602		ABC transporter substrate binding protein
738,116	T/C	Intergenic					
758,103	T/C	GCT/GCC	Alanine	PA14_08830	PA4265	*tufA*	Elongation factor Tu
1,052,018	G/T	GGC/GTC	Glycine/Valine	PA14_12160	PA3992		Murin transglycosylase
1,135,419	C/T	Intergenic					
1,185,260	T/C	TAC/CAC	Tyrosine/Histidine	PA14_13780	PA3875	*narG*	Respiratory nitrate reductase alpha subunit
1,289,849	G/T	GCC/TCC	Alanine/Serine	PA14_15210	PA3788		LysR family transriptional regulator
1,298,491	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_15310	PA3770	*guaB*	Inosine 5'-monophosphate dehydrogenase
1,337,210	G/T	GGC/TGC	Glycine/Cysteine	PA14_15720	PA3764		Transglycosylase
1,345,617	T/G	GTG/GGG	Valine/Glycine	PA14_15790	PA3760		Phosphoenolpyruvate-protein phosphotransferase
1,382,883	C/T	CAG/TAG	Glutamine/STOP	PA14_16190	PA3728		Hypothetical
1,421,131	C/T	CCG/CTG	Proline/Leucine	PA14_16600	PA3695		Alpha/beta hydrolase
1,518,742	Deletion of G	Frameshift		PA14_17660	PA3605		Hypothetical

1,611,088	Insertion of G	Intergenic					
1,696,730	Deletion of G	Intergenic					
1,731,558	T/C	CTC/CCC	Leucine/Proline	PA14_20080	PA3402		Hypothetical
1,760,186	T/G	GTG/GGG	Valine/Glycine	PA14_20491	PA3368		Acetyltransferase
1,897,804	Insertion of G	Intergenic					
1,955,322	G/T	GGG/GTG	Glycine/Valine	PA14_22460	PA3226		Alpha/beta hydrolase
1,968,127	T/G	TAC/GAC	Tyrosine/Aspartic acid	PA14_22620	PA3217	*cyaB*	Hypothetical
2,054,407	C/T	CCG/CTG	Proline/Leucine	PA14_23610	PA3132		Hydrolase
2,078,818	C/T	ACC/ATC	Threonine/Isoleucine	PA14_23920	PA3108	*purF*	Amidophosphoribosyltransferase
2,101,504	A/G	ATG/GTG	Methionine/Valine	PA14_24220	PA3088	*ppnK*	Inorganic polyphosphate/ATP-NAD kinase
2,189,991	Deletion of GC	Frameshift		PA14_25030	PA3018		Hypothetical
2,199,501	G/A	CTG/CTA	Leucine	PA14_25130	PA3010		Hypothetical
2,266,101	C/T	GCC/GTC	Alanine/Valine	PA14_25970	PA2944	*cobN*	Cobaltchelatase subunit
2,488,972	Insertion of G	Frameshift		PA14_28830			Hypothetical
2,563,135	A/G	GAT/GGT	Aspartic acid/Glycine	PA14_29560	PA2670		Hypothetical
2,636,539	Insertion of G	Frameshift		PA14_30440	PA2602		Hypothetical
2,717,231	Insertion of G	Intergenic					
2,823,869	G/T	GCC/TCC	Alanine/Serine	PA14_32490	PA2485		Hypothetical
2,902,168	A/G	AGC/GGC	Serine/Glycine	PA14_33130	PA2435		Cation transporting P-type ATPase
2,906,836	Deletion of G	Frameshift		PA14_33200			Hypothetical
2,967,058	A/G	AGC/GGC	Serine/Glycine	PA14_33610	PA2402		Peptide synthase
2,967,078	C/G	GCC/GCG	Alanine	PA14_33610	PA2402		Peptide synthase
2,967,133	T/C	TTG/CTG	Leucine	PA14_33610	PA2402		Peptide synthase
2,967,154	T/C	TTG/CTG	Leucine	PA14_33610	PA2402		Peptide synthase
2,997,125	T/G	TAC/GAC	Tyrosine/Aspartic acid	PA14_33750	PA2391		Outer membrane protein
3,104,307	Insertion of G	Frameshift		PA14_34870		*chiC*	Chitinase
3,137,923	Deletion GCG	Frameshift		PA14_35290		*gnd*	Gluconate dehydrogenase
3,298,597	G/T	GCC/TCC	Alanine/Serine	PA14_37030	PA2130	*cupA3*	Usher
3,300,176	G/T	GTG/TTG	Valine/Leucine	PA14_37040	PA2129	*cupA2*	Chaperone

3,355,674	Insertion of C	Frameshift		PA14_37680			Hypothetical
3,436,453	T/C	TTC/CTC	Phenylalanine/Leucine	PA14_38510	PA2009	*hmgA*	Homogentisate 1,2-dioxygenase
3,566,821	Insertion of G	Frameshift		PA14_40020	PA1895		Hypothetical
3,639,578	G/A	GCG/GCA	Alanine	PA14_40780	PA1837		Hypothetical
3,687,905	G/A	GCC/ACC	Alanine/Threonine	PA14_41350	PA1796	*folD*	Bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase,/5,10-methylene-tetrahydrofolate cyclohydrolase
3,711,601	Deletion of C	Frameshift		PA14_41560	PA1779		Assimilatory nitrate reductase
3,765,487	Deletion of C	Frameshift		PA14_42220			Membrane sensor domain containing protein
3,799,535	A/C	ATC/CTC	Isoleucine/Leucine	PA14_42710	PA2170		Hypothetical
3,934,559	T/C	CTG/CCG	Leucine/Proline	PA14_44210	PA1567		Glycine/D amino acid oxidase
3,943,570	A/G	Intergenic					
4,159,861	Deletion of G	Frameshift		PA14_46660	PA1363		RNA polymerase ECF subfamily sigma 70 factor
4,391,772	C/A	CCG/ACG	Proline/Threonine	PA14_49420	PA1158		Two component sensor
4,460,999	C/A	GCC/GAC	Alanine/Aspartic acid	PA14_50220	PA1097	*fleQ*	Transcriptional regulator
4,488,205	T/C	Intergenic					
4,502,169	T/G	GTG/GGG	Valine/Glycine	PA14_50680	PA1059	*shaA*	
4,532,506	Insertion of G	Intergenic					
4,533,297	T/C	Intergenic					
4,595,853	C/A	GCG/GAG	Alanine/Glutamic acid	PA14_51730	PA0971	*tolA*	
4,636,034	T/C	CTG/CCG	Leucine/Proline	PA14_52260	PA0928		Sensor/response regulator hybrid
4,787,261	Deletion of two Cs	Frameshift		PA14_53980	PA0793		Hypothetical
4,848,971	T/C	CTC/CCC	Leucine/Proline	PA14_54690	PA0741		Hypothetical
5,003,595	G/A	CGC/CAC	Arginine/Histidine	PA14_56030	PA4311		Hypothetical
5,009,310	T/C	Intergenic					
5,033,370	T/G	GTA/GGA	Valine/Glycine	PA14_56430	PA4341		Transcriptional regulator
5,036,924	C/A	CTG/ATG	Leucine/Methionine	PA14_56480	PA2922		Hydrolase
5,100,605	C/A	GCC/GCA	Alanine	PA14_57240	PA4404		Hypothetical
5,150,583	C/A	ACG/AAG	Threonine/Lysine	PA14_57850	PA4454		Hypothetical
5,235,267	G/A	GGC/GAC	Glycine/Aspartic acid	PA14_58750	PA4526	*pilB*	Type 4 fimbrial biogenesis protein
5,252,021	T/C	TCC/CCC	Serine/Proline	PA14_58910			Chromosome partioning like protein
5,295,976	G/A	CGC/CAC	Arginine/Histidine	PA14_59530			Hypothetical

5,412,824	A/C	AAC/ACC	Asparagine/Threonine	PA14_60730	PA4589		Outer membrane protein
5,589,447	G/A	Intergenic					
5,709,739	G/A	GCC/ACC	Alanine/Threonine	PA14_64060	PA4844		Chemotaxis transducer
5,836,844	T/C	Intergenic					
5,889,223	C/A	ACC/AAC	Threonine/Asparagine	PA14_66110	PA1390		Glycosyl transferase family protein
6,152,721	C/A	GGC/GGA	Glycine	PA14_68980	PA5223	*ubiH*	2-octaprenyl-6-methoxyphenyl hydroxlase
6,156,782	Deletion of CG	Frameshift		PA14_69040	PA5228		5-formyltetrahydrofolate cyclo-ligase
6,208,103	T/C	CTG/CCG	Leucine/Proline	PA14_69580	PA5269		Hypothetical
6,458,917	A/C	ATC/CTC	Isoleucine/Leucine	PA14_72490	PA5493	*polA*	DNA polymerase I

Appendix Table 5. Mutations in common between the red and brown pigmented isolate.

Location	**Sequence Change**	**Codon change**	**Amino acid change**	**Gene Number PA14**	**Gene Number PAO1**	**Gene I.D**	**Product Annotation**
155,680	C/T	GCC/GCT	Alanine	PA14_01690	PA0138		ABC transporter permease
1,423,505	A/G	GAT/GGT	Aspartic acid/Glycine	PA14_16640	PA3691		Lipoprotein
1,635,489	Insertion of C*	Intergenic					
1,794,770	A/G	GAC/GGC	Aspartic acid/Glycine	PA14_20850	PA3341		MarR family transcriptional regulator
2,520,703	A/G	GAG/GGG	Glutamic acid/Glycine	PA14_29130	PA2707		ATPase
3,994,253	Insertion of C**	Intergenic					
4,085,427	A/C	CAT/CCT	Histidine/Proline	PA14_45960	PA1430	*lasR*	Transcriptional regulator
4,435,660	G/A	GAA/AAA	Glutamic acid/Lysine	PA14_49890	PA1120	*tpbB*	Diguanylate cyclase
4,807,520	C/T	CTC/CTT	Leucine	PA14_54180	PA0781		Hypothetical
4,937,922	G/T	GCG/GCT	Alanine	PA14_55460	PA0684	*hxcZ*	
5,467,721	C/T	CCC/TCC	Proline/Serine	PA14_61250	PA4628	*lysP*	ABC family lysine-specific permease
6,457,982	C/A	GAC/GAA	Aspartic acid/Glutamic acid	PA14_72490	PA5493	*polA*	DNA polymerase I

* 2 Cs in red

**2 Cs in brown

General Discussion

General Discussion

The successful clinical management of chronic respiratory disease, in particular Cystic Fibrosis (CF), has been hindered by the rapid emergence of antimicrobial resistance (1, 2). Uncontrollable cycles of infection and inflammation resulting in irreversible lung damage contribute to progressive lung function decline (3-6). To date strategies have primarily focused on the alleviation of the symptomatic consequences of disease. This includes a combination of chest physiotherapy to clear the airways of viscous mucus, anti-inflammatories and antibiotics (7-10). However, the chronic colonisation of the CF airways by pathogens, frequently *Pseudomonas aeruginosa*, marks a switch in the emphasis of patient treatment to improving quality of life (QoF). Therefore, early and aggressive eradication strategies are essential in the prevention of pathogen establishment which have been shown to correlate with improved patient outcomes (11-14). Unfortunately, these strategies are not always effective in the prevention of chronic infection/inflammation, therefore alternative interventions must be sought (15). The design of such strategies requires a comprehensive understanding of the environmental and host factors contributing to the progression of chronic respiratory disease. Gastro-oesophageal reflux (GOR) is one such host factor which has been implicated as a key comorbidity in CF and a range of other respiratory conditions (16-20). Gastro-oesophageal reflux disease (GORD) was shown to correlate with increased disease severity and the development of bronchiectasis (21). However, the underlying mechanism through which GORD associated lung damage occurs had yet to be fully elucidated. It was proposed that GORD derived bile aspiration was the potential underlying causative agent responsible, with the focus of this PhD centred on establishing the molecular mechanisms through which bile exerts its impact on the biodiversity of the lung microbiota and on the key CF associated pathogen *P. aeruginosa.*

From a clinical perspective, the prevalence of GORD in CF and the resulting aspiration of bile into the airways has been confirmed as a significant comorbidity modulating the progression of lung disease, with evidence from both *in vitro* and *in vivo* studies to support this (12, 22-28). In fact, though this thesis has focused on CF, GORD has been described as a key comorbidity for a range of other respiratory conditions (as outlined in the general introduction). Therefore, there are far reaching consequences for the findings reported here. The microbiology of the CF lung and the changes that occur throughout disease progression have been the subject of intensive research efforts in the past number of years. These studies have shown that signature diverse microbial communities are present in the CF airways with a shift towards pathogen dominated, reduced microbial diversity as patients' transition to adulthood (29-34). Initial studies have predominantly focused on the characterisation of the lung microbiota, however the emphasis must now shift to the clinical implications of shifts in the microbiota and to the range of factors contributing to the restructuring of these communities.

The longitudinal clinical analysis of an Australian paediatric cohort revealed a correlation between the presence of bile acids and pathogen dominated microbiota. There was a shift to a reduced biodiversity pathogen dominated microbiota in patients for which bile acids were present. This reduction in diversity of the lung microbiota is a hallmark of CF disease progression and has been reported in several studies of the CF respiratory microbiota (30, 31, 35-37). Inter patient differences in terms of bile acid concentrations were present within the cohort. Interestingly, low concentrations of both bile and bile acids have been demonstrated to impact microbial communities and bacterial behaviour *in vitro* (12, 25-27). The differences in bile acid profiles between patients is also a subject worthy of further investigation, as individual bile acids have been demonstrated to exert differential biological effects (38). These studies support the hypothesis

that bile acids are capable of influencing the composition of the lung microbiota associated with increased disease severity. While these clinical studies are certainly informative, further studies and potentially animal models will be required in order to establish causality.

The observation that bile acid status correlated strongly with CF pathophysiology in paediatric patients is highly significant, as bile acid profiling may provide a rapid and effective early prognostic biomarker for the identification of high-risk patients predisposed to the onset of chronic respiratory infections. Where this is the case, advances in bile acid profiling technologies, possibly to the stage where point of care devices are routine in hospitals and clinics, would have significant clinical benefit. Most importantly, bile may represent a potential therapeutic target for the development of novel therapeutics, providing an opportunity for effective early clinical intervention.

While this research no doubt has clinical implications, the advances in CF molecular therapeutics such as Orkambi (39, 40) and to a lesser extent gene therapy which have been revolutionary for CF patients, would question the need for such research. However, the availability and administration of these therapies is not without its limitations. The primary limitation is the age at which patients' may commence treatment (40). Until recently this stood at 6 years of age with the FDA recently approving treatment in paediatric patients as young as two (40). Unfortunately, by this time the dysregulated cycles of chronic inflammation and chronic infection which are central to the pathogenesis of CF have been initiated with patients as young as 1 already exhibiting bronchiectasis (5, 6, 41, 42). Hence, there is a degree of pre-existing lung damage prior to the administration of these treatments. Furthermore, the acquisition of pathogens and subsequent microbial dysbiosis occurs in infancy, prior to the commencement of these therapies (32, 43). This further highlights the need for alternative earlier intervention strategies which may be used in

conjunction with these molecular therapeutics with the goal of hindering the progression of lung disease.

It is clear that bile has the potential to play a role in shaping the respiratory microbiota, with individual respiratory pathogens shown to respond to bile by modulating the expression of virulence related signal transduction systems (26). Deciphering the mechanisms through which pathogens such as *P. aeruginosa* respond to bile is key to the development of targeted strategies for the prevention of chronic bacterial infection which cannot be eradicated by conventional antibiotic treatment strategies. This research predominantly focused on *P. aeruginosa* which is the primary pathogen associated with morbidity and mortality in CF (21, 44). However, other respiratory pathogens such as *Staphylococcus aureus* and *Stenotrophomonas maltophilia* were also shown to respond to bile by transitioning towards a biofilm lifestyle. It was interesting to note that while clinical isolates of these species from the lungs of patients with respiratory disease exhibited an increased biofilm state in response to bile, model isolates from culture collections exhibited a contrasting phenotype of markedly reduced biofilms. This would suggest a degree of adaptation within the challenging ecosystem of the host pulmonary system. Transcriptomic and phenotypic analysis of the *P. aeruginosa* response to bile challenge revealed alterations in virulence, including enhanced antibiotic resistance and biofilm formation, potentially linked to alterations in central metabolism and redox flux. The connection between virulence potential and metabolic flux has been previously reported in a range of pathogens including *P. aeruginosa* (45, 46), *S. aureus* (47), *Salmonella enterica* (48) and *Listeria monocytogenes* (49). A comprehensive understanding of the behavioural changes induced by bile will facilitate the design of increasingly effective treatment plans for the management of disease.

The adaptations of bile tolerant bacteria residing within the GI tract is a valuable source of information regarding the mechanisms of bile response systems which may be conserved between enteric and respiratory bacteria. There is a wealth of literature describing the numerous strategies employed by enteric bacteria to colonise the gut and withstand bile and bile acid stress. This includes modification to the outer membrane, the lipopolysaccharide (LPS) moiety in particular (50-53). An array of multidrug efflux pumps, such as the AcrAB and EmrAB pumps, have been implicated in the bile tolerance of these bacteria, facilitating the removal of bile that permeates the outer membrane (53-57). Porin modification has also been shown to contribute to bile tolerance by decreasing the ability of bile salts to penetrate the membrane (55, 58). Bile acids are also capable of inducing a transcriptional response with generalised and oxidative stress responses described in a variety of organisms (59, 60). It is therefore possible that bacteria residing in the airway may employ some of these strategies in order to survive and persist in the airways.

The primary phenotype associated with the switch to chronicity is the production of a biofilm with bile shown to increase biofilm formation in the *P. aeruginosa*. Bile has been previously shown to induce biofilm in the enteric organisms *Salmonella* and *Vibrio cholerae* (61-64) Understanding how the pathogen senses and responds to this host factor would facilitate the design of novel therapeutic strategies and facilitate the design of targeted molecular therapeutics preventing the induction of biofilm. Therefore, the identification of sensory pathways involved in the bile induced chronic switch would be a significant advance. This translational research would provide many opportunities for the development of novel therapeutics, which could potentially target the sensory pathways in these respiratory pathogens orchestrating this critical response to bile. The mechanisms through which enteric bacteria sense and respond to bile and/or bile acids

in the environment has been previously investigated and is still not fully understood. The two component system PhoPQ (65) and transcriptional activators; RpoS and SigB are reported to play a role (66, 67). There may also be an indirect detection of bile acids by detection of alterations in the bacterial cell membrane.

The DctB-DctD two component system regulates C4-dicarboxylate uptake in the cell (68) and the GtrS-GltR two component system regulates glucose uptake (69). Mutations within these genes hindered but did not completely abolish the ability of *P. aeruginosa* to enhance biofilm formation in the presence of bile. Hence, there appears to be a degree of redundancy in the regulatory controls underpinning the bile response. Additionally, protein interaction studies would be required to investigate the regulation and activation of these systems and whether there is any interaction between them. Further elucidation of the hierarchical intricacies of the molecular mechanism controlling the response of the pathogen to this key host factor may facilitate the design of signal transduction blockers (70, 71). However, the absence of an identifiable master regulator controlling the bile response makes the design of molecules to block the response much more challenging.

The combined transcriptomic and phenotypic approach undertaken identified additional signal transduction components and the possible molecular mechanism controlling the bile response. Though there was components identified in common with a generalised stress response, the combination of systems altered appeared to indicate that there is a unique bile specific response. This revealed a connection between flux through central metabolism, key metabolic two component systems, outlined above, and the mounting of an effective bile response. These analyses also indicated that the response to bile may involve several other regulatory systems including small RNAs and cyclic di-GMP with cyclic di-GMP implicated in *V. cholerae'*

response to bile (72). Further work is required to elucidate the complexity of these systems and to decipher how the cell integrates these various regulatory circuits to mount an effective response to this emerging host factor which has the ability to modulate the progression of chronic respiratory disease.

Reports of *P. aeruginosa* acquisition via an aerodigestive route is significant considering the bile responsiveness of *P. aeruginosa* (73, 74). The gastrointestinal tract is known to act as a reservoir for *P. aeruginosa* and other potential respiratory pathogens with acid suppressed of CF patients suffering from GORD contributing to bacterial overgrowth (75-77). These isolates may have had prior exposure and adaptation to bile within the GI tract with the potential subsequent transmission of pre-adapted isolates into the lower airways, equipping these organisms with a further competitive advantage within the CF airways (74, 78, 79). Therefore, the wide spread administration of acid suppression therapies in these patient cohorts should be re-evaluated for their efficacy due to the inadvertent impact on GI tract bacterial overgrowth which may have potential negative repercussions on the progression of chronic lung disease (80). The administration of probiotics may be a viable strategy to counter these effects (76).

The fact that bile itself is a complex mixture consisting of a mixture bile acids, phospholipids, fatty acids and bilirubin adds a further layer of complexity when deciphering the response of *P. aeruginosa*. Hence, the bacterial response to bile may be a culmination of response to these individual components. Bile acids have emerged as a key active component underpinning chronic biofilm formation in *P. aeruginosa*, with CDCA in particular eliciting the strongest response. Specificity in the interaction between bile acids and a potential receptor system would enhance the feasibility of designing a targeted molecular therapy. However, further work is needed to establish

the degree to which individual bile acids contribute to antibiotic tolerant biofilm formation in *P. aeruginosa* and other respiratory pathogens.

Of course, in pursuing a molecular mechanism and in seeking to understand the factors that govern chronicity in the lungs of patients with respiratory disease, one must also take note of the genotypic and phenotypic heterogeneity that pervades in the lungs (81-83). Several studies have highlighted the evolution of *P. aeruginosa* and other pathogens within the lungs of patients with respiratory disease (84-86). These studies have highlighted extensive diversification and adaptation to the CF lung environment, underpinned by the emergence of hyper-mutator strains (87) with mutations in key quorum sensing genes, such as *lasR* (88), and antibiotic resistance genes such as the *mex* genes (89) described. As a result, any intervention would need to target a community of variants rather that a single clonal population. The hyper induction of pyocyanin and PQS signalling in response to bile was particularly interesting given the central role these systems play in the host-pathogen and microbe-microbe interactions, with QS expression demonstrated to cause global metabolic changes within the cell, similarly to that observed within the bile transcriptome (90-96). The question as to why pigmented variants emerge following long-term bile exposure could potentially be attributed to the requirement for the population to manage what are essentially toxic levels of pyocyanin and PQS within the community (92). Increasing PYO and PQS upon initial exposure may offer a competitive advantage to *P. aeruginosa* as it seeks to establish itself within the mixed microbial community, responding to what it has evolved to recognise as an important host signal (97). The pigments themselves may also confer an increased tolerance to stresses encountered during colonisation, such as oxidative stress and redox imbalance (98-100). Further work will be needed to establish the impact of these variants on the dynamics of *P. aeruginosa* within

respiratory microbiomes, and to elucidate the extent to which mutations in these systems are propagated within the clinical microbiota.

Though not in the scope of this thesis, bile has been shown to both directly and indirectly impact the initiation of inflammation, indirectly through its promotion of colonisation by pro-inflammatory pathogens and directly through its impact on the host inflammatory response. The presence of bile acids has been correlated with increased levels of neutrophils, neutrophil elastase and the pro-inflammatory cytokine interleukin-8 (24, 101, 102). Bile acids have also been shown to destabilise the transcription factor HIF-1 required for the resolution of acute inflammation (103, 104). The dual targeting of airway infection and airway inflammation by bile and bile acids outlined in Figure 1 below makes it an ideal target for early preventative measures.

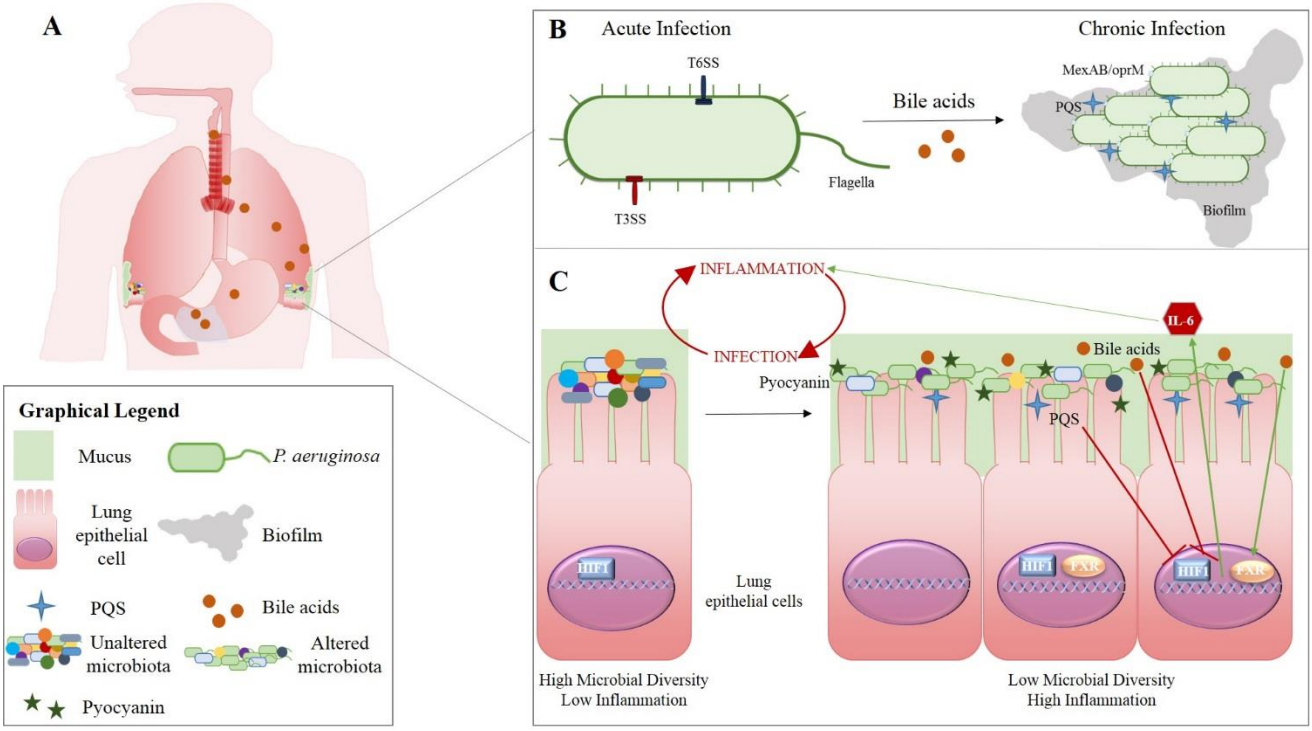


Figure 1; Overview of the bile acid-microbiota axis within the CF lung. (A) The accumulation of bile acids within the lungs of patients with CF as a consequence of aspiration leads to changes in the structure of the lung microbiota. This results in a transition from a high diversity stable community (left lung) to a pathogen dominated low diversity population (right lung). (B) The impact of bile acids on the behaviour of the key CF associated pathogen *P. aeruginosa* is consistent with a switch towards a chronic antibiotic tolerant biofilm lifestyle. (C) The dual impact of bile acids on the lung microbiota (promoting chronicity) and the inflammatory response (e.g. FXR-dependent induction of pro-inflammatory cytokines) underscores the unifying principle of bile acids as a major host factor promoting the progression of chronic respiratory disease. Modulation of host signalling through the HIF-1 transcription factor by bile acids and bile-induced *P. aeruginosa* derived PQS serve to further promote dysregulated inflammation in this patient cohort.

Early intervention is crucial in the long term prevention of bile induced lung damage, with a concerted effort to identify biomarkers for early prognosis. There are many possible therapeutics aimed at the control of aspiration. The most common approach currently undertaken is a surgical approach in the form of a laparoscopic Nissen Fundoplication (105, 106). As with all surgical

procedures, however, there are associated risks hence there is limited practice of this surgery in paediatric patients (107, 108). Therefore, there are calls for less invasive anti-GORD techniques. Pro-kinetic macrolides are one such strategy which target bile aspiration at the source by enhancing gastric emptying and consequently reducing the frequency of aspiration events (109, 110). The administration of inhaled bile acid sequestrants (111) may prove to be a viable alternative which could silence the biological effects of bile acids on both bacterial and host cells. As bile acids display differential biological activity with the gut microbiota influencing the bile acid profiles present, manipulation of the gut microbiota with probiotics may be worthy of further investigation.

Taken together, the new knowledge presented in this thesis provides a new perspective on the factors governing the chronic persistence of *P. aeruginosa* in the lungs of patients with CF. It offers evidence of a correlation between lung bile acids and changes in community structure, both from a cross-sectional and longitudinal perspective. The *in vitro* studies support the hypothesis that bile, and bile acids, can shift the behaviour of *P. aeruginosa* and other respiratory pathogens towards a chronic persistent lifestyle. However, further clinical and animal model studies will be required to establish a causal link. The aspiration of bile acids is another example of how the role of bile, and particularly bile acids, in host cellular physiology extends far beyond the classical understanding. With increasing sensitivity, the detection of bile acids at physiologically relevant concentrations has expanded our appreciation for their role in previously unforeseen clinical conditions. The realisation that microbially derived bile acids can impact on chronic diseases from CF to Cancer has opened new horizons in the search for better more effective clinical interventions, beyond their classical role in human physiology. The next horizon for bile acid research will be deciphering and manipulating their bioactivity for control of the pathophysiology of a range of clinical conditions.

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Publication List

Publications

1. **Flynn S**, Woods D.W., Ní Chróinín M, Mullane D, Adams C, Reen F.J. and O'Gara F. **Bile Aspiration; a host factor modulating chronic respiratory infection.** (2016) In Bile Acids: Biosynthesis, Metabolic Regulation and Biological Functions. Edited by Murphy A. (Nova Science Publishers).

2. **Flynn S**, Reen F.J., Woods D.W., Dunphy N, Ní Chróinín M, Mullane D, Stick S, Adams C and O'Gara F. **Bile signalling promotes chronic respiratory infections and antibiotic tolerance.** *Sci. Rep,* 2016, 6, 29768.

3. Ulluwishewa D, Wang L, Pereira C, **Flynn S**, Cain E, Stick S, Reen FJ, Ramsay JP and O'Gara F. **Dissecting the regulation of bile-induced biofilm formation in *Staphylococcus aureus.*** Microbiology, 2016, **162**(8): 1398-1406.

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