Using an *in vitro* model of human cystic fibrosis airways to investigate bacterial strategies which inactivate defence mechanisms and increase infection

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Thesis Summary

Cystic fibrosis (CF) is the most common inherited genetic condition amongst Caucasians and arises due to mutations in the cystic fibrosis transmembrane conductance regulator, a chloride channel expressed upon the apical surface of epithelia. Whilst CF is a multi-organ disease, the inability to clear dehydrated mucus from the airways predisposes individuals to the development of chronic bacterial infections, the main cause of morbidity and mortality in CF. Infection of CF airways is highly ordered, with *Staphylococcus aureus* predominating in the first decade of life, followed by *Pseudomonas aeruginosa* during adulthood. Two obstacles to the development of better treatments stem from an incomplete understanding of the polymicrobial nature of CF airway infection and its impact upon interspecies and host-pathogen interactions, alongside the need for models which more closely mimic the CF lung and its unique environment.

After characterising a panel of *P. aeruginosa* CF clinical isolates, this study sought to determine the impact of oxygen availability upon *S. aureus-P. aeruginosa* interspecies interactions, in light of evidence that mucus plugging within CF airways leads to regions of anoxia. Anoxia was shown to modulate *S. aureus-P. aeruginosa* community composition in planktonic co-culture and mixed species biofilms in an isolate-dependent manner. Further investigations into the mechanisms facilitating *P. aeruginosa* dominance suggest that the anti-staphylococcal agent is extracellular, >3 kDa in size and heat-resistant.

Whilst pulmonary inflammation is a hallmark of CF, how airways respond to stimuli received during polymicrobial airway infections is poorly understood. Monolayers of CF and non-CF bronchial epithelia were challenged with *S. aureus* and/or *P. aeruginosa* extracellular products. CF airway epithelia exhibited a hyper-inflammatory phenotype at baseline compared to non-CF epithelia. Furthermore, only co-stimulation of non-CF epithelia with both pathogens, enhanced the IL-6 and IL-8 response beyond that measured following single bacterial challenges. Finally, CF and non-CF airway epithelia grown at air-liquid interface in the presence of fibroblasts were used to mimic the sequential nature of CF infection. Binding studies demonstrated that prior infection with *S. aureus* enhanced *P. aeruginosa* binding to the CF airway model in an isolate-specific manner, a finding not seen in the non-CF airway model.

These studies demonstrate that *S. aureus-P. aeruginosa* interactions are likely to influence the CF microbiome, airway inflammation, airway colonisation and ultimately, disease progression. It is hoped that the models used here can be employed in future studies to understand the complex interspecies and host-pathogen interactions that occur in CF, with the aim to identify novel targets and treatments to combat these life limiting infections.

Key words: cystic fibrosis, *in vitro,* pathogen-pathogen, air liquid interface, host-pathogen

For you Mom.

Thank you for loving me unconditionally.

Everything I am, everything I have achieved is because of your guidance, your fierce love and the sacrifices you have made.

You will forever be my everything

and I miss you more than words can ever say.

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Abbreviations

3-oxo-C_{12}-HSL	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
α1-AT	α1-antitrypsin
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
A. fumigatus	*Aspergillus fumigatus*
aGM1	Asialoganglioside GM1
AHL	Acyl homoserine lactone
ALI	Air-liquid interface
ANOVA	Analysis of variance
AMP	Adenosine monophosphate
ASL	Airway surface liquid
ATCC	American tissue culture collection
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
Bcc	*Burkholderia cepacia* complex
BSA	Bovine serum albumin
C_4-HSL	*N*-butanoyl-L-homoserine lactone
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CI	Competitive index
Cl^-	Chloride ion
ClfA	Clumping factor A
CO_2	Carbon dioxide
Cbp	Collagen binding protein
CRD	Carbohydrate recognition domain
CTB	CellTiter-Blue®
DMEM:F12	Dulbecco's modified eagle medium: Nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
Ebp	Elastin binding protein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
EMEM	Eagle's minimum essential medium
ENaC	Epithelial sodium channel
ELISA	Enzyme-linked immunosorbent assay
Ex	Excitation
FBS	Foetal Bovine Serum
Fe^{3+}	Ferric ion
FEV_1	Forced expiratory volume in one second
FnbpA	Fibronectin binding protein A
g	Gram
g	Gravity
h	Hour
H_2	Hydrogen
HCN	Hydrogen cyanide
HCO_3^-	Bicarbonate ion
HBD	Human beta defensin
HO-1	Heme oxygenase-1

HPLC	High performance liquid chromatography
HQNO	2-heptyl-4-hydroxyquinoline N-oxide
HUVEC	Human umbilical vein endothelial cell
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IMS	Industrial methylated spirit
KC	Keratinocyte chemoattractant
kDa	Kilodaltons
LasA	Elastase A
LasB	Elastase B
LB	Luria bertani
LBN	Luria bertani with nitrate
LES	Liverpool epidemic strain
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
µ	Micro
m	milli
M	Molar
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney cells
min	Minutes
mg	Milligram
MIC	Minimum inhibitory concentration
MIP-2α	Macrophage inflammatory protein-2α
MOI	Multiplicity of infection
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MSA	Mannitol salt agar
MSD	Membrane spanning domain
MYD88	Myeloid differentiation primary response gene 88
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding domain
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor of kappa B
n	Nano
NS	Not significant
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCL	Periciliary liquid
p	Pico
PIA	*Pseudomonas* isolation agar
PQS	*Pseudomonas* quinolone signal
PRR	Pattern recognition receptor
PVL	Panton-Valentine leucocidin
QS	Quorum sensing
RANTES	Regulated upon activation in normal T-cell, expressed and secreted
RFU	Relative fluorescence units
RIR	Relative increase ratio
ROS	Reactive oxygen species
SCN^-	Thiocyanate ion
SCV	Small colony variant
Sec	Seconds

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SC	Secretory component
sIgA	Secretory immunoglobulin A
SLPI	Secretory leukoprotease inhibitor
SP	Surfactant protein
SPLUNC1	Short palate lung and nasal epithelium clone 1
TEER	Transepithelial electrical resistance
TSB	Tryptic soy broth
TW	Transwell® insert
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
V	Volts
v/v	Volume per volume
w/v	Weight per volume
wt	Wildtype

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1 Introduction

1.1 Cystic Fibrosis: Epidemiology, economic impact and aetiology

Cystic fibrosis (CF) is the most common inherited genetic condition amongst Caucasians. With a carrier rate of 1 in 25 (Ratjen and Doring, 2003), CF affects approximately 80,000 people worldwide, 25,000 of which are registered in Europe. Approximately 10,000 people are living with CF in the United Kingdom (European Lung Foundation, 2009), with Ireland having the highest prevalence in Europe (Cystic Fibrosis Ireland, 2013). Figure 1 shows the prevalence of CF across Europe.

Figure 1. Estimated prevalence of CF across Europe. The UK has one of the highest incidences of CF across 21 European countries. Data was collected by the European Cystic Fibrosis Society between 2007-2009 (European Lung Foundation, 2009).

Improvements in the diagnosis and treatment of CF over the last sixty years has significantly increased life expectancy. Whilst over three quarters of individuals with CF born in 1938 would die within their first year of life, average life expectancy in the 1980's increased to ten years of age (Child Life Society, 2018). Today, life expectancy of an individual with CF in the UK is 41 years of age, whilst those born in 2000 are expected to live to 47 years of age (Cystic Fibrosis Trust, 2017, Trust, 2018). This success can be attributed to numerous factors including patient segregation, improvements in treatments and treatment regimens, a multidisciplinary approach regarding individual care and earlier diagnosis (Dodge *et al.*, 2007). The median age for the diagnosis of CF in the UK following birth is 26 days (Cystic Fibrosis Trust, 2018).

A gender disparity remains within the CF population. Whilst one study reported that life expectancy is 3.7 years lower for women with CF compared to men (Harness-Brumley *et al.*, 2014), according to the UK CF Trust Registry, the median predicted survival for women in the UK is 6.5 years lower than men (Cystic Fibrosis Trust, 2018). Although further research is required to address this, females with CF are more likely to be underweight, a factor known to influence survival (Fogarty *et al.*, 2012, Corey *et al.*, 1988). Furthermore, oestrogen has been associated with a worsening of CF symptoms (Chotirmall *et al.*, 2012). In addition to greatly impacting upon an individual's quality of life, CF is also associated with a significant economic burden. Despite its low disease prevalence, a study conducted in 2012 demonstrated that the average annual healthcare cost per individual with CF was £48,603. This has been shaped by costs associated with medications for symptom management, acute hospitalisations and primary healthcare visits (Angelis *et al.*, 2015).

CF is an autosomal recessive, single gene, inherited disorder, which arises due to a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989). Composed of 250,000 base pairs and encoded on the long arm of chromosome 7, the CFTR is a cAMP-dependent anion channel belonging to the ATP-Binding Cassette (ABC) transporter family of membrane proteins (Tsui and Dorfman, 2013). Present upon the apical surface of epithelial cells and the newly discovered and uncommon cell type, pulmonary ionocytes (Plasschaert *et al.*, 2018), the CFTR is involved primarily in the transport of chloride (Cl^-) and to a lesser degree, bicarbonate (HCO_3^-) and thiocyanate (SCN^-) ions (Quinton, 2008, Fragoso *et al.*, 2004, Quinton, 1983). By exerting an inhibitory effect upon the epithelial sodium channel (ENaC) (Konig *et al.*, 2001), the CFTR regulates the movement of water onto the apical surface of epithelial cells.

1.2 CFTR: Structure and Function

The CFTR is a 1,480 amino acid glycoprotein (170 kDa), belonging to the ABC transporter family. Composed of a tandem repeat of the characteristic ABC motif, it consists of two membrane spanning domains (MSDs), each located next to a nucleotide binding domain (NBD). When phosphorylated, the binding of adenosine triphosphate (ATP) to NBD-1 and its consequent hydrolysis induces a conformational change, opening the chloride channel, whilst the binding of ATP to NBD-2, closes it. The activity of the channel is also regulated by the regulatory (R) domain, known to block the ATP binding sites on NBD-1 through de-phosphorylation of its serine residues (Lyczak *et al.*, 2002, Gadsby and Nairn, 1999). When open, chloride and bicarbonate ions move from the cytosolic side, through the CFTR pore by passive diffusion onto the epithelial cell surface. The CFTR also inhibits ENaC, important in initiating sodium reabsorption (Ismailov *et al.*, 1996). The structure of CFTR protein is illustrated in Figure 2**.**

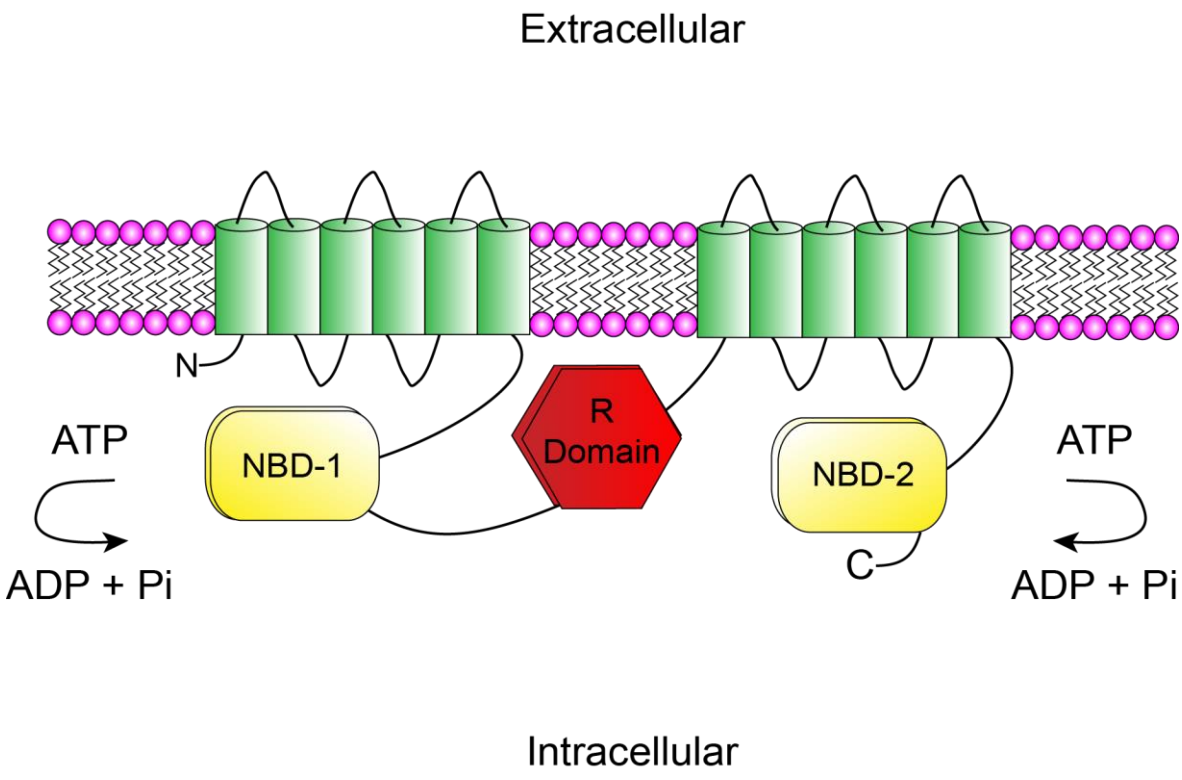

Figure 2. Overview of the structure of the cystic fibrosis transmembrane conductance regulator (CFTR). Each of the two, six membrane spanning domains of the CFTR (green) are bound to a nuclear binding domain (yellow). The regulatory domain (red) is comprised of numerous charged amino acids. Activation of this chloride channel requires phosphorylation of serine residues in the R domain by protein kinase A or C and the hydrolysis of ATP by the two nuclear binding domains, giving rise to adenosine diphosphate (ADP) and inorganic phosphate (Pi). Adapted from (UK Cystic Fibrosis Gene Therapy Consortium, 2018).

1.3 CFTR Mutations

A total of 2,031 mutations in the CFTR have been identified on the Cystic Fibrosis Mutation Database (SickKids, 2018) and are categorised into five classes according to their effect upon protein synthesis, maturation, regulation, chloride conductance and trafficking (Fanen *et al.*, 2014). The type of CF mutation influences CF disease severity and whilst a number are well studied, the vast majority remain poorly characterised. A brief description of each class is outlined in Table 1 below.

Table 1. CFTR mutation classes and their intracellular effect. Adapted from (CFTR.info, 2017).

Class	Mutation	Example
I	Frameshift and nonsense mutations which introduce premature stop codons, preventing the expression of CFTR at the apical membrane of epithelia.	Trp1282X
II	Missense and inframe mutations affect protein folding, causing it to remain trapped at the endoplasmic reticulum and preventing its trafficking to the apical membrane of epithelial cells. Instead it becomes a target for ubiquitination and is subsequently sent to the proteasome for degradation.	Phe508del
III	Full length CFTR is synthesised and is incorporated into the cell membrane. However, a gating defect caused by substitutions in the amino acid sequence prevents the channel from opening in response to cAMP and other agonists.	Gly551Asp
IV	A conductance defect caused by missense mutations. The introduction of amino acid substitutions alters the structure of the channel's pore, affecting the movement of chloride anions.	Arg117His
V	Missense mutations introducing alternative splicing of mRNA. Reduced amounts of functional CFTR reach the apical cell membrane of respiratory epithelia.	Ala445Glu
VI	Mutations which cause an increased turnover of CFTR at the apical cell surface, due to its instability.	Rescued Phe508del

As outlined in Table 1, the Phe508del mutation, caused by a deletion of a single phenylalanine residue in NBD-1 (at position 508) is a classic example of a class II mutation. Here the CFTR protein is ubiquitinated and degraded by the endoplasmic reticulum (Farinha and Amaral, 2005). In addition to being the first identified CF mutation, it remains the most prevalent, being present on at least one allele in 90% of all individuals with CF worldwide (Bobadilla *et al.*, 2002) and 89.5% in the UK (Cystic Fibrosis Trust, 2018). In the UK, 49.1% of CF patients are homozygous for Phe508del, whilst 40.4% are heterozygous (Cystic Fibrosis Trust, 2018). This prevalence is likely to be due to this region being particularly susceptible to mutation (Lyczak *et al.*, 2002). No other mutations account for more than 5% of CF mutations (O'Sullivan and Freedman, 2009, Cystic Fibrosis Trust, 2018). Class I and II mutations result in the most severe CF phenotypes due to the absence of the CFTR in the apical membrane of epithelia (Welsh and Smith, 1993).

1.4 Clinical manifestations of CF

As the CFTR is expressed upon the surface of epithelia lining the respiratory, digestive and reproductive tracts, CF is a multi-organ disease. Its absence, impaired functioning, reduced presence or instability at the apical cell membrane decreases chloride secretion onto the epithelial cell surface, coupled with increases in the reabsorption of sodium and water (Matsui *et al.*, 1998). Ultimately water reabsorption gives rise to high-viscosity dehydrated mucus. Mucus obstructions can form within the gastrointestinal tract, with distal intestinal obstruction syndrome occurring in 5.3% of individuals with CF (Cystic Fibrosis Trust, 2018). Mucus plugs in exocrine glands lead to approximately 29.8% of individuals with CF being on treatment for CF-related diabetes mellitus and 12.9% developing liver disease (Cystic Fibrosis Trust, 2018). Blockages within the vas deferens also causes 98% of males to be infertile (Taussig *et al.*, 1972), which is further hindered by reductions in sperm quality (Wang *et al.*, 2003).

Despite these systemic effects, pulmonary manifestations present the most severe symptoms and are the main cause of morbidity and mortality in CF (Lyczak *et al.*, 2002, Ciofu *et al.*, 2013). The formation of mucus plugs within the airways facilitates the development of chronic bacterial infections which typically begin early in life and are accompanied by an extensive, yet ineffective airway inflammatory response. Individuals display bronchiectasis, accompanied by shortness of breath, chest pain and a chronic productive cough. Extensive fibrosis of the airways caused by chronic airway infections and inflammation leads to narrowing of the airway lumen and reductions in pulmonary function overtime, ultimately leading to hypercapnia, respiratory failure and death (Flume *et al.*, 2010). Respiratory failure due to chronic pulmonary infections is the main cause of mortality in CF (Lyczak *et al.*, 2002, Ciofu *et al.*, 2013).

1.5 Mutant CFTR and impairments in pulmonary innate immunity

CFTR is expressed upon numerous cell types within the lungs, including the surface of ciliated airway epithelia (Kreda *et al.*, 2005), cells of the submucosal glands (Engelhardt *et al.*, 1992) and professional phagocytes (Painter *et al.*, 2006, Di *et al.*, 2006). Together these cell types play essential roles within pulmonary innate immunity and provide protection against inhaled pathogens. In addition to forming a physical barrier, airway epithelia secrete antimicrobial peptides and proteins and can initiate an airway inflammatory response (Hiemstra, 2001, Bartlett *et al.*, 2008). Whilst submucosal glands produce mucus which traps inhaled pathogens and particles, airway epithelia facilitate its removal from the airways due to the expression of motile hair-like projects known as cilia (Wanner *et al.*, 1996). Furthermore, professional phagocytes including alveolar macrophages and circulating neutrophils are recruited to the site of infection in order to permit bacterial killing and clearance. Mutations in the CFTR are known to impair many aspects of pulmonary innate immunity, permitting the development of chronic airway infections and inflammation.

1.5.1 Impaired mucociliary clearance

Ciliated epithelia account for around half of all epithelia within the airways and are abundant in mitochondria (Spina, 1998). Each cell contains approximately 200-300 cilia upon their apical cell surface, with there being approximately 10^9 cilia per cm^2 of the respiratory tract (Livraghi and Randell, 2007). Interspersed between ciliated epithelia at a ratio of 1:5 are goblet cells, containing acidic, mucin-rich granules and protrude surface microvilli (Ganesan *et al.*, 2013).

Goblet cells constitutively secrete mucus into the lumen of the large airways, governing not only its depth, which can range from 7-70 µm, but also its rate of production, acidity and viscosity (Jayaraman *et al.*, 2001, Tarran, 2004). Consisting primarily of water (97%), in addition to ions, mucus also contains mucins , large anionic molecular glycoproteins (Thornton *et al.*, 2008). Whilst there are currently twenty known mucins, five are secreted into the airways (Williams *et al.*, 2006, Davis, 2002, Rogers, 2007), of which MUC5AC and MUC5B are the most predominant (Groneberg *et al.*, 2002). Structurally related and present in similar concentrations, MUC5AC is secreted by tracheal-bronchial goblet cells, whilst MUC5B is secreted by glands in the submucosal connective tissue (Groneberg *et al.*, 2002, Hovenberg *et al.*, 1996). The ability of secreted mucins to undergo extensive cross-linking via disulphide bridges, gives rise to characteristic viscous gel-like properties of mucus (Ridley *et al.*, 2014, Voynow and Rubin, 2009), with sialic acid residues also contributing to its viscoelastic properties (Shiomi *et al.*, 2002). As well as trapping inhaled pollutants and inhaled pathogens, mucins are also able to specifically bind to particular pathogens, serving as adhesion decoys and preventing bacterial binding directly to airway epithelia. MUC1 (a transmembrane mucin present upon the surface of respiratory epithelia) has been shown to possess this ability,

whereby it forms interactions with the two most prevalent pathogens known to colonise CF airways, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Linden *et al.*, 2008).

The synchronised unidirectional beating of ciliated epithelia help drive overlying mucus (containing trapped inhaled pollutants and pathogens), propelling it from the lower airways and out of the trachea, preventing tissue damage and infection (Livraghi and Randell, 2007). To permit this rapid beating, the cilia are bathed in periciliary fluid (also referred to as the periciliary liquid), which has a depth of approximately 5-7 µm (Tarran, 2004). Collectively referred to as the airway surface liquid (ASL), the ability to maintain the two distinct phases of high viscosity mucus and low viscosity periciliary liquid (PCL) arises due to the presence of membrane spanning mucins. These large mucins form a mesh between cilia, which is effective at preventing MU5AC and MUC5B from collapsing into the PCL. Mucociliary clearance is effective at removing approximately 90% of all inhaled particles and thus an essential innate defence mechanism of the airways (Widdicombe, 2002, Vareille *et al.*, 2011).

Mutations within the CFTR however, deplete the PCL and thus decrease the volume of the ASL. These mechanisms consequently bring the once overlying mucus layer into direct contact with ciliated respiratory epithelia (Matsui *et al.*, 1998). Whilst non-CF airways have a mucus flow of ~60 µm/s, the dehydration of mucus within CF airways cause the cilia to flatten, leading to mucostasis (Button *et al.*, 2016, Henderson *et al.*, 2014). Inhaled pathogens are no longer cleared from the airways and can go on to colonise and establish an infection. This is summarised in Figure 3**.**

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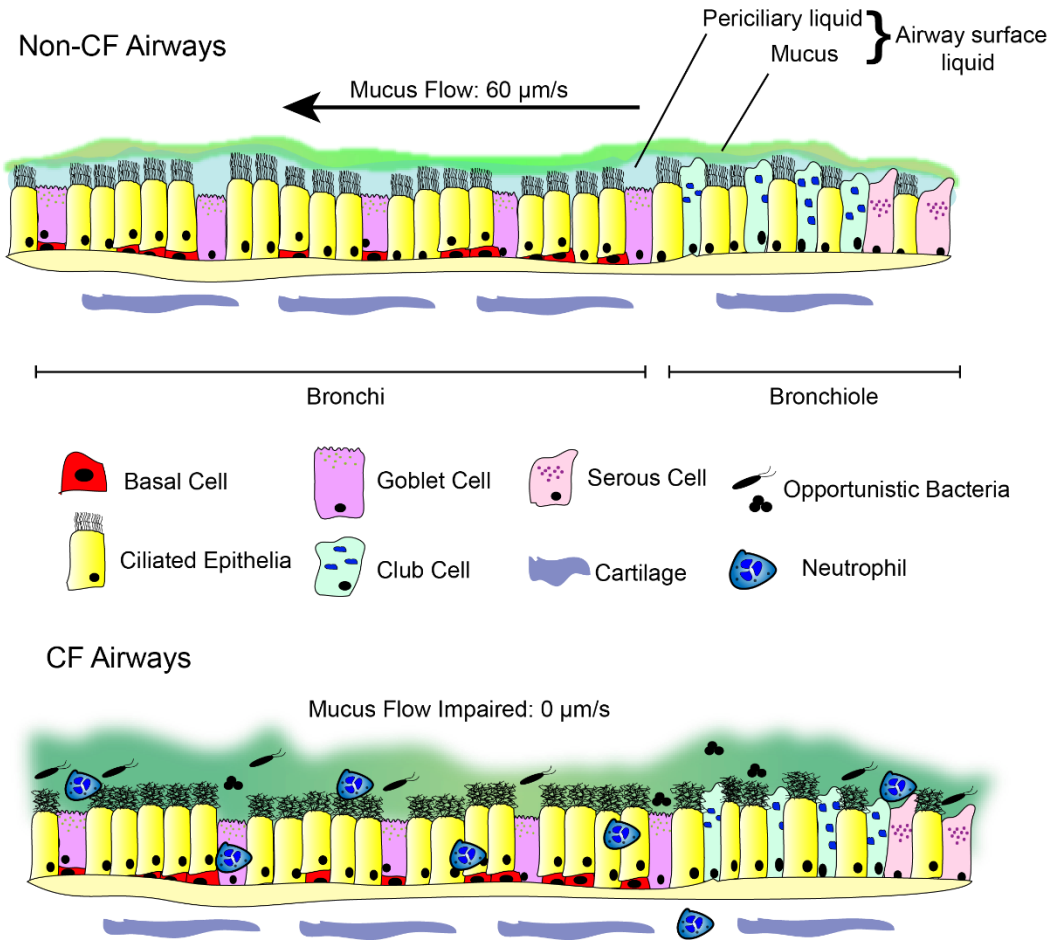


Figure 3. Non-CF vs. CF airways. In non-CF airways (top), cilia beat within the low-viscosity periciliary liquid in a unidirectional manner, propelling the overlying mucus at a rate of 60 µm/s from the bronchioles to the pharynx. Inhaled pathogens, pollutants and irritants are thus removed from the lower airways, preventing damage and limiting infection. In CF airways (bottom), the absence, improper functioning or instability of the CFTR causes sodium and water hyperabsorption, leading to a loss of periciliary liquid. Airway mucus dehydrates and comes into direct contact with the surface of respiratory epithelia. Cilia become flattened and mucociliary clearance becomes impaired. Inhaled pathogens are able to colonise the lungs, leading to an unresolved inflammatory response, which results in the significant infiltration of neutrophils. Adapted from (Ratjen and Doring, 2003).

Mutations in the CFTR also influence mucus production, through its role in regulating bicarbonate ion secretion. Bicarbonate has been shown to play a role in the hydration of intestinal mucins in CF mice (Garcia *et al.*, 2009, Gustafsson *et al.*, 2012). It has been hypothesised that this inability to transport bicarbonate ions in CF airways may also impact upon mucus hydration, increasing its viscosity (Quinton, 2008), as well as acidifying the ASL (Coakley *et al.*, 2003). It is possible that this acidification alters the electrostatic charge on the side chains of mucins, increasing mucus viscosity (Bhaskar *et al.*, 1991).

The continual secretion of mucus and inability to clear it from the airways facilitates the formation of thick mucus plaques and plugs which can occlude the airway lumen (Worlitzsch *et al.*, 2002, Hamutcu *et al.*, 2002). Coupled with the increased oxygen consumption by CF epithelia (Stutts *et al.*, 1986), respiring pathogens and host phagocytes (Worlitzsch *et al.*, 2002, Kolpen *et al.*, 2010), this gives rise to steep oxygen gradients within airway mucus, which range from normoxia to anoxia (Cowley *et al.*, 2015, Worlitzsch *et al.*, 2002). In addition to anoxia permitting the growth of obligate anaerobes within the CF lung (Rogers *et al.*, 2003), mucus plugs are likely to provide a protected niche to CF pathogens such as *P. aeruginosa*, which have been reported to exhibit a higher tolerance to antibiotics under anoxia (Schobert and Jahn, 2010, Schaible *et al.*, 2012).

1.5.2 Defects within microbial detection

Airway epithelia are also able to sense and respond to microbial challenges within the airway lumen, through the expression of Toll-like receptors (TLRs). Existing as either monomers, hetero- or homodimers, these pattern recognition receptors (PRR) are able to recognise and bind to diverse array of pathogen-associated molecular patterns (PAMPs), microbial ligands which can be either external or internal in origin (Akira *et al.*, 2006). PAMPs are typically essential for bacterial survival and are thus highly conserved.

Expressed at low levels on the surface of bronchial epithelia and up-regulated in the presence of an infection, TLR4 recognises and binds to lipopolysaccharide (LPS), a microbial product derived from the outer membrane of Gram-negative bacteria (Poltorak *et al.*, 1998). TLR5 is another major receptor, recognising flagellin from the bacterium *P. aeruginosa* (Zhang *et al.*, 2005), whilst TLR2 recognises lipoproteins derived from the cell wall of the bacterium *S. aureus* (Hashimoto *et al.*, 2006). Following TLR activation and intracellular signalling, airway epithelia release pro-inflammatory messengers which initiates the host immune response (Adamo *et al.*, 2004).

Immortalised CF epithelial cell lines and primary bronchial biopsies obtained from individuals with CF have both been used to show that CF epithelia express lower amounts of surface TLR4 compared to non-CF epithelia, a finding believed to be due to the receptor remaining within the cell (Hauber *et al.*, 2005, Chillappagari *et al.*, 2014, John *et al.*, 2010). Thus, reductions in the ability of airway epithelia to detect *P. aeruginosa*-derived LPS may in part facilitate *P. aeruginosa* persistence within the CF lung. Furthermore, TLR4 activation leads to the production of the stress-induced protein heme oxygenase-1 (HO-1). Activation of HO-1 leads to the subsequent breakdown of haem into iron and bilirubin, which is known to exert an anti-inflammatory effect (Chillappagari *et al.*, 2014). Thus, a lack of TLR4 may also contribute to excessive inflammation in CF airways due to reductions in HO-1 production. TLR2 however, was shown to be heavily involved in the recognition of pathogens within the airways (Muir *et*

al., 2004). Its upregulation in CF epithelia compared to non-CF epithelia may contribute to the characteristic excessive inflammation seen within the CF lung (Balloy *et al.*, 2015).

An additional study proposed that the CFTR is also a PRR, involved in the detection and internalisation of *P. aeruginosa*-derived LPS, which serves to initiate an inflammatory response (Schroeder *et al.*, 2002). Thus, mutations in the CFTR may impair the ability of CF airway epithelia to internalise *P. aeruginosa* LPS and in turn mount an inflammatory response. Wildtype CFTR has also be reported to bind to *P. aeruginosa*, leading to its internalisation within airway epithelia and removal by epithelial cell desquamation (Pier *et al.*, 1997, Pier *et al.*, 1996). However, more studies addressing the adherence of CF pathogens to the CF lung are needed.

1.5.3 Defects in antimicrobial peptides and proteins

1.5.3.1 Defensins

Defensins are small (3-6 kDa) cationic peptides secreted by airway epithelia, possessing a broad spectrum of antimicrobial activity against a range of bacteria, enveloped viruses and fungi (Ganz, 2005). The most abundant peptides found within ASL, human β-defensins 1-3 (HBD1-3), play an essential role in airway defence (McCray and Bentley, 1997, Zhao *et al.*, 1996, Singh *et al.*, 1998). The positive charge of these small peptides enables binding to the negatively charged bacterial membrane. By becoming embedded within the membrane and forming a pore, they facilitate bacterial killing by osmotic-mediated lysis. HBD1-3 are effective against a number of Gram-negative bacteria, with HBD-2 and -3 both demonstrating a particular potency against *P. aeruginosa*. However, only HBD-3 exerts bacteriostatic activity against the Gram-positive pathogen *S. aureus* (Harder *et al.*, 2001, Harder *et al.*, 2000).

Whilst the synthesis and secretion of β-defensins into the airway lumen is not influenced by mutations in the CFTR, their bacteriostatic and bactericidal abilities are severely reduced in CF airways. β-defensins are subjected to degradation by macrophage derived cathepsins released during the chronic inflammatory response characteristically seen in CF (Taggart *et al.*, 2003). Moreover, there is evidence that the high salt environment associated with CF inactivates constitutively active HBD-1. This is likely to be due to the sodium concentration competitively inhibiting interactions between the cationic peptide and the negatively charged bacterial membrane (Goldman *et al.*, 1997, Lehrer *et al.*, 1993).

1.5.3.2 Lysozyme

Lysozyme is secreted into the ASL by surface epithelia and glandular serous cells (Konstan *et al.*, 1981). This small cationic protein is known to target the β1-4 glycosidic bonds between N-acetylglucosamine and N-acetyl-muramic acid. Bound to airway mucins, lysozyme is an effective antimicrobial agent against several Gram-positive pathogens, where its ability to

degrade the peptidoglycan cell wall facilitates bacterial killing by osmotic-mediated lysis. The Gram-positive bacterium *S. aureus* is resistant to the hydrolytic activity of lysozyme however, due to modifications within N-acetyl-muramic acid at the C-6 position (Bera *et al.*, 2005, Bera *et al.*, 2007).Whilst its activity against Gram-negative bacteria is reduced (Coonrod, 1986), lysozyme is able to kill the bacterium *P. aeruginosa* (Akinbi *et al.*, 2000). It has also been demonstrated that the antimicrobial activity of lysozyme is not solely dependent upon its hydrolytic activity, as lysozyme works synergistically with a number of other antimicrobial compounds secreted in the airway lumen, including lactoferrin (Ellison *et al.*, 1988, Ellison and Giehl, 1991, Akinbi *et al.*, 2000).

Whilst lysozyme activity is believed to be similar, if not elevated within CF airways (Sagel *et al.*, 2009b), *P. aeruginosa* colonisation impairs the function of this antimicrobial protein. Whilst acidification of the ASL is believed to have a minimal effect upon its function (Davies *et al.*, 1969), elastase E produced by *P. aeruginosa* (but not neutrophil elastase) targets pulmonary lysozyme, leading to its cleavage and loss of its bacteriolytic ability (Jacquot *et al.*, 1985).

1.5.3.3 Lactoferrin

Lactoferrin is constitutively secreted into the ASL by serous cells (Brogan *et al.*, 1975, Bowes *et al.*, 1981). With iron being important for bacterial metabolism, the ability of lactoferrin to reversibly sequester free ferric ions (Fe^{3+}), deprives both Gram-positive and Gram-negative bacteria of this essential micronutrient (Bullen *et al.*, 1974). Whilst lactoferrin is believed to be present in similar levels in non-CF and CF airways (Pezzulo *et al.*, 2012), it is subject to proteolytic cleavage within the CF lung. Unlike lysozyme however, lactoferrin is degraded by both neutrophil elastase (NE) and proteases secreted by *P. aeruginosa*, which serves to liberate the iron (Britigan *et al.*, 1993). Iron has been shown to be elevated in both sputum and bronchioalveolar lavage (BAL) fluid obtained from individuals with CF (Stites *et al.*, 1999, Reid *et al.*, 2004) and its increased availability has been linked to facilitating *P. aeruginosa* persistence within CF airways (Reid *et al.*, 2007).

The ability of lactoferrin to bind Fe^{3+} however, does not appear to be the main mechanism in which this protein exerts its antimicrobial function. Lactoferrin has also been shown to bind to LPS upon the surface of Gram-negative bacteria. Binding of lactoferrin destabilises LPS, leading to its removal from the outer membrane. Consequent changes within the makeup and stability of the outer membrane of bacteria increases its permeability, making it more susceptible to osmotic-mediated lysis, and killing mediated by lysozyme and antibiotics (Farnaud and Evans, 2003, Ellison *et al.*, 1988). Reducing osmotic-mediated lysis of *P. aeruginosa* through the degradation of lactoferrin and increasing the availability of iron may protect *P. aeruginosa* and provide it with a more favourable environment within the lung in which to colonise and persist.

1.5.3.4 PLUNC

Palate, Lung, Nasal Epithelial Clone (PLUNC) belongs to a superfamily of proteins with very little sequence homology (Bingle *et al.*, 2011). Termed 'short' or 'long', SPLUNC1 is a 25 kDa glycoprotein secreted by tracheobronchial epithelia and submucosal glands (Campos *et al.*, 2004). In addition to its role in regulating the volume of ASL (Garcia-Caballero *et al.*, 2009), its hydrophobicity and surfactant like properties allows it to reduce surface tension within the large airways (Bartlett *et al.*, 2011). Induced by TLR2 (Thaikoottathil and Chu, 2011), SPLUNC1 has been shown to exert an antimicrobial effect against a number of bacteria, including *P. aeruginosa* (Bartlett *et al.*, 2011, Zhou *et al.*, 2008). The ability of PLUNC to inhibit aggregation and the formation of microcolonies, makes it one of a number of innate molecules alongside lactoferrin, which exert an anti-biofilm effect within the airways (Gakhar *et al.*, 2010). Chronic infection of CF airways is facilitated by the secretion and formation of bacterial biofilms (Costerton, 2001). The upregulation of PLUNC in response to bacterial infection makes it an effective defence mechanism (McGillivary and Bakaletz, 2010, Sayeed *et al.*, 2013).

Analysis of the CF airway secretome identified that SPLUNC1 was one of a few innate immune proteins downregulated in the ASL, compared to non-CF airways (Bingle *et al.*, 2007). SPLUNC is also known to be degraded by NE (Jiang *et al.*, 2013). A study infecting SPLUNC knockout mice demonstrated an increased susceptibility to *P. aeruginosa* airway infection and an increase in *P. aeruginosa* biofilm formation (Liu *et al.*, 2013), whilst another study demonstrated that SPLUNC inhibited the growth of *P. aeruginosa in vitro* (Zhou *et al.*, 2008). SPLUNC also appears to be important in providing protection against *S. aureus*, where it has been shown to reduce *S. aureus* biofilm formation (Yu *et al.*, 2018). As NE is elevated in the CF lung, reductions in SPLUNC due to its degradation are likely to assist the growth and biofilm production of *S. aureus* and *P. aeruginosa*.

1.5.3.5 Secretory IgA and Secretory Component

Another important component of airway defence is the 385 kDa globular glycoprotein, secretory IgA (sIgA). Existing as a dimer, covalently linked to a J chain, this chief antibody of the respiratory mucosa is able to bind to inhaled pathogens within the upper airways, in addition to their exotoxins (Corthesy, 2013, Johansen *et al.*, 2001). By blocking bacterial adhesion to airway epithelia, it facilitates their retention within airway mucus and removal by the mucociliary escalator. The binding of sIgA to a 70 kDa polypeptide referred to as secretory component (SC) (Mostov, 1994) protects the antibody from proteolysis (Crottet and Corthesy, 1998, Lindh, 1975), in conjunction with it acting as an adhesion decoy (Hammerschmidt *et al.*, 1997).

The neutrophil dominated immune response within the chronically-inflamed CF lung compromises SC and sIgA mediated defences, with NE being shown to degrade sIgA (Doring *et al.*, 1986). Increasing concentrations of NE have been detected within CF sputum of children with CF (1.0-1.8 log µg/mL) (Sagel *et al.*, 2012), which is below the limit of detection in healthy volunteers (Birrer *et al.*, 1994). NE has also been shown to be highest in individuals colonised by *P. aeruginosa* (Chalmers *et al.*, 2017, Weldon *et al.*, 2009). In *P. aeruginosa* keratitis, alkaline protease and elastase secreted by *P. aeruginosa* have been shown to partially degrade SC, giving rise to the question as to whether this also occurs in CF airways colonised by *P. aeruginosa* (Lomholt and Kilian, 2008). Proteolytic degradation of SC whether by *P. aeruginosa* derived or neutrophil derived proteases are likely to abolish its antimicrobial function in the CF lung.

1.5.3.6 Alveolar Surfactants

Whilst the highly-branched structure of the airways helps to prevent large particles (>5 µm) from reaching the distal air spaces, smaller particles such as bacteria can become deposited at the air liquid interface (ALI) within the lower airways. Whilst this thin layer of liquid is abundant in two small hydrophobic surfactant proteins (SP), SP-B and SP-C, helping maintain a low surface tension interface (Whitsett and Weaver, 2002), surfactants SP-A and SP-D form an essential part of innate immunity (Hartshorn *et al.*, 1998). The carbohydrate recognition domain (CRD) of SP-A allows it to bind to the lipid-A component of membrane anchored LPS and thus promote binding to both Gram-positive bacteria such as *S aureus* (Geertsma *et al.*, 1994) as well as Gram-negative bacteria, including *P. aeruginosa* (Mariencheck *et al.*, 1999, Giannoni *et al.*, 2006). The less abundant SP-D also plays an important part in bacterial clearance, binding to the LPS core sugars on Gram-negative bacteria including *P. aeruginosa* (Kuan *et al.*, 1992), whilst binding to lipoteichoic acid and peptidoglycan upon the cell wall of Gram-positive bacteria (van de Wetering *et al.*, 2001). SP-D has been shown to act as a chemotactic factor, encouraging neutrophil migration to sites of infection (Cai *et al.*, 1999). By binding to carbohydrates upon the pathogen's surface, both surfactants have been shown to enhance macrophage and neutrophil-mediated phagocytosis and thus clear bacteria from the lungs (Gaynor *et al.*, 1995, Tino and Wright, 1996, Hartshorn *et al.*, 1998, Madan *et al.*, 1997).

During chronic inflammation of the CF lung, the large and characteristic shift in the protease-anti-protease balance leads to the degradation of these surfactants by host proteases (Delacourt *et al.*, 1995). Whilst SP-A is known to be degraded by neutrophil serine proteases present in high concentrations in the CF lung (Schochett *et al.*, 1999, von Bredow *et al.*, 2001), SP-D is degraded by chronically challenged alveolar macrophages (von Bredow *et al.*, 2003). Degradation of both SP-A and SP-D may impair the phagocytic clearance of *S.*

aureus and *P. aeruginosa* by airway neutrophils and macrophages and facilitate their survival within the CF lung.

1.5.4 Neutrophils – the foot solider

Following airway infection, the release of the pro-inflammatory mediator interleukin-8 (IL-8) from airway epithelia and inflamed endothelia, encourages circulating neutrophils to tightly roll across and bind the surface of pulmonary capillaries and enter the infected airways (Lawrence and Springer, 1991). Once primed at the site of infection, these professional phagocytes utilise a potent arsenal of bactericidal mechanisms to permit bacterial clearance from the lung. This ranges from bacterial phagocytosis and the release of reactive oxygen species (ROS) as part of the respiratory burst, to the secretion of proteases and peptides including NE and lysozyme (Segal, 2005, Cascao *et al.*, 2009). The secretion of web-like neutrophil extracellular traps (NETs) also serves to agglutinate bacteria, inhibiting their ability to disseminate and colonise (Brinkmann *et al.*, 2004). Following their short lifespan, neutrophils undergo apoptosis (programmed cell death), where they are eventually cleared by another professional phagocyte, the macrophage (Savill *et al.*, 1989). As this process is anti-inflammatory, alterations to the ability or rate of clearance can cause dying neutrophils to become 'leaky.' Spillage of the neutrophil intracellular contents can consequently have a severe impact upon lung health, leading to chronic and excessive inflammation.

CF is characterised by a neutrophil-dominated immune response, where in some instances, these granulocytes account for nearly two-thirds of all immune cells found within CF airways (Hartl *et al.*, 2006). Their accumulation within the airways leads to the excessive release NE within CF sputum (Goldstein and Doring, 1986). NE also perturbs the fine protease to anti-protease balance within the lung. Whilst protease inhibitors α1-antitrypsin (α1-AT) and secretory leukoprotease inhibitor (SLPI) protect airway epithelia from NE-mediated damage, the concentrations found within CF airways not only overwhelm the concentration of these protease inhibitors, but they can also degrade them (Cantin *et al.*, 1989, Baumstark *et al.*, 1977).

NE damages the airway architecture through the degradation of extracellular matrix proteins including collagen (type I and type V), elastin and fibronectin, with cleavage of fibronectin also enhancing *P. aeruginosa* binding (Taggart *et al.*, 2000, Walsh *et al.*, 2001, Suter *et al.*, 1988). Elastin and collagen breakdown products have been detected in the urine of individuals with CF (Stone *et al.*, 1995). NE has also been shown to lead to the proteolytic cleavage of ENaC, leading to sodium hyperabsorption and mucus dehydration (Ji *et al.*, 2000, Caldwell *et al.*, 2005). It has also been shown to further impair already compromised innate defence mechanisms, due to its ability to degrade the immunoglobulin IgG, complement proteins and the airway antimicrobial peptide LL-37 (Nadel, 1991, Bergsson *et al.*, 2009). Furthermore, NE

is known to elevate the expression of the pro-inflammatory chemokine IL-8 (Nakamura *et al.*, 1992) and is associated with decreased pulmonary function (Mayer-Hamblett *et al.*, 2007). The cleavage of key cell surface receptors due to protease imbalance also impairs the ability of neutrophils to phagocytose common CF bacteria, through the loss of cell surface CD14, CD16 and IL-8R (CXCR1) (Tirouvanziam *et al.*, 2008).

Whilst the survival of neutrophils in CF patients are known to be prolonged (Moriceau *et al.*, 2010), the cleavage of phosphatidylserine receptors upon the surface of macrophages also impairs the removal of apoptotic neutrophils from the CF lung (McKeon *et al.*, 2008, Vandivier *et al.*, 2002b). The release of neutrophil DNA from both necrotic cells and from activated neutrophils as extracellular traps, serve to increase mucus viscosity (Lethem *et al.*, 1990, Marcos *et al.*, 2010). The release of the neutrophil's intracellular contents is also likely to exasperate the pro-inflammatory response seen within CF airways. This heightened inflammatory response has also been shown to prime neutrophils to release granules early (Koller *et al.*, 1995). The premature release of proteases such as gelatinase have been shown to damage the basement membrane, aiding neutrophil migration to the site of infection, whilst inflammation amplifies the release of ROS from neutrophils, which subsequently damage airway epithelia (Delacourt *et al.*, 1995, Brockbank *et al.*, 2005).

Mutations in the CFTR also affect the ability of neutrophils to kill ingested bacteria (Zhou *et al.*, 2013). The inability to transport chloride ions into the phagolysosome protects *P. aeruginosa* from hypochlorous acid-mediated killing (Painter *et al.*, 2008). Moreover, the failure for secondary and tertiary granules to fuse into the phagolysosome also protect the bacteria from intracellular killing (Pohl *et al.*, 2014). Airway neutrophils in individuals with CF have also been shown to express elevated levels of TLR5 upon their cell surface compared to circulating neutrophils in the same individuals, along with neutrophils isolated from healthy controls and individuals with bronchiectasis (a permanent widening of the airways, leading to mucus accumulation) (Koller *et al.*, 2008). As TLR5 recognises *P. aeruginosa* flagellin, it suggests that TLR5 may be important in neutrophil interactions with *P. aeruginosa.* The production of thick alginate biofilms by *P. aeruginosa* also impairs neutrophil function, causing immobilisation, premature granule release and cell rounding (Jesaitis *et al.*, 2003). This impairment of neutrophils by biofilms may not be limited to *P. aeruginosa,* as *S. aureus* also forms biofilms within CF airways (Hirschhausen *et al.*, 2013). Additionally, it has been reported that the phagocytosis of *S. aureus* by CF neutrophils isolated from sputum is lower than that of CF circulating neutrophils, although the underlying mechanisms require further study (Timmis *et al.*, 2011).

1.5.5 Alveolar Macrophages – The sentry

Whilst neutrophils have a typical lifespan of 4-6 hours, macrophages can reside within the lung for many months (Murphy *et al.*, 2008). They arise either through division of tissue resident macrophages, or through pre-cursor monocytes. Following their release from the bone marrow, monocytes circulate in blood vessels and migrate into tissues where they differentiate into monocyte-derived macrophages (Thomas *et al.*, 1976, Sawyer *et al.*, 1982). Interstitial macrophages are present within the lung parenchyma, and whilst they are poor phagocytes, they play key roles in lung inflammation, antigen presentation and the development of pulmonary fibrosis (Byrne *et al.*, 2015, Schneberger *et al.*, 2011, Weinberg and Unanue, 1981). However, alveolar macrophages release oxygen free radicals, lysozyme and defensins to permit bacterial killing. Unlike neutrophils however, macrophage mediated phagocytosis serves many functions, from promoting the clearance of bacteria, to the removal of tissue debris and apoptotic neutrophils (Cox *et al.*, 1995).

It has been shown previously that macrophages, unlike neutrophils, require wtCFTR to effectively phagocytose complement-coated bacteria (Van de Weert-van Leeuwen *et al.*, 2013). Thus, mutations in the CFTR, particularly class I mutations leading to its absence from the apical membrane, may attenuate the phagocytic ability of CF macrophages. Another study using human macrophages, demonstrated whilst both CF and non-CF effectively ingested and killed *P. aeruginosa* over time, a higher percentage of viable *P. aeruginosa* was seen in the phagolysosome of CF macrophages after four hours (Del Porto *et al.*, 2011). *S. aureus* has also been shown to survive within CF macrophages due to reduced phagolysosome fusion (Li *et al.*, 2017). Thus, CF macrophages may promote the intracellular survival of both *S. aureus* and *P. aeruginosa*, not only protecting both species from the hosts immune system and antibiotics, but it may also act as a reservoir for chronic infection.

Furthermore, research into the effect of exposure to LPS obtained from *P. aeruginosa* 10 upon alveolar macrophages have yielded some interesting results. CF murine macrophages and *ex vivo* alveolar macrophages demonstrated an enhanced pro-inflammatory response upon exposure to LPS, including IL-1α, IL-6 and IL-8 and therefore may contribute to the hyper-inflammatory phenotype of CF airways (Bruscia *et al.*, 2009). In spite of this, other groups have illustrated that more CF macrophages are alternatively-activated, consequently reducing their bactericidal activity (Murphy *et al.*, 2010).

Excess damage to CF airways coupled with reduced killing of CF pathogens by professional phagocytes are likely to promote bacterial survival and the development of chronic infections.

1.5.6 Pro-inflammatory cytokines

Detection of a microbial challenge within the airways results in the release of an array of pro-inflammatory cytokines and chemokines. These small proteins (8-30 kDa) serve to not only attract inflammatory cells to the site of infection and increase vascular permeability, but also aid in the upregulation and expression of several host defence proteins, such as HBD-2 and -3 (Greene and McElvaney, 2005, Ryu *et al.*, 2010, Hiratsuka *et al.*, 1998). In response to infection and injury, airway epithelia are known to release large quantities of the potent pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α). TNF-α release leads to an increase in the expression of adhesion molecules upon the apical surface of lung endothelia, facilitating the binding of circulating neutrophils to the inflamed capillary (Lauterbach *et al.*, 2008).

1.5.6.1 Interleukin-6

The pro-inflammatory cytokine interleukin-6 (IL-6) activates antibody production by B cells and the production of acute phase proteins by liver hepatocytes (such as C-reactive protein) (Muraguchi *et al.*, 1988, Bode *et al.*, 2012, Kopf *et al.*, 1994). IL-6 is believed to induce the transcription and expression of the major airway mucins MUC5AC and MUC5B (Chen *et al.*, 2003b), as well as induce the expression of receptors upon endothelial cells, to facilitate neutrophil binding to the site of infection (Cronstein, 2007), and activate downstream mediators such as prostaglandins, which play a role in priming neutrophils (Biffl *et al.*, 1994).

IL-6 is an inflammatory marker in CF airways and has been detected in exhaled breath condensate (Carpagnano *et al.*, 2003) and bronchoalveolar lavage fluid (BALF) (Noah *et al.*, 1997). IL-6 may also be involved in the recruitment of leukocytes to the inflamed airways (Romano *et al.*, 1997), as well as in the priming of neutrophils (Biffl *et al.*, 1994).

1.5.6.2 Interleukin-8

Produced by a range of cell types, including airway epithelia, endothelial cells, fibroblasts and neutrophils, IL-8 plays an essential role in airway innate immunity (Eckmann *et al.*, 1993, Bazzoni *et al.*, 1991, Rolfe *et al.*, 1991). As a chemokine, it facilitates the binding and migration of circulating neutrophils to the site of infection, helping phagocytose and clear pathogens (Wardlaw, 1990). It also plays an additional role in neutrophil activation, increasing their antimicrobial capacity (Baggiolini and Clark-Lewis, 1992).

Excessive neutrophil infiltration into their airways and high levels of pro-inflammatory cytokines such as IL-8 are often detected in CF children following newborn screening and often in the absence of any detectable infection (Rosenfeld *et al.*, 2001b, Khan *et al.*, 1995). High levels of leukotriene B4 (LTB4), required for neutrophil vascular adhesion and extravasation into

tissues, is also seen (Bodini *et al.*, 2005). Thus, whether this pro-inflammatory phenotype is a result of CFTR dysfunction (Stecenko *et al.*, 2001), an exaggerated response to bacterial infection (DiMango *et al.*, 1995, Kube *et al.*, 2001), or a combination of both, is the focus of further research.

1.5.6.3 Interleukin-10

Whilst a pro-inflammatory immune response is essential for clearing airway infections, the ability to resolve inflammation is essential for physiological function. Restoring tissue homeostasis is a very active process, involving both non-professional and professional phagocytes. The constitutive secretion of interleukin-10 (IL-10) by airway epithelia serves to inhibit the release of pro-inflammatory mediators by monocytes and macrophages (Bonfield *et al.*, 1995a, de Waal Malefyt *et al.*, 1991, Ding *et al.*, 1993).

Low levels of IL-10, have been detected within the CF lung (Bonfield *et al.*, 1995a, Sagel *et al.*, 2012, Dosanjh *et al.*, 1998). Due to the ability of IL-10 to resolve inflammation, such as inhibiting cytokine production by activated macrophages (Fiorentino *et al.*, 1991), its low levels within CF airways are likely to contribute to chronic inflammation, a hallmark of CF.

1.5.6.4 Transforming growth factor beta-1

Transforming growth factor beta (TGF-β) is a multi-functional cytokine which exists as three isoforms, TGF-β_1, TGF-β_2 and TGF-β_3 and is encoded by three separate genes (Thomas *et al.*, 2016). All isoforms have been detected in heathy bronchial airway epithelia (Magnan *et al.*, 1994). TGF-β_1 is involved in mediating rapid wound repair following damage to airway bronchial epithelia (Howat *et al.*, 2002) and in stimulating fibroblast proliferation *in vitro* (Nakamura *et al.*, 1995). It has been shown to elevated in the BALF of individuals with CF (Harris *et al.*, 2009), in lung tissue isolated from individuals with CF (Corrin *et al.*, 1994), as well as in conditioned media obtained from cultured CF epithelia (Perkett *et al.*, 2006). This increase in TGF-β_1 in CF airways is likely to be mediated by NE (Lee *et al.*, 2006), regional hypoxia within the CF lung (Nicola *et al.*, 2011) and persistent injury to airway epithelia due to chronic infection and inflammation (Hilliard *et al.*, 2007).

Select polymorphisms in TGF-β_1 in individuals with CF is associated with worsened disease severity, including a worsening of pulmonary function (Brazova *et al.*, 2006, Drumm *et al.*, 2005, Arkwright *et al.*, 2000) and neutrophilic inflammation (Harris *et al.*, 2009), with TGF-β_1 being known to be a potent neutrophil chemoattractant (Parekh *et al.*, 1994). TGF-β_1 has also been shown to impair the rescue of CFTR in primary CF epithelia (Snodgrass *et al.*, 2013), as well as impair mucociliary clearance and deplete the ASL volume in polarised CF primary epithelia (Manzanares *et al.*, 2015). The cytokine may also enhance fibrosis within CF airways by driving myofibroblast differentiation (Harris *et al.*, 2013).

1.5.7 The impact of CFTR dysfunction upon pulmonary innate immunity is severe

It is evident that mutations in the CFTR exhibit devastating effects upon many different aspects of pulmonary innate immunity, particularly in relation to CF airway epithelia. This ranges from impairments in mucociliary clearance and in the detection of microbes, to the degradation of epithelial-derived antimicrobial peptides and proteins. Despite this, a number of key questions remain. Impaired mucociliary clearance leads to mucus plugging, which can give rise to regions of anoxia. However, the impact of environmental conditions such as oxygen availability upon CF pathogens is poorly understood. Furthermore, whilst inflammation is a hallmark of CF, does infection with more than one CF pathogen influence the inflammatory response of CF airway epithelia? Furthermore, whilst mutated CFTR has been reported to be both a PRR and an adhesion ligand for bacteria, studies addressing bacterial adhesion to CF airway epithelia are lacking.

1.6 Bacterial infections of CF Airways

CF airways are colonised by a complex polymicrobial community of aerobic and anaerobic bacteria (Zhao *et al.*, 2012, Rogers *et al.*, 2009, Tunney *et al.*, 2008), viruses (Etherington *et al.*, 2014) and fungi (Willger *et al.*, 2014). Bacteria are known to predominate in CF airways, forming 99% of the microbial community, whilst viruses and fungi form the remaining 1% (Moran Losada *et al.*, 2016). Most bacterial infections within the CF lung are environmental or commensal bacteria which exploit impairments in innate immunity, whilst patient-to-patient transmission also plays a key role in the spread of CF adapted pathogens (Saiman and Siegel, 2004).

Despite the complex microbial community known to colonise CF airways, *S. aureus* and *P. aeruginosa* are the two most prevalent pathogens (Cystic Fibrosis Trust, 2018, Filkins *et al.*, 2015, Moran Losada *et al.*, 2016). As shown in Figure 4, infection of the CF lung is known to occur in a highly sequential order and one that is heavily age dependent. Whilst *S. aureus* and non-capsulated *Haemophilus influenzae* (*H. influenzae*) colonises and infects the airways in the first decade of life, *P. aeruginosa* predominates in the second and third decades (Talwalkar and Murray, 2016, Lyczak *et al.*, 2002).

P. aeruginosa is considered to be the most important of the three main “classic” CF pathogens, where it is associated with increased morbidity, hospitalisations and greater decreases in pulmonary function (Emerson *et al.*, 2002, Kerem *et al.*, 1990, Com *et al.*, 2014). Other important emerging pathogens include methicillin-resistant *Staphylococcus aureus* (MRSA), *Stenotrophomonas maltophilia, Mycobacterium abscessus* and *Prevotella spp*. (Parkins and Floto, 2015).

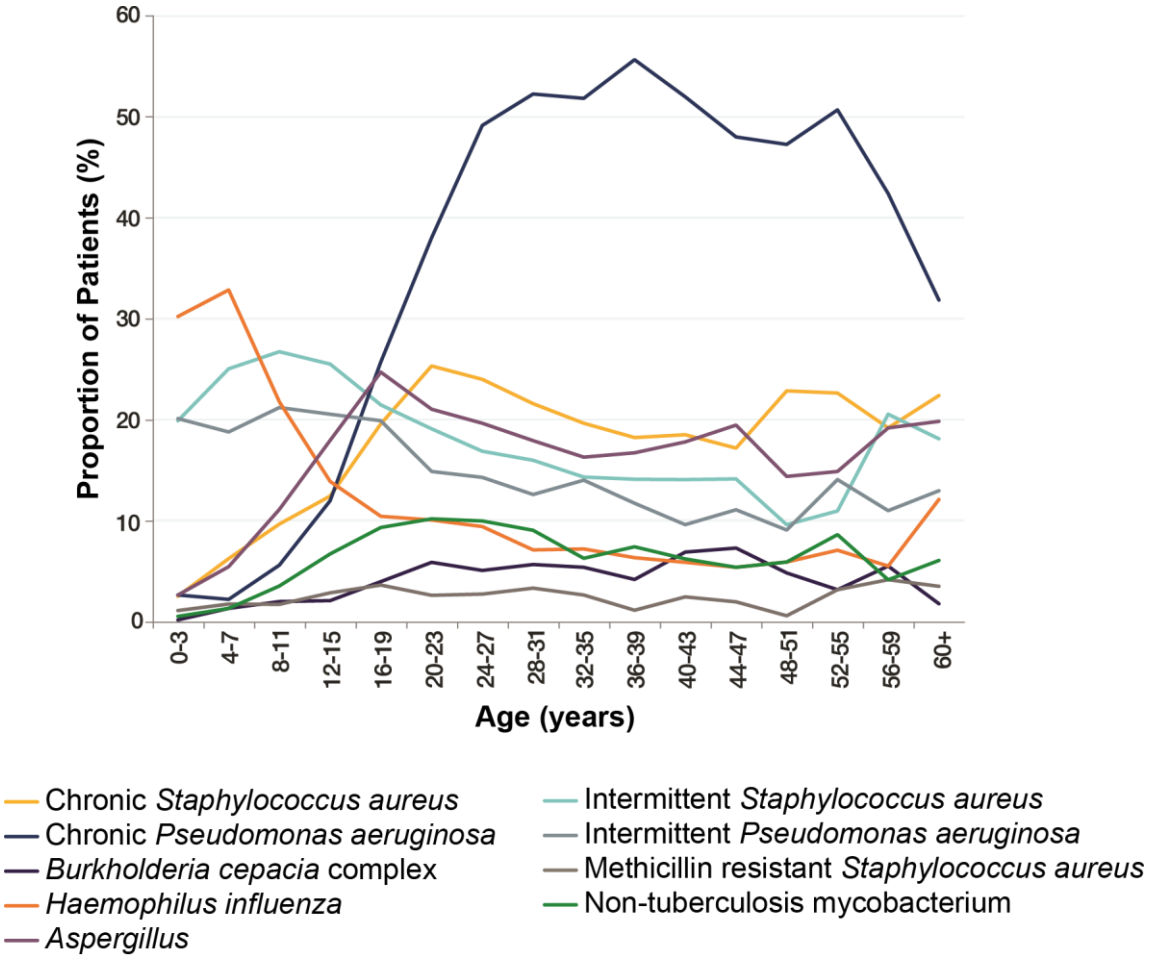


Figure 4. Colonisation of the CF lung is highly sequential and age dependent. This graph illustrates the percentage of patients who are culture positive for a particular bacterial species, categorised by age. *S. aureus* (yellow and light blue lines) and *H. influenzae* (orange) typically colonise CF airways within the first decade of life, with *S. aureus* being the predominant organism. However, as an individual progresses through adolescence and into adulthood, *P. aeruginosa* (grey and navy lines) predominates. Graph modified from the 2017 Cystic Fibrosis Trust registry report (Cystic Fibrosis Trust, 2018).

1.6.1 *Staphylococcus aureus*

S. aureus is often the first bacterium to colonise the CF lung, typically during infancy (Armstrong *et al.*, 1997, Kahl, 2010). A ubiquitous organism as well as a commensal of the anterior nares (in approximately 30% of the UK population) (Gamblin *et al.*, 2013)this Gram-positive coccus is responsible for causing chronic infections of the respiratory tract (Lyczak *et al.*, 2002). Prior to the use of antibiotics, *S. aureus* was associated with high mortality rates in children (Ahlgren *et al.*, 2015). However, the routine administration of anti-staphylococcal agents such as prophylactic flucloxacillin following initial diagnosis until the age of three is administered in the UK (CysticFibrosisTrust, 2009). Despite this, a Cochrane review highlighted that there is no agreement on how to best treat chronic *S. aureus* infection (Ahmed and Mukherjee, 2016). Furthermore, there are concerns as to whether prophylactic antibiotic use can lead to earlier *P. aeruginosa* acquisition (Elborn, 1999).

Intermittent *S. aureus* infection is detected in 24.5% of the paediatric UK CF population, compared to 16.8% of the UK CF population over the age of 16 (Cystic Fibrosis Trust, 2018). Furthermore, chronic *S. aureus* infection is detected in approximately 7.7% of the paediatric CF population in the UK and 21.5% in those over the age of 16 (Cystic Fibrosis Trust, 2018). The emergence of antibiotic resistant strains of *S. aureus* to antibiotics such as methicillin and flucloxacillin was first identified in 1960's (Jevons *et al.*, 1963, Barber, 1961). This led to the circulation of methicillin resistant *S. aureus* (MRSA) within the CF population. The prevalence of MRSA is relatively low in the UK, with a prevalence of approximately 1.8% within the CF population under 16 years and 2.7% of the adult population being colonised. This is contrast to the USA which has a reported MRSA prevalence of 26% within the CF community (Cystic Fibrosis Foundation, 2008). The presence of MRSA has previously been reported to be linked to a more rapid decrease in pulmonary function (Dasenbrook *et al.*, 2008). Despite this, other authors have reported that the presence of MRSA appears to have a minimal impact upon pulmonary function (Boxerbaum *et al.*, 1988, Thomas *et al.*, 1998, Miall *et al.*, 2001).

1.6.1.1 *S. aureus* virulence

The role of *S. aureus* within CF disease pathogenesis and progression is poorly understood, in addition to the mechanisms which allow it to become the predominant organism early in life (Lyczak *et al.*, 2002). *S. aureus* is known to possess a range of virulence factors which allow it to effectively colonise the CF airway epithelium. This is summarised in Figure 5. The increased presence of the cellular receptor asialoganglioside 1 (aGM1) upon the surface of CF respiratory epithelia compared to non-CF, are likely to enhance the bacterium's ability to bind to the tetrasaccharide sugar moiety of this receptor and effectively adhere to host cells (Imundo *et al.*, 1995), whilst the binding of *S. aureus* to aGM1 and tumour necrosis factor-α receptor initiates airway inflammation, including the release of IL-8 (DiMango *et al.*, 1998, Gomez *et al.*, 2004).

The presence of fibronectin binding protein (FnbpA), clumping factor A (ClfA), collagen binding protein (Cbp) and elastin binding protein (Ebp) upon the bacteria's surface allow it to adhere to airway epithelia (Lynch and Bruce, 2013), as well as components of the extracellular matrix (Sinha *et al.*, 1999, Patti *et al.*, 1992). *S. aureus* has also been shown to bind to MUC1**,** a transmembrane mucin present upon the surface of respiratory epithelia (Linden et al., 2008). Thus, the inability to clear high viscosity dehydrated mucus from CF airways further enhances the ability of this trapped opportunistic pathogen, to colonise the airways and cause infection in CF (Saggers and Lawson, 1970, Sanford et al., 1989).

S. aureus is also known to secrete a diverse range of exotoxins, including α- and β-toxins which damage airway epithelia and induce inflammation (Dragneva *et al.*, 2001, Ratner *et al.*, 2006), with the β-toxin Panton-Valentine leucocidin (PVL), causing neutrophil and macrophage lysis (Gladstone and Van Heyningen, 1957). The release of other extracellular products including lipoteichoic acid and peptidoglycan are known to induce airway inflammation (Fournier and Philpott, 2005).

Whilst the *S. aureus* golden carotenoid pigment impairs neutrophil killing (Liu *et al.*, 2005), the production of a biofilm provides resistance against phagocytosis and antibiotic-mediated killing (Singh *et al.*, 2010, Thurlow *et al.*, 2011). *S. aureus* is also known to have a low susceptibility to the effects of HBD-1 and HBD-2, with the latter only demonstrating a bacteriostatic effect, even at high concentrations (Harder *et al*., 1997, Singh *et al*., 1998). *S. aureus* can also produce a biofilm during chronic infection, facilitating its persistence and protecting it from antibiotics as well as the cellular and humoral components of the immune system (Gotz, 2002, Jones *et al*., 2001).

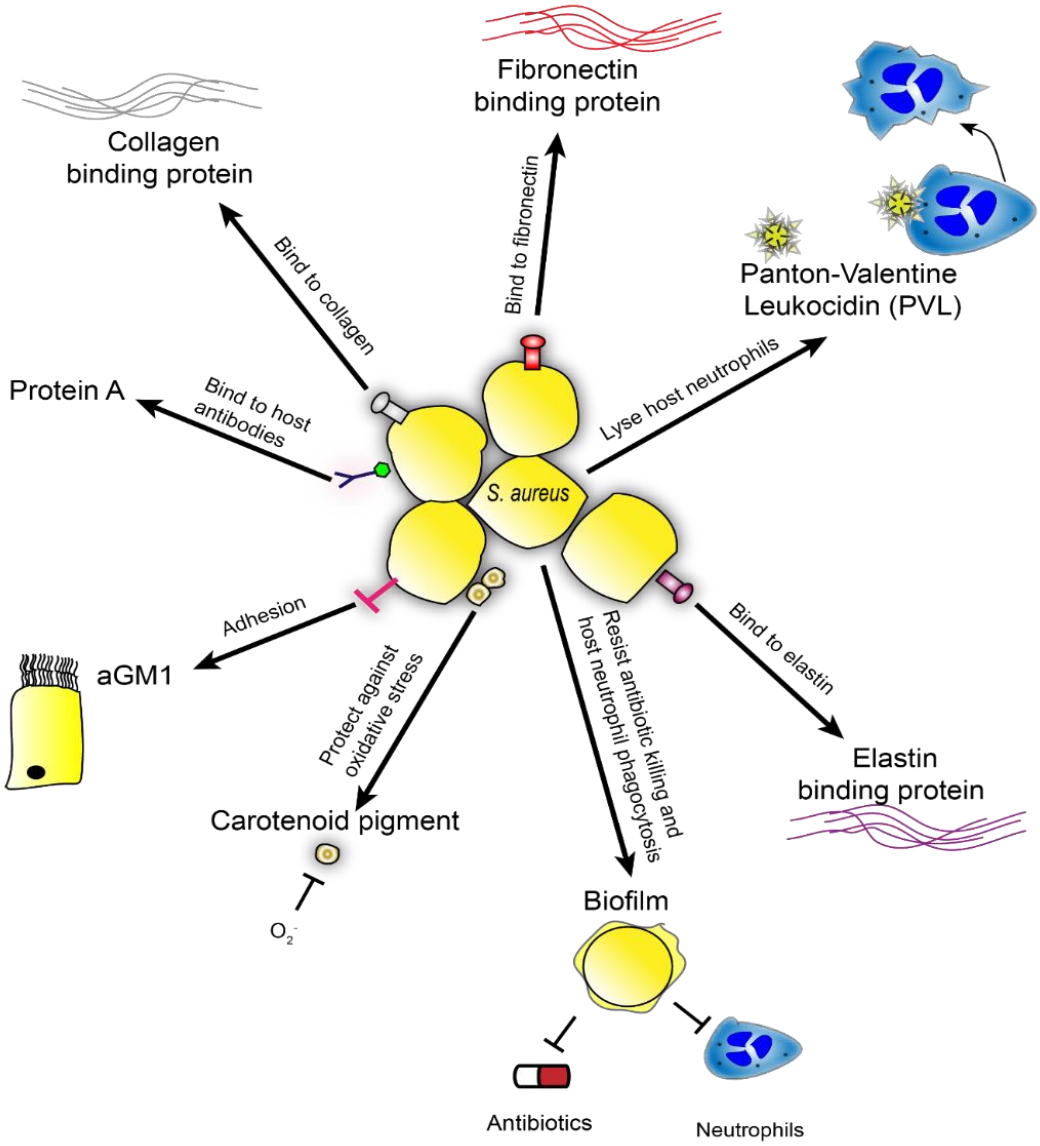


Figure 5. *S. aureus* utilises an extensive array of virulence factors. The cell associated aGM1, collagen-, elastin- and fibronectin- binding proteins facilitate bacterial adhesion to airway epithelia and the extracellular matrix. Protein A is effective in inhibiting opsonisation by host antibodies. Carotenoid pigment provides resistance against damaging reactive oxygen species (ROS), whilst biofilms inhibit bacterial killing by antibiotics and the host neutrophils. The extracellular virulence factor Panton-Valentine Leukocidin (PVL) is effective in causing host neutrophil lysis.

S. aureus has also been shown to grow as small colony variants (SCV's) within CF airways (Kahl *et al.*, 1998, Sadowska *et al.*, 2002, Gilligan *et al.*, 1987). This reversible switch in phenotype causes *S. aureus* to grow as small, non-pigmented colonies, which are able to produce thymidine and haemin (Proctor *et al.*, 2006). In addition to being able to become internalised within host cells (Vaudaux *et al.*, 2002, von Eiff *et al.*, 1997), they exhibit a heightened resistance to anti-staphylococcal antibiotics (Chuard *et al.*, 1997, Besier *et al.*, 2007). The prevalence of SCV's within the CF population has been estimated to be between 8-33% (Yagci *et al.*, 2013, Kahl *et al.*, 1998). *S. aureus* SCV's have also been associated with a worsening of pulmonary function (Besier *et al.*, 2007).

1.6.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod-shaped organism ubiquitously found in water supplies and soil and is a non-dominant member of normal skin flora (Green *et al.*, 1974, Franzetti *et al.*, 1992). Whilst it optimally grows at 37 °C, it can survive at temperatures up to 42 °C. Like *S. aureus, P. aeruginosa* is also facultative in its oxygen requirements and whilst it grows preferably under normoxia, it can grow under anoxia through the use of arginine (Vander Wauven *et al.*, 1984) and nitrate as end terminal electron acceptors (Line *et al.*, 2014). *P. aeruginosa* also has one of the largest bacterial genomes known, consisting 6.3 million base pairs, of which 9.1% encode regulatory proteins (Stover *et al.*, 2000). As an opportunistic pathogen, it typically causes infections in those who are immunocompromised, including burn victims, cancer and AIDS patients and neonates, as well as individuals with CF (Lyczak *et al.*, 2000).

P. aeruginosa first colonises CF lungs as early as 6 months of age, where it is predominantly acquired from the environment (Burns *et al.*, 2001, Cystic Fibrosis Trust, 2016b), whilst patient-patient contact has also been identified as a source of bacterial transmission; particularly in epidemic strains (Doring *et al.*, 1996, Hoogkamp-Korstanje *et al.*, 1995, Scott and Pitt, 2004). This includes the Liverpool Epidemic Strain (LES), first identified in 1996 and associated with increased virulence, enhanced resistance to antibiotics and a faster decline in pulmonary function (Ashish *et al.*, 2012, Salunkhe *et al.*, 2005, Fothergill *et al.*, 2007b).

Initial infection is typically acute and intermittent, where *P. aeruginosa* can be eliminated by an aggressive course of aerosolised antibiotics (Geller, 2009). Re-infection may be caused by one or more *P. aeruginosa* strains (Burns *et al.*, 2001). Approximately 20.1% of the paediatric CF population in the UK and 14.2% of the adult population are intermittingly colonised by *P. aeruginosa* (Cystic Fibrosis Trust, 2018). However, the infection eventually becomes chronic. Approximately 5.4% of the paediatric and 44.5% of the adult CF population in the UK are chronically colonised by *P. aeruginosa* (Cystic Fibrosis Trust, 2018). CF sputa positive for *P. aeruginosa* are associated with a worse clinical score, than those positive for *S. aureus* (Ahlgren *et al.*, 2015). Furthermore, young children who are culture positive for *P. aeruginosa* have a 2.6-fold increased risk of mortality over the subsequent eight years compared to those who are culture negative (Emerson *et al.*, 2010). According to Lee *et al.* chronic *P. aeruginosa* infection in the context of CF is the detection of *P. aeruginosa* in over half of sputum samples over the last twelve months (Lee *et al.*, 2003).

1.6.2.1 *P. aeruginosa* virulence

P. aeruginosa possesses an impressive arsenal of cell-associated and extracellular virulence factors which allow it to cause chronic infections in the CF lung. This is summarised in Figure 6. Cell associated pili and fimbriae facilitate adhesion to lung epithelial cell surface receptors (de Bentzmann *et al.*, 1996a, Saiman and Prince, 1993, Giltner *et al.*, 2006), internalisation by airway epithelia (Plotkowski *et al.*, 1999) and biofilm formation (O'Toole and Kolter, 1998).

Whilst flagella are required for bacterial swimming (Drake and Montie, 1988) it has been shown to bind to MUC1 mucin within the airways (Lillehoj *et al.*, 2002). However, flagellin is also highly immunogenic (Mizel *et al.*, 2003). Another potent activator of the immune system is LPS upon the surface of *P. aeruginosa* (Cryz *et al.*, 1984). LPS is composed of the hydrophobic lipid A domain embedded within the bacterial cell membrane, linked to a core polysaccharide and a O-antigen, with the latter being immunogenic (Goldberg and Pler, 1996). Subsequent modifications and losses of the O-antigen of LPS during the course of chronic airway infection facilitates evasion from the innate immune system, thus promoting long-term survival (Cigana *et al.*, 2009), whilst losses in flagellin may also evade TLR5 activation (Blohmke *et al.*, 2008). The ability of some CF isolates of *P. aeruginosa* to also synthesise specific forms of Lipid A containing aminoarabinose and palmitate, may provide protection against antimicrobial peptides present within the airways (Ernst *et al.*, 1999).

P. aeruginosa also secretes several virulence factors. The yellow-green siderophore pyoverdine competes with host transferrin and binds to the micronutrient iron, facilitating its uptake (Cox, 1986). The blue secondary metabolite pyocyanin inhibits cilia beating in airway epithelia, inhibits cellular respiration and induces neutrophil apoptosis (Munro *et al.*, 1989, Allen *et al.*, 2005). Furthermore, it has also been shown to cause imbalances within the protease-antiprotease balance within the lungs, by inhibiting the alpha-1 protease inhibitor (Britigan *et al.*, 1999). Rhamnolipids induce necrosis of host neutrophils (Van Gennip *et al.*, 2009) and disrupt tight junctions between airway epithelia (Zulianello *et al.*, 2006), whilst hydrogen cyanide can inhibit aerobic respiration of epithelia by targeting cytochrome c oxidase (Gallagher and Manoil, 2001).

P. aeruginosa is known to secrete numerous proteases. Elastase B (Las B) degrades elastin within the CF lung, along with laminin, collagen III, collagen IV and fibrin, in addition to airway surfactants -A and -D (Mariencheck *et al.*, 2003, Heck *et al.*, 1986, Morihara, 1964). Furthermore, Las B has been shown to degrade antimicrobial proteins of the immune system, including lysozyme, IL-8 and sIgA (LaFayette *et al.*, 2015, Jacquot *et al.*, 1985, Diebel *et al.*, 2009). Tissue damage serves to increase nutrient availability, facilitate bacterial spread and induce lung fibrosis, whilst degrading components of the hosts immune response provides a survival advantage. Individuals with CF have been reported to excrete an increased

concentration of desmosine (cross-linked amino acids), a by-product of elastin degradation (Viglio *et al.*, 2000). In addition to also degrading elastin, Elastase A (LasA) is a protease involved in the degradation of the *S. aureus* peptidoglycan cell wall (Kessler *et al.*, 1993b). A study of *P. aeruginosa* clinical isolates demonstrated that 75% of those studied produced detectable levels of elastase (Kuang *et al.*, 2011). Alkaline protease has previously been shown to cause tissue destruction within the CF lung (Suter, 1994), whilst Protease IV degrades pulmonary surfactants -A and -D (Malloy *et al.*, 2005).

P. aeruginosa is known to also inject a number of its cytotoxic effector proteins directly into airway epithelia. Employing a type III secretion system (TTSS): exoenzymes S, T and U induce cytoskeletal rearrangement, along with cell lysis (Vance *et al.*, 2005). The ability of *P. aeruginosa* to induce apoptosis of airway epithelia (Losa *et al.*, 2014, Rajan *et al.*, 2000) and degrade tight junction proteins facilitates its survival and dissemination into the airways (Azghani, 1996). *P. aeruginosa* virulence is summarised in Figure 6.

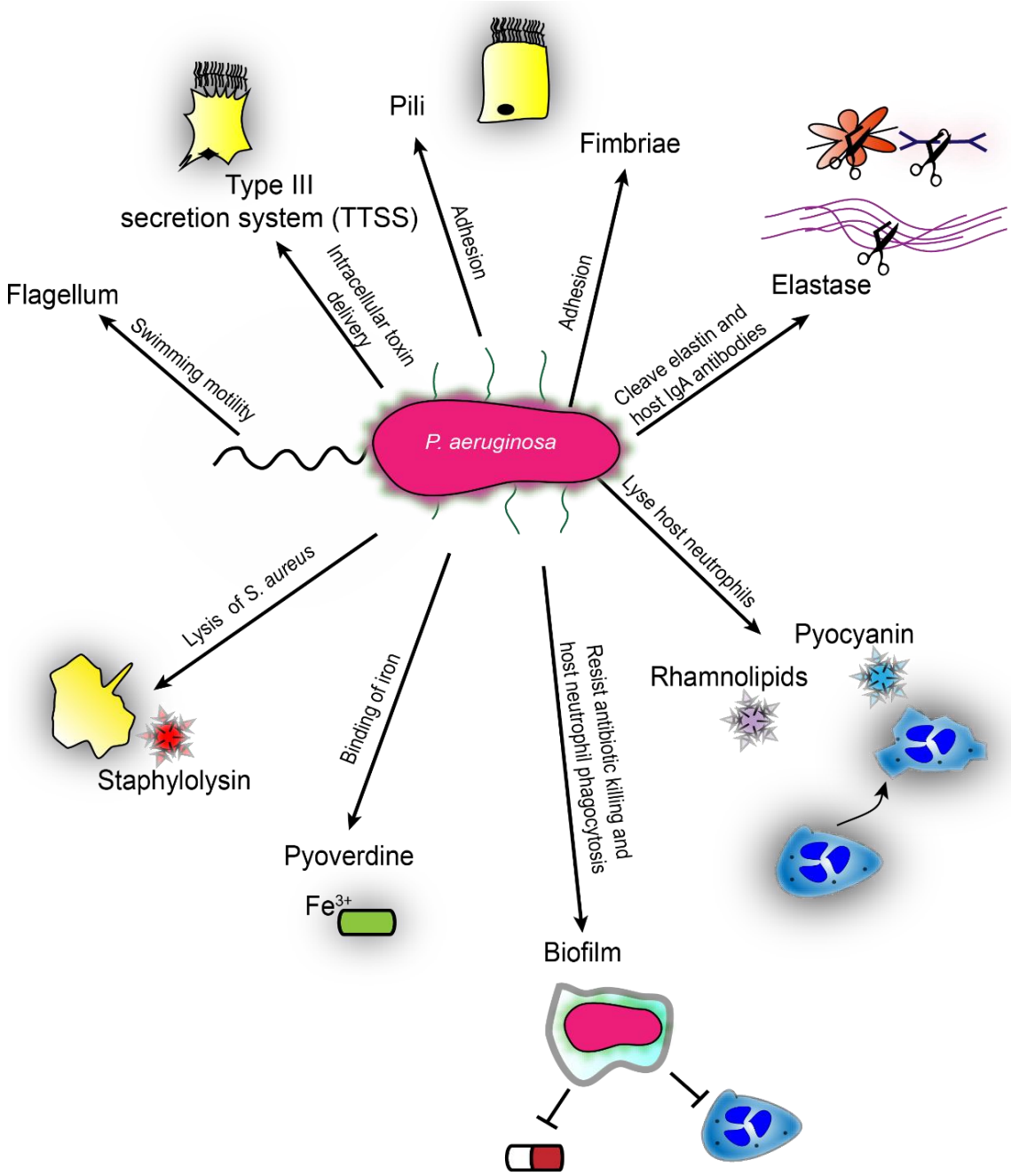


Figure 6. *P. aeruginosa* is armed with a potent arsenal of virulence factors. Cell associated virulence factors fimbriae and pili promote bacterial adhesion, whilst the type III secretion system permits the delivery of bacterial toxins into the cytoplasm of airway epithelia. Flagellum facilitates swimming motility, whilst staphylolysin is involved in the lysis of *S. aureus.* Pyoverdine binds to iron, whereas biofilm inhibits bacterial killing by antibiotics and phagocytosis by host neutrophils. Rhamnolipids and pyocyanin both kill host neutrophils. Elastase permits the degradation of elastin and host IgA antibodies.

1.6.2.2 Adaptation to the CF lung

During chronic infection, *P. aeruginosa* is known to adapt to the unique niche of the CF lung. Whilst selective pressures of the host's immune response and antibiotics drive this (Ciofu *et al.*, 2005, Nair *et al.*, 2013), *P. aeruginosa* variants within the CF lung have a hypermutable phenotype (Oliver *et al.*, 2000), with genetic recombination and its accessory genome driving diversity (Darch *et al.*, 2015). Overtime, this subsequently leads to the co-existence of clonal lineages which differ in phenotype from one another, (Feliziani *et al.*, 2014, Williams *et al.*, 2015, Chung *et al.*, 2012, Smith *et al.*, 2006a). During the course of chronic infection *P. aeruginosa* exhibits an adapted phenotype, which differs significantly to those isolates recovered from the environment, or early infection of CF airways. Such changes include losses in motility (Mahenthiralingam *et al.*, 1994) and the acquisition of a mucoid phenotype (Martin *et al.*, 1993). This is summarised in Figure 7 below.

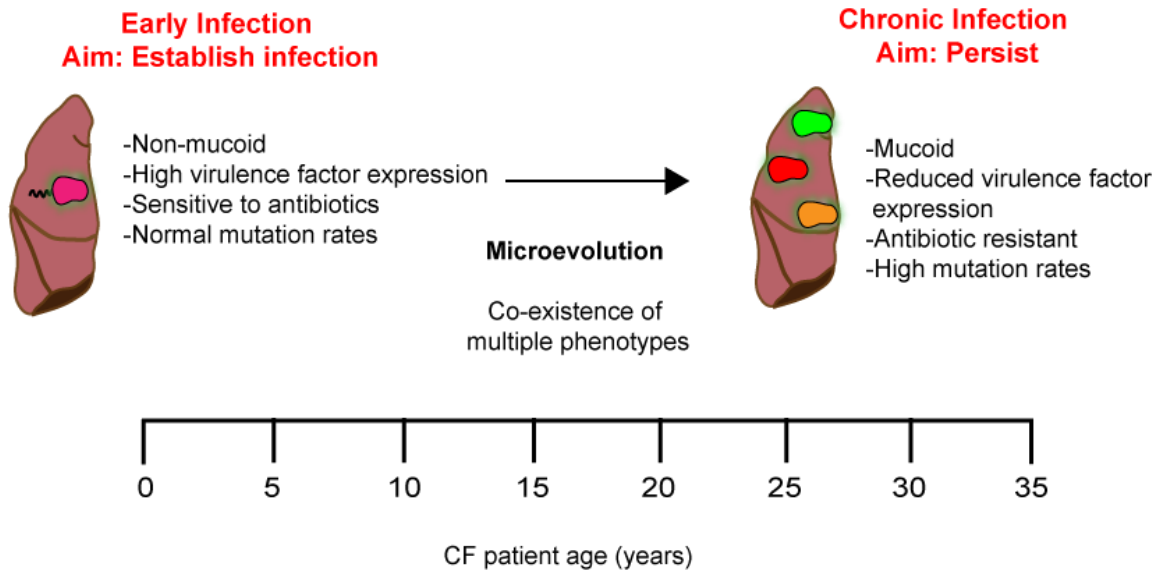


Figure 7. *P. aeruginosa* microevolution in the CF lung. During early infection, *P. aeruginosa* (indicated in pink in the lung on the left) is non-mucoid, secretes an arsenal of extracellular factors (e.g. pyocyanin and proteases), exhibits motility, is sensitive to antibiotics and has a normal mutation rate. During chronic infection, increases in *P. aeruginosa* genotypic diversity gives rise to phenotypic diversity which express a variety of adaptations (represented by *P. aeruginosa* shown in green, red and orange on the right), such as alginate overproduction (mucoid phenotype), reduced virulence factor expression, resistance to antibiotics and an increased mutation rate. Adapted from (Sousa and Pereira, 2014).

1.6.2.3 Biofilm production

During chronic infection in the CF lung, *P. aeruginosa* typically adopts a mucoid phenotype which is coupled to the production of a biofilm (Hoiby *et al.*, 2001, Fick *et al.*, 1992). Consisting of a large encapsulated community of one or more bacterial species, biofilms are three-dimensional structures rich in alginate, mucoid exopolysaccharide (MEP), proteins, extracellular DNA and cellular debris (Nivens *et al.*, 2001). Whilst extracellular DNA not only chelates cations, it also cross-links the exopolysaccharides and is involved in bacterial adhesion and cellular aggregation (Mulcahy *et al.*, 2008, Das *et al.*, 2010). The overproduction of alginate however mainly provides protection against host neutrophils and macrophages and acts as an effective scavenger against ROS, but also reduces apoptotic cell clearance (Mathee *et al.*, 1999, McCaslin *et al.*, 2015, Pedersen *et al.*, 1990, Leid *et al.*, 2005). The protection provided against antibiotics varies. Whilst biofilm limits the penetration of β-lactam antibiotics (Gordon *et al.*, 1988), low metabolic activity and oxygen levels provide protection against ciprofloxacin and tobramycin (Walters *et al.*, 2003). *P. aeruginosa* has been shown to give rise to a small subpopulation of metabolically dormant cells referred to as 'persister cells', which are phenotypically distinct, yet genetically identical to most of the bacterial population and are able to withstand high concentrations of bactericidal antibiotics (Mulcahy *et al.*, 2010, Koeva *et al.*, 2017). Once the concentration of antibiotic falls, the persister cells are able to repopulate the biofilm, leading to recurrent infection.

The formation of a biofilm is a highly ordered process. *P. aeruginosa* initially uses flagella mediated swimming to attach itself to a solid surface (e.g. airway mucus). This attachment is influenced by several factors within the CF lung microenvironment including temperature, pH, ionic concentration and nutrient availability (Hall-Stoodley *et al.*, 2004). *P. aeruginosa* will then undergo irreversible attachment, followed by co-ordinated growth and biofilm maturation and eventual dispersal, where a subset of planktonic (free-swimming) *P. aeruginosa* bacteria are then able to colonise distance sites of the CF lung, with this dissemination being encouraged by changes within nutrient availability (Sauer *et al.*, 2002, Tolker-Nielsen *et al.*, 2000, Hunt *et al.*, 2004, Sauer *et al.*, 2004). The biofilm formation process in CF airways is illustrated in Figure 8.

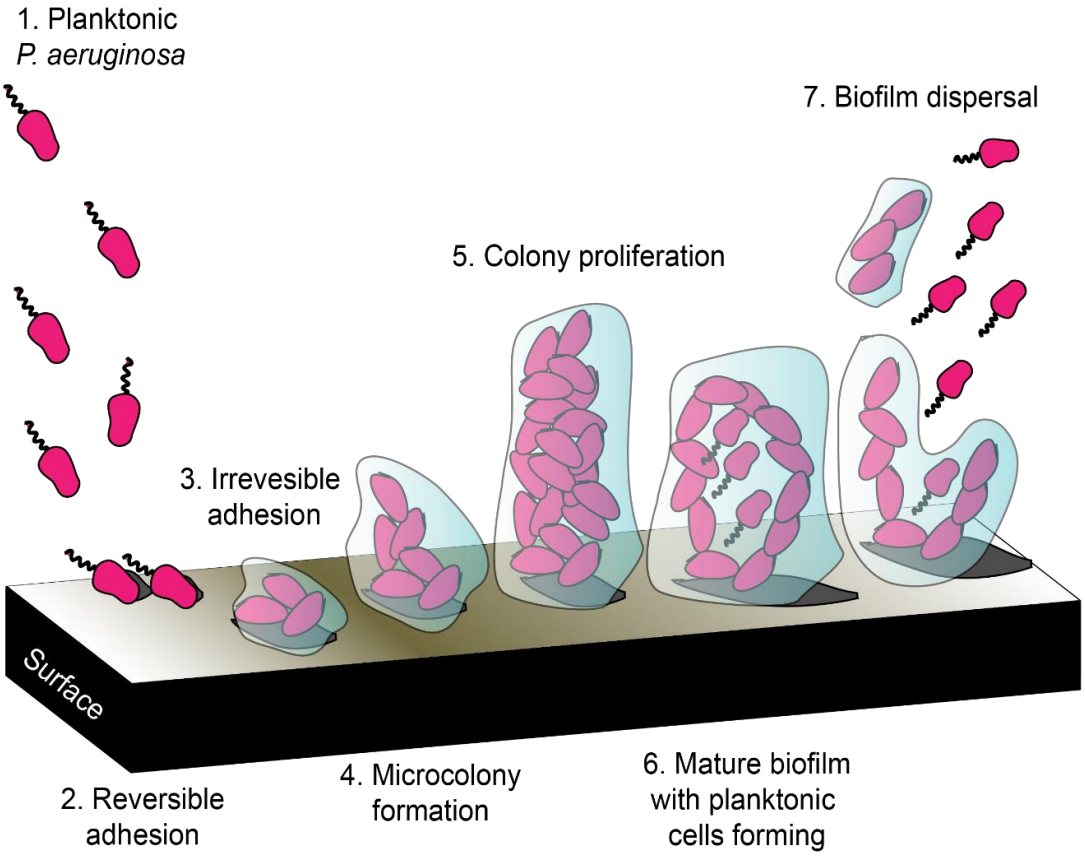


Figure 8. Mechanism of *P. aeruginosa* biofilm formation. After planktonic *P. aeruginosa* has formed weak interactions with a surface (such as airway mucus), the bacterial population eventually forms a microcolony, consisting largely of non-motile bacteria. As *P. aeruginosa* replicates and the biofilm matures, water channels develop, facilitating the circulation of nutrients and oxygen. During dispersal, a subset of motile planktonic bacteria are liberated, which can then colonise distant sites of the CF lung. Adapted from (Perfectus Biomed, 2017).

1.6.2.4 Quorum Sensing (QS)

P. aeruginosa biofilm production, motility and virulence gene expression are regulated by quorum sensing (QS), a system that produces and detects signals in a density-dependent manner (Pesci *et al.*, 1997, Whiteley and Greenberg, 2001, Rutherford and Bassler, 2012). This form of bacterial communication is found across a wide variety of bacterial species and plays an important role in opportunistic infections (de Kievit and Iglewski, 2000). The ability to regulate tightly and synchronise virulence gene expression through QS is critical for *P. aeruginosa* survival. Producing highly immunogenic virulence factors early in an infection would facilitate bacterial clearance. Virulence factor production by large bacterial communities are likely to facilitate colonisation and persistence.

P. aeruginosa releases acyl homoserine lactones (AHL's) which act as diffusible extracellular signalling molecules (autoinducers) (Rutherford and Bassler, 2012). At low bacterial densities, the levels of autoinducers are under the limit of detection. As *P. aeruginosa* divides and the concentration of autoinducers increases and eventually reaches a threshold concentration, they cross the bacterial membrane and bind to their cognate receptor and subsequently regulate gene transcription (Rutherford and Bassler, 2012, de Kievit and Iglewski, 2000).

The regulatory network of *P. aeruginosa* is highly complex and dependent upon three systems: las, rhl and pseudomonas quinolone signal (PQS). The las system consists of a transcriptional regulator LasR, which binds to the autoinducer molecule 3-oxo-C_{12}-HSL (Pesci *et al.*, 1997). The rhl system contains the transcriptional regulator RhlR which binds to the autoinducer C_4-HSL (Pesci *et al.*, 1997). Whilst the las system is at the top of the QS hierarchy and can regulate the rhl system, both of these in turn are able to regulate the PQS system (Schuster and Greenberg, 2006, Latifi *et al.*, 1996, Pesci *et al.*, 1997). Whilst the las system regulates LasA protease, LasB elastase and biofilm production (Gambello and Iglewski, 1991, Anderson *et al.*, 1999) the rhl system regulates the synthesis of pyocyanin, siderophores, rhamnolipids and hydrogen cyanide (Brint and Ohman, 1995, Latifi *et al.*, 1996). The PQS signal (2-heptyl-3-hydroxy-4-quinolone) acts as a link between the las and rhl systems and rather than being involved in sensing cell density, it is produced during times of stress (McKnight *et al.*, 2000). This complex regulatory network is illustrated in Figure 9.

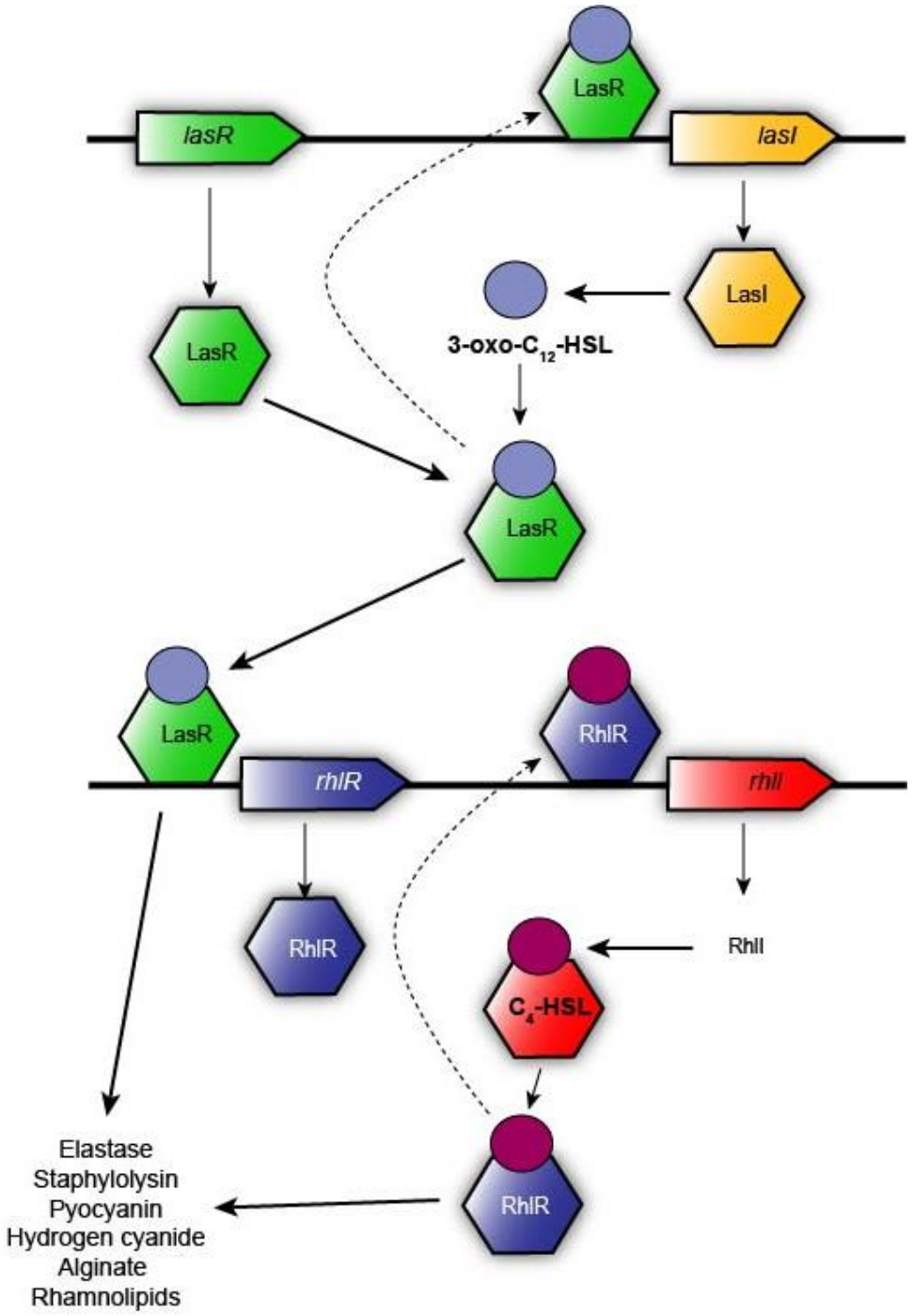


Figure 9. Quorum sensing in *P. aeruginosa* virulence. Transcription of the *lasR* gene leads to the production of the transcriptional regulator LasR (green haxgon). This in turn binds to the homoserine lactone signalling autoinducer molecule 3-oxo-C_{12}-HSL (blue circle), produced by the LasI protein (yellow hexagon). This LasR-autoinducer complex (green hexagon and blue circle) has a number of roles, from acting as an autoinducer in the production of LasI (dashed line), to binding to a series of virulence factor promoters, such as elastase. The LasR-autoinducer complex also activates the rhI system, leading to the production of the RhIR transcriptional regulator (dark blue hexagon). The binding of RhIR to the autoinducer C_4-HSL (purple circle) also acts as an autoinducer, and activates the transcription of other virulence factor genes downstream, including alginate, pyocyanin and hydrogen cyanide. Adapted from (Jimenez *et al.*, 2012).

Periodically, individuals with CF experience an acute, intermittent worsening of their respiratory symptoms referred to as a 'pulmonary exacerbation'. This is typically associated with an increase in inflammation, increased malaise and lethargy, increased sputum production, weight loss, a worsening cough, increased dyspnoea and a decrease in pulmonary function by ≥10% (Flume *et al.*, 2009, Rosenfeld *et al.*, 2001a, Goss and Burns, 2007). Individuals may also present with pneumothorax and haemoptysis which warrants treatment and both are associated with increased 2-year mortality rates (Flume *et al.*, 2005a, Flume *et al.*, 2005b)

Pulmonary exacerbations are linked to a failure to return to baseline lung function (Sanders *et al.*, 2010), a reduced quality of life (Britto *et al.*, 2002) and increased mortality (Liou *et al.*, 2001, de Boer *et al.*, 2011). Whilst not associated with changes in bacterial density (Stressmann *et al.*, 2011b), treatment typically warrants a course of oral, inhaled or intravenous antibiotics based upon recent microbiological antibiotic sensitives, coupled with airway clearance techniques (Wagener *et al.*, 2013, Justicia *et al.*, 2015). Currently, the ability to detect the advent of pulmonary exacerbations is unreliable (van Horck *et al.*, 2017).

1.7 Symptom Management - clearance, inflammation, infection and transplantation

1.7.1 Airway clearance

Due to the extensive mucostasis that occurs within CF airways, the main goal of airway maintenance therapy is to help dislodge and clear mucus from the lungs. Clearance is clinically recommended upon diagnosis, and aims to not only reduce the bacterial burden and improve airflow, but also reduce airway inflammation and damage (Rand *et al.*, 2013). Typical strategies include a combination of exercise, physiotherapy and breathing techniques, to the use of devices such as flutter and oscillating positive expiratory pressure devices. This serves to vibrate the airways, helping to dislodge the mucus and stimulate ciliary beating, allowing for expectoration (Tarran *et al.*, 2005, Hess, 2001).

1.7.2 DNase

Individuals with CF also take a series of medications, with the average individual being prescribed seven daily treatments (Rand *et al.*, 2013). These include the use of a nebulised recombinant human DNase enzyme, which breakdowns free neutrophil extracellular DNA within the mucus. Reducing mucus viscosity, DNase has shown long term benefits in improving lung function, reducing pulmonary exacerbations and airway inflammation (Lieberman, 1968, Jones and Wallis, 2010, Paul *et al.*, 2004).

1.7.3 Hypertonic saline

Inhaled hypertonic saline (~6%) and mannitol both serve to create an osmotic gradient within CF airways, helping to draw water onto the epithelial cell surface and thus hydrating airway mucus (Button *et al.*, 2012, Ratjen, 2006). In addition to being inexpensive and well tolerated, it serves to improve mucocililary clearance, lung function, reduce neutrophil influx, IL-8 concentration and the frequencies of lung exacerbations (Robinson *et al.*, 1997, Elkins *et al.*, 2006, Dellon *et al.*, 2008, Reeves *et al.*, 2011). There may be potential benefits of taking hypertonic saline and mannitol before or during airway clearance, although the evidence is conflicting (Dentice and Elkins, 2016, Elkins and Dentice, 2016).

1.7.4 Targeting airway inflammation

Whilst there is some controversy regarding the drugs that should be given to CF patients to combat chronic and excessive airway inflammation, individuals are often prescribed Azithromycin. It is well tolerated, and helps reduce *P. aeruginosa* bacterial burden and also slows the decline in lung function and frequency of pulmonary exacerbations (Saiman *et al.*, 2003, Wolter *et al.*, 2002). A number of anti-proteases are also being developed to combat airway inflammation. Both aerosolised α1-AT and nebulised SPLI have shown promise by inhibiting NE, reducing NE activity and decreasing the concentration of IL-8 (McElvaney *et al.*, 1991, McElvaney *et al.*, 1992, Griese *et al.*, 2007).

1.7.5 Antimicrobial Strategies

Current strategies for treating infections of the CF lung are based on the results of traditional microbiological culture and susceptibility testing. However, it is often the case that these results do not translate *in vivo*. Often the strategy chosen by clinicians is not reliant upon the results from antimicrobial susceptibility testing, but by factors such as regimens which have previously been effective and strategies which reduce side effects such as toxicity. Furthermore, the method of antimicrobial delivery is also important if the treatment is to have an effect. Whilst nebulised antibiotics only reach high concentrations in the conductive zone, intravenous and oral antibiotics provide high concentrations in the respiratory zone.

In the UK, *S. aureus* positive cultures in children require the prescription of the narrow-spectrum antibiotic flucloxacillin, which is successful in reducing the lungs bacterial burden (Smyth and Walters, 2012, Cystic Fibrosis Trust, 2009). The presence of *S. aureus* whilst an individual is on flucloxacillin may warrant the use of a second oral antibiotic such as rifampicin for 2-4 weeks, or the intravenous administration of flucloxacillin (Cystic Fibrosis Trust, 2009).

P. aeruginosa colonisation is associated with an increase in hospital admissions, along with decreases in survival, pulmonary function and general health (Doring *et al.*, 2012). There is an international consensus that identification of *P. aeruginosa* from CF sputum typically warrants

an aggressive antibiotic regimen aimed at eradicating the bacterium, thus preventing chronic colonisation. Oral ciprofloxacin and nebulised colistin is typically recommended for acute infections with *P. aeruginosa* for up to three months (Cystic Fibrosis Trust, 2009), whilst chronic infection with *P. aeruginosa* typically requires nebulised colistin (Cystic Fibrosis Trust, 2009).

Current recommendations also include nebulised tobramycin (Gibson *et al.*, 2003), which is often used in conjunction with a fluoroquinolone to combat antibiotic resistance (Dudley *et al.*, 2008). Inhaled fluoroquinolones are effective against *P. aeruginosa* biofilms and under anoxic conditions (a current limitation of tobramycin) and phase III trials have demonstrated that this formulation improves lung function and is well tolerated (Geller, 2009). Whilst Aztreonam has been used for many years to treat *P. aeruginosa,* its re-formulation as a lysine salt has shown that Aztreonam is not only effective in reducing *P. aeruginosa* burden within the sputum of chronically infected patients, but also improves lung function and is well tolerated (Oermann *et al.*, 2010, Retsch-Bogart *et al.*, 2009, McCoy *et al.*, 2008). Other anti-pseudomonal antibiotics recommended clinically include the β-lactam antibiotics ceftazidime and meropenem (Cystic Fibrosis Trust, 2009).

New antimicrobial drugs and optimal treatment strategies are required to effectively combat CF airway infections, particularly in the case of *P. aeruginosa.* Once established it is almost impossible to eradicate, resulting in a clinical shift from eradication, to infection control to prevent pulmonary exacerbations.

1.7.6 Lung Transplantation

Despite adherence to airway clearance and antimicrobial treatment strategies, chronic infection and inflammation eventually lead to large decreases in lung function. During end-stage lung disease, a number of individuals with CF may be eligible for lung transplantation. Whilst there is an endemic shortage of available organs and strict acceptance criteria (such as rapidly declining pulmonary function, increased pulmonary exacerbation and recurrent pneumothorax), to those who are eligible, it provides enormous benefits regarding overall lung function, fitness and long-survival. Whilst in its very early infancy, advances within biomedical engineering may show some promise in the field of CF, in the form of a tissue engineered lung. Decellularisation of the lung scaffold and its repopulation with healthy cells may one day provide long-term benefits to individuals with CF (Petersen *et al.*, 2010) .

1.8 Future developments

1.8.1 Targeting the CFTR mutation (gene therapy)

Gene therapy has been the focus of several clinical trials for inherited diseases, with the aim of delivering a healthy gene to target cells and providing either long-term therapy or ultimately, a cure. The CF lung however, is a particularly hostile environment, largely due to the presence of mucus plugs, large immune cell populations, polymicrobial biofilms, and hydrolytic enzymes. Moreover, there is uncertainty concerning whether the levels of gene transfer achieved within the CF lung will bring about improvements in lung function. One *in vitro* study showed that mixing 6-10% of non-CF epithelia with CF epithelia restored chloride secretion to the levels seen in non-CF (Johnson *et al.*, 1992). However, another study identified that wtCFTR must be expressed in a quarter of epithelial cells for mucus transport to be restored (Zhang *et al.*, 2009). It is likely that different levels of CFTR expression will be required dependent upon CFTR mutation severity.

Lentivirus trials have shown some promise with regard to restoring CFTR function both in murine models of CF and in human derived *in vitro* polarised airway epithelial models (Mitomo *et al.*, 2010, Sinn *et al.*, 2008). Whilst they provide benefits in relation to the ability for re-administration (Sinn *et al.*, 2008), there are a number of challenges too. These include the high dose required to ensure effective delivery, the need to include adjuvants to open tight junctions and facilitate virus entry and concerns regarding safety, due to random integration of the viral genome (Stocker *et al.*, 2009, Sinn *et al.*, 2008, Cmielewski *et al.*, 2010).

Non-viral vectors have been used in several phase I safety trials. Often consisting of cationic lipids and polymers, modified mRNA and DNA nanoparticles their benefit over viral vectors regarding chloride transport has yet to be established (Pichon *et al.*, 2010, Griesenbach and Alton, 2012, Alton *et al.*, 1999). Non-viral vectors have been shown to be suitable candidates for regular and routine administration to the nasal epithelium of individuals with CF (Hyde *et al.*, 2000). The ability to re-administer gene therapy is an essential requirement if treatment is to be successful. Gene transfer to the superficial, terminally-differentiated airway epithelia is not only less efficient than incorporation into dividing cells, but it will only provide a short-term solution due to cell turnover. Thus, repeated delivery is required due to provide a lasting benefit.

1.8.2 Targeting the CFTR protein (pharmacological drugs)

As previously mentioned, the most common mutation is the Phe508del (class II mutation), affecting approximately 90% of the CF population in the UK. The loss of this highly conserved phenylalanine amino acid from NBD1 of the CFTR results in the protein becoming misfolded, leading to its ubiquitin mediated degradation. To correct this defect, the development and screening of numerous drug candidates has been undertaken. This not only includes chaperones, which help to rescue misfolded proteins, allowing them to reach the epithelial cell surface, but also drugs which target proteins within the epithelial cell responsible for inhibiting CFTR tracking and degradation.

Ivacaftor (VX-770) is a potentiator drug, increasing the time the CFTR is open, subsequently facilitating the transport of chloride ions (Ramsey *et al.*, 2011). Vertex Pharmaceuticals Inc. recently released its result from two phase 3 trials. The 'Evolve' study evaluated the safety and efficacy of a dual therapy consisting of the CFTR potentiator Ivacaftor, with the CFTR corrector Tezacaftor. The study was conducted across North America and Europe in CF patients aged 12 and older who had two copies of the Phe508del mutation. Results demonstrated significant improvements in pulmonary function compared to placebo (Vertex Pharmaceuticals Incorporated, 2017). The results from the phase 3 'Expand' study compared the safety and efficacy of the Ivacaftor-Tezacaftor dual therapy with ivacaftor monotherapy. The study was conducted in individuals with CF over the age of 12, with one copy of Phe508del and another mutation that results in residual CFTR function. The results demonstrated that the dual therapy was significantly improved increased in pulmonary function compared to those receiving ivacaftor alone (Vertex Pharmaceuticals Incorporated, 2017). In both studies the most common adverse effects were a cough and infective pulmonary exacerbations.

Correcting the CFTR at the protein level is transforming CF, improving overall health and help to make CF a better managed disease. However, further work is required to better understand the impact this treatment is having on CF airway microbiology. Adverse effects of infective pulmonary exacerbations in the latest clinical trials are evidence that despite improvements in lung function, CF microbiology remains an essential area of research.

1.8.3 Other areas of research

Whilst most of the research focus within the CF community is targeting the lung, numerous research groups have made progress treating the comorbidities commonly seen in CF patients. Whilst bone marrow transplants may potentially correct the immune defects seen in neutrophils and macrophages, transplanting cells from a healthy pancreas aim to address the extrapulmonary manifestations of the disease, such as pancreatic insufficiency, diabetes mellitus and pancreatitis (Kessler *et al.*, 2010).

1.9 Modelling CF

1.9.1 Animal Models of CF

Mice, ferrets and pigs have all been used since the early 1990's as models to study CF (Fisher et al., 2011). Together, this three species approach has provided a greater understanding of the disease, from the mechanisms underlying increased susceptibility to infection, to the function of the CFTR. The use of CF animal models, however, are limited in their ability to recapitulate fully the human disease, in terms of severity and systemic disease.

1.9.1.1 Murine Model

The first murine model CFTRtm1UNC (homozygous for a mutated CFTR gene) was developed in 1992, to which thirteen subsequent models have since followed. The majority predominantly display an intestinal phenotype, characterised by goblet cell hyperplasia, a failure to thrive and the formation of mucus plugs and blockages within the ileum (Fisher *et al*., 2011). The nasal phenotype of CF mice closely resembles that seen in humans, with the models also replicating severe malabsorption and weight loss seen in humans, largely due to clogging of the gastro-intestinal (GI) tract (Fisher *et al*., 2011). In spite of this, there are several limitations to this model. Whilst mucus accumulation and obstruction in the ileum are similar in adults with CF, the phenotype of weaning mice and newborns with CF varies. The biggest drawback however, is the inability of these models to display spontaneous bacterial infection and inflammation, key hallmarks of CF (Fisher *et al*., 2011). The use of agar beads containing *P. aeruginosa* have been used as an attempt to overcome this (Bragonzi *et al.*, 2012, Cigana *et al.*, 2018, Bayes *et al.*, 2016). The use of beads however fails to mimic the nature of bacterial colonisation seen in humans. Later models even exhibited the ability to spontaneously clear a large inoculum of *S. aureus* and *P. aeruginosa*, a phenomenon not seen in humans (Fisher *et al*., 2011, Cohen and Prince, 2012). Additional limitations of the CF murine model are that mice do not express IL-8, but produce two homologs: macrophage inflammatory protein-2α and keratinocyte chemoattractant. Studying these inflammatory markers consequently makes it difficult to translate them to IL-8 production in humans.

1.9.1.2 Porcine Model

The drawbacks of murine models have consequently led to the use of large animals to study CF, namely pigs and ferrets. The development of pigs homozygous for the most common mutation Phe508del more closely mimics the human phenotype, particularly between new-born piglets and neonates with CF (Rogers et al., 2008). In addition to this, porcine models demonstrate a similar bioelectric property of the nasal mucosa (which closely resembles that of children and adults with CF), including the formation of bile and mucus plugs within the gall bladder. Moreover, unlike mice, pigs develop spontaneous infection and inflammation, which

they are unable to clear once established (Rogers *et al*., 2008, Fisher *et al*., 2011). However, as with murine models, there are several limitations. All piglets born with the mutation develop meconium ileus within two days, a phenomenon which only affects a small percentage of children with CF. Subsequent obstructions of the ileum require surgical intervention to ensure survival of the animal, which consequently impact upon the overall usefulness of this model (Rogers *et al*., 2008, Stoltz *et al*., 2013, Fisher *et al*., 2011). Moreover, whilst they mimic defects in the transport of chloride ions apically across epithelial cells, there is no hyperabsorption of sodium, or a decrease in the height of the ASL (Chen *et al*., 2010).

1.9.1.3 Ferret Models

Ferret models, in addition to being used to study influenza, are used within the field of CF largely due to the extraordinary similarity in lung biology to humans. Like pigs, mutations in the CFTR generate a similar bioelectric profile to humans, with ferrets also being susceptible to spontaneous infection early in life, particularly by *S. aureus* (Keiser and Engelhardt, 2011). Despite this, 75% of newborn ferrets develop meconium ileus, which like the porcine model, limit its benefits as a model organism due to low survival rates and shortened longevity. This is further complicated by the fact that the lung phenotype of adult ferrets is still under investigation (Fisher *et al*., 2011).

This three species approach to study CF has paved the way for preclinical testing of both gene therapies (Sinn *et al.*, 2008, Mitomo *et al.*, 2010) and in meeting the regulatory requirement to bring pharmaceutical drugs such as Ivacaftor to clinical trials. Whilst *in vivo* models have provided results relating to therapeutic responses to treatments, they are less suited to studies addressing mechanistic insight, such as host-pathogen interactions. *in vitro* models of CF airways are also required in CF research as they provide a top-down approach to understanding CF progression, as opposed to the bottom-up whole organism *in vivo* approach. They consequently allow individual aspects of pathogen-pathogen and host-pathogen interactions to be investigated, which in turn help to piece together a complex picture of what happens *in vivo.* Thus, the inherent limitations of both *in vivo* and *in vitro* models serve to complement one another.

1.9.2 Primary and immortalised cell lines

Immortalised cell lines are often used to conduct initial studies into CF airway research, largely due to their cost-effectiveness and homogeneity. Either immortalised through viral transformation or naturally due to becoming cancerous, they continue to provide many benefits. Classic examples of immortalised cell lines used within airway research include the CF cell lines, IB31, CF3BEo- and the non-CF cell lines C38, Calu-3, Beas2-B and A549. Despite this, immortalised cell lines have several limitations too. Their abnormal growth

patterns may influence their response to stimuli, whilst the process of immortalisation may also influence their phenotype. Once such example is the airway epithelial cell line Calu-3, which not only displays exceedingly high transepithelial electrical resistance (TEER) values compared to most airway epithelial cell lines, but it also secretes the mucin MUC5AC, with evidence that cilia has not formed through β-tubulin staining (Stewart *et al.*, 2012).

Primary cells isolated from individuals with CF (nasal or bronchial brushings) provide a closer representation of the human environment of the CF lung (Randell *et al.*, 2011). Although they also more closely resemble the heterogeneity of the CF population, they have a number of limitations, from their limited availability and their finite lifespan, to their cost. Moreover, primary cells typically exhibit wide donor variability and typically require a larger sample size to be used. Whilst attempts to immortalise primary cell lines have been conducted, this is often with limited success. Not only do some cells fail to survive the transformation, others lose their ability to become polarised (Gruenert *et al.*, 1988).

1.9.2.1 Submerged culture

The plating of primary and immortalised cell lines into submerged monolayers continue to play a major role within CF airway research, including the study of pathogen-pathogen and host-pathogen interactions. Although this approach is relatively inexpensive, the lack of cell differentiation and polarisation in both immortalised and primary cell lines means they fail to closely mimic human physiology and morphology of the CF airways. Examples include the lack of cilia, mucus production, polarised secretion of cytokines, and antimicrobial factors. The use of submerged cultures to study host-pathogen interactions can also compromise the expression of cell surface receptors. Together, such disadvantages often mean that host-pathogen studies can only be conducted over a short period in submerged cultures, before host cells undergo monolayer detachment (Moreau-Marquis *et al.*, 2010).

1.9.2.2 3D Organoids

First developed in 2009, organoids are 3D tissues of aggregated cells which more closely mimic the phenotype of the organ from which they were derived (Sato *et al.*, 2009). Cells are derived from blocks of human respiratory tissue and grown in flasks under constant, gentle agitation. Unlike submerged culture, these 3D models of the airways are positive for mucus production (Ulrich and Doring, 2004). Whilst this approach has yielded some insightful results and has been used previously to study host-pathogen interactions, their long-term differentiation (over a month) make them time consuming and low throughput. Moreover, the formation of aggregates as a result of agitation can vary significantly across organoids, which affects reproducibility.

1.9.2.3 Air-Liquid Interface (ALI)

To overcome the aforementioned limitations of submerged models (cell differentiation and polarisation) and 3D organoids (reproducibility due to donor variability and factors relating to agitation), the culturing of cells onto porous membrane supports at an air-liquid interface (ALI) more closely resemble the *in vivo* conditions found in airway epithelium. This methodology was first developed in 1988 as a means to differentiate guinea pig epithelial cells (Whitcutt *et al.*, 1988), first being used in respiratory research in 1990 in the differentiation of bronchial epithelia (Wu *et al.*, 1990).

Exposure to cell culture media on the basolateral side and air on the apical side forces primary or immortalised epithelia to undergo mucociliary differentiation. Whilst this process is poorly understood, it gives rise to the characteristic formation of tight junctions between adjacent epithelia, expression of specific cell surface markers, mucin production (such as MUC5AC and MUC5B) and the formation of cilia. This differentiation also overcomes limitations in submerged culture regarding their ability to withstand higher inocula of bacterial infection for longer periods, thus enabling long term infection studies. The major drawback of ALI culture is the length of time they take to establish, with cultures typically taking over three weeks to a month to completely differentiate and form an impermeable barrier.

A novel co-culture model of non-CF and CF airways was established in Dr Lindsay Marshall's laboratory (Bielemeier, 2012b). Being physiologically representative of human CF airways, it enables investigations to be conducted relating to host-pathogen interactions. Each co-culture model (CF or non-CF) consists of a suspended transwell®, coated in human collagen type IV. This aims to mimic the upper layer of the basement membrane within airways. The basement membrane is known to serve several fundamental roles, from facilitating epithelial adhesion and migration, to being important for inducing cell differentiation and encouraging their characteristic polarised phenotype.

Seeded on top of this is a monolayer of pulmonary fibroblasts. These mesenchymal cells play an active role within the airways, rather than acting solely as a structural support. Known to form interactions with overlying epithelial cells, pulmonary fibroblasts are responsible not only for modulating epithelial proliferation and differentiation, but also for the deposition of extracellular matrix components, such as fibronectin, tenascin and collagen I and III (Knight, 2001). Their ability to also secrete matrix metalloproteinases such as collagenase in response to inflammatory cytokines such as IL-6, highlights their importance in airway remodelling following damage. There is also evidence that these cells are involved in airway inflammation (Manuyakorn *et al.*, 2016, Fitzgerald *et al.*, 2003, Knight, 2001).

Seeded on top of these fibroblasts, are one of two epithelial cells lines, IB3-1 or C38. IB3-1 cells are an immortalised bronchial epithelial cell line used to model CF airways. Isolated from a paediatric patient with CF, these cells are a compound heterozygote containing an allele for the most common CF mutation, Phe508del, along with a nonsense mutation allele, W1282X (Zeitlin *et al.*, 1991). C38 epithelial cells are used to model non-CF airways, derived from IB3-1 cells, where the CF phenotype has been corrected using an adeno-associated viral vector to replace both mutant CFTR alleles with a wild-type CFTR (Egan *et al.*, 1992). A haematoxylin and eosin stained cross section of this *in vitro* ALI co-culture transwell® model of CF and non-CF airways is provided, along with a section of the human bronchi obtained from non-CF airways for comparison, as shown in Figure 10.

A.

B.

C.

Figure 10. Formalin fixed cross sections of non-CF and CF co-culture models. Airway epithelia were stained with haematoxylin and eosin, whilst fibroblasts were stained with vimentin. Non-CF C38 (A) and CF IB3-1 (B) epithelia grew as a pseudostratified layer on top of a population of sub-epithelial fibroblasts. Images were taken from the thesis of (Bielemeier, 2012b) and represents *N*=2. The transwell® membrane was lost for the IB3-1 co-culture. (C) represents the pseudostratified epithelium of the *in vivo* bronchial epithelium and is taken from (Wandsworth, 2018).

A schematic of this *in vitro* co-culture model is illustrated in Figure 11.

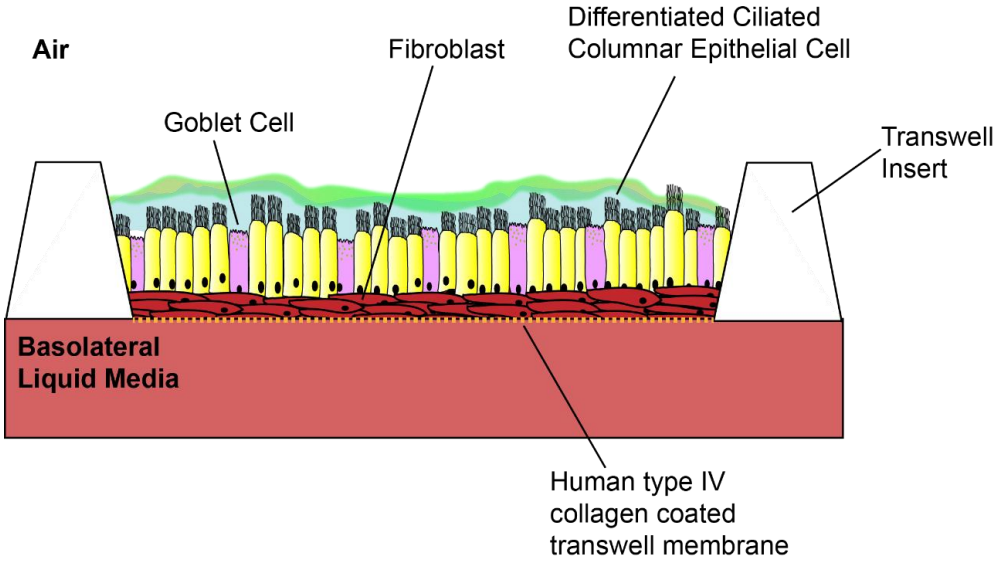


Figure 11. Schematic diagram of the *in vitro* human airway co-culture model. Human pulmonary fibroblasts are seeded onto a collagen coated transwell® membrane. After 4 days, IB3-1 (CF) or C38 (CF phenotype corrected) bronchial epithelial cells are seeded on top of the fibroblasts. After a further 4 days, the apical media is aspirated and the basal media is refreshed, introducing cells to an air liquid interface (ALI). This forces cells to differentiate overtime into the characteristic morphology seen within the human airways, as depicted above.

1.10 Aims and Objectives

How mutations in the CFTR impair pulmonary immune defences have been extensively studied, alongside the mechanisms major CF pathogens use to colonise and persist within the CF lung. Despite advances in the development of pharmacological drugs and overall CF care, chronic pulmonary infections and inflammation continue to be the main cause of morbidity and mortality in CF. Two obstacles to the development of more effective therapeutics and treatment strategies stem from an incomplete understanding of the polymicrobial nature of CF airway infection and its impact upon interspecies and host-pathogen interactions, alongside the need for models which more closely mimic CF airways and its unique microenvironments.

S. aureus and *P. aeruginosa* are the two most prevalent pathogens in CF airways and the most problematic. As mucus plugging within CF airways gives rise to regions of anoxia, this research aimed to investigate the impact of oxygen availability upon *S. aureus-P. aeruginosa* interspecies interactions and the possible mechanisms which influence bacterial community composition. As chronic inflammation is a hallmark of CF, this study sought to also determine the impact of *S. aureus-P. aeruginosa* co-infection upon the airway inflammatory response of CF and non-CF airway epithelia, comparing it to mono-infection. Finally, as infection of the CF lung is highly sequential, the impact of prior infection with *S. aureus* upon subsequent *P. aeruginosa* airway colonisation was investigated.

Aim 1: Characterise CF clinical isolates of *P. aeruginosa*

Adaptation of *P. aeruginosa* to CF airways is accompanied by extensive phenotypic changes. Prior to studying *S. aureus-P. aeruginosa* interactions, chapter 3 aimed to phenotypically characterise eight novel *P. aeruginosa* CF clinical isolates obtained from Birmingham Children's Hospital. Assays ranged from determining colony morphology, to the production of extracellular virulence factors and biofilm.

Aim 2: Explore the effects of static growth and anoxia upon *S. aureus-P. aeruginosa* interactions

Although a number of studies have investigated the interspecies interactions between *S. aureus* and *P. aeruginosa,* the bacteria are typically grown under normoxia with vigorous culture aeration. It is now appreciated that steep oxygen gradients exist within the CF lung, largely due to decreases in pulmonary function, the presence of thick mucus plugs and the consumption of oxygen by airway epithelia, bacteria and host phagocytes. Confirmation of this has been made through the detection of obligate anaerobes within CF airways. Chapter 4 aimed to investigate the effects of normoxia and anoxia upon *S. aureus-P. aeruginosa* interactions in both planktonic co-culture and mixed species biofilms, as well as begin to

explore the mechanisms which are likely to govern interspecies interactions and community composition.

Aim 3: Elucidate the effects of *S. aureus-P. aeruginosa* co-stimulation upon the airway inflammatory response

The impact of shed and secreted bacterial products upon the CF airway inflammatory response has been widely studied. However, how airway epithelia integrate and respond to stimuli from polymicrobial infections is poorly understood. Chapter 5 aimed to determine the effects of *S. aureus-P. aeruginosa* co-stimulation upon the production of major pro- and anti-inflammatory cytokines in both CF and non-CF airway epithelia, comparing this to stimulation with either *S. aureus* or *P. aeruginosa* exoproducts alone.

Aim 4: Determine whether prior *S. aureus* infection influences *P. aeruginosa* airway colonisation

Infection of CF airways is highly sequential, with *S. aureus* predominating in the first decade of life, followed by *P. aeruginosa* dominance during adolescence and adulthood. Despite this, the impact of *S. aureus* infection upon CF airway pathogenesis is poorly understood, with concerns that *S. aureus* could prime CF airways to subsequent *P. aeruginosa* colonisation. The use of *in vitro* ALI models of CF airways to date have focused upon infection with a single infection, namely *P. aeruginosa.* The final aim of this research sought to employ co-culture ALI models of CF and non-CF airways to address the role of bacterial adhesion in the CF lung as well as sequential nature of infection, to determine whether prior colonisation by *S. aureus* enhances subsequent *P. aeruginosa* colonisation.

2 Materials and Methods

In this chapter the materials and methodologies used to achieve the key aims are described, including equipment, software, reagents, chemicals and other consumables used.

2.1 Equipment and Software

Below is a list of the equipment and software used in this research.

- Anaerobic chamber (Don Whitley Scientific, UK)
- Benchtop 1-14 Microfuge (Sigma Aldrich, Germany)
- Benchtop Centrifuge (Eppendorf, Germany)
- Biosafety cabinet (Thermo Fisher Scientific, UK)
- Dionex 3000 (Thermo Fisher Scientific, UK)
- Electrophoresis apparatus and Western Blot wet transfer system (Bio-Rad, UK)
- Heat-block (Thermo Fisher Scientific, UK)
- Human placental collagen type IV (Sigma Aldrich, UK)
- Improved Neubauer Haemocytometer (CamLab, UK)
- Inverted microscope (Nikon Eclipse, Europe)
- Inverted fluorescence microscope (Zeiss Axiovert 200M, Zeiss, UK)
- Mascot deamon (MatrixScience, UK)
- Mass spectrometer (5600 Triple Tof, ABSciex, UK)
- Microbiology Incubator (Sanyo Biomedical, Europe)
- Mini Incubator (Labnet, Europe)
- Mr Frosty™ Freezing container (Nalgene, UK)
- Nano high performance liquid chromatography analytical column (AcclaimTM PepMapTM C18, 3 µm, 100 Å, 75 µm x 150 mm, Thermo Fisher Scientific,UK)
- Nano high performance liquid chromatography trap column (PepMapTM C18, 5 µm, 100 Å, 300 µm x 1 mm, Thermo Scientific, UK)
- Nano high performance liquid chromatography with automated autosampler (nLC, 3000 Dionex, ThermoFisher Scientific, UK)
- Orbital shaker (ThermoFisher Scientific, UK)
- Plate reader (MULTISKAN GO spectrophotometer, Thermo Scientific, UK)
- Plate reader (Spectramax Gemini XS, Molecular Devices, UK)
- Spectrophotometer 6315 (Beckman Instrument Ltd, UK)
- TripleTof Mass Spectrometer System (AB Sciex, UK)
- Ultrasonicator (Ultrasonic Cleaner, USC-TH, VWR, UK)

2.2 Reagents, chemicals and consumables

Below is a table of all the reagents, chemicals, kits and consumables used in this research, along with the name of the manufacturer.

Table 2. List of reagents, chemicals, kits and consumables used.

Reagent	Manufacturer
0.22 µm polyethersulfone membrane sterile filter	Corning
1.5 mL microfuge tubes	Eppendorf
2.0 mL microfuge tubes	Fisher Scientific
0.5M Tris pH6.8	Bio-Rad Laboratories
1.5M Tris pH8.8	Bio-Rad Laboratories
3 kDa Amicon filter centrifugal units	Millipore
3 kDA FITC-dextran	Sigma Aldrich
24-well plates (tissue culture treated)	Corning
96-well plates (tissue culture treated)	Corning
96-well Maxisorp Enzyme-linked immunosorbent assay plates	Nunc
Acetic Acid	Fisher Scientific
Acetonitrile	Fisher Scientific
40% Acrylamide/Bisacrylamide solution 37.5:1	Fisher Scientific
Agar	Fisher Scientific
Ammonium bicarbonate	Sigma Aldrich
Ammonium persulphate	ThermoFisher
Antibiotic-Antimycotic (100x)	Gibco
Black 96-well plate	Corning
Bradford protein assay	Biorad
Breathe-Easy® membrane	Sigma Aldrich
CellTiter-Blue®	Promega
CHAPS	
Ciprofloxacin	ACROS Organics
Coomassie G-250 stain	VWR
Crystal violet	ACROS Organics
Diethanolamine buffer	Sigma Aldrich
Dimethyl sulfoxide	Sigma Aldrich
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12	Gibco
Eagle’s Minimum Essential Medium	Gibco

Ethylenediaminetatraacetic acid (EDTA)	Sigma Aldrich
Formic acid	Fisher Scientific
Glacial acetic acid	VWR
Glucose, powder	ThermoFisher
Glycerol	Sigma Aldrich
Glycine	Fisher Scientific
Heat-Inactivated Foetal Bovine Serum	Gibco
Human collagen type IV	Sigma Aldrich
IL-6 ELISA ready-set-go® kit	e-Bioscience
IL-8 ELISA ready-set-go® kit	e-Bioscience
IL-10 ELISA ready-set-go® kit	e-Bioscience
Instant dried skimmed milk powder	Tesco
Isopropanol	Fisher Scientific
L-glutamine solution	Gibco
Laemmli buffer	Sigma Aldrich
Lipopolysaccharide from *Escherichia coli* 0111:B4	Sigma Aldrich
Mannitol Salt Agar	Oxoid
Methanol	VWR
Multiwell plate sealing films	Sigma Aldrich
$(NH_4)_2S_2O_8$ (APS)	Sigma Aldrich
N,N,N′,N′-tetramethylethylenediamine (TEMED)	Sigma Aldrich
Nutrient Agar	Oxoid
Nutrient Broth	Oxoid
Pierce Prestained Protein Molecular Weight Marker	ThermoFisher
Potassium nitrate	ACROS Organics
Pseudomonas Isolation Agar	Oxoid
Pseudomonas C-N selective supplement	Oxoid
Sodium dodecyl sulphate	Sigma Aldrich
Sulphuric acid	Fisher Scientific
T-25 Tissue Culture Flasks	Nunc
T-75 Tissue Culture Flasks	Nunc
TEMED	Fisher Scientific
Thioureua	Sigma Aldrich
Tobramycin	ACROS Organics
Transwell® companion plates	BD-Falcon
Trichloroacetic acid	ThermoFisher

Tris	Fisher Scientific
Triton X-100	Sigma Aldrich
Trypan Blue	Gibco
Trypsin-EDTA (0.25%), phenol red	ThermoFisher
Trypsin Gold; sequencing grade	Promega
Urea	Fisher Scientific
White 96-well plate	Corning

2.3 Microbiology

2.3.1 Bacterial strains

Laboratory reference strains and CF clinical isolates used in this study are listed in Table 3. The eight CF clinical isolates of *P. aeruginosa* were originally obtained and purified from paediatric CF sputum samples at Birmingham Children's Hospital, England between 1990-1999.

Table 3. Bacterial strains used in this study.

Strain	Species	Source	Reference
ATCC 6538	*S.aureus*	American Tissue Culture Collection (ATCC)	(Forbes *et al.*, 2015)
PAO1	*P.aeruginosa*	Wound exudate Melbourne, Australia	(Holloway, 1955)
Isolate 1	*P.aeruginosa*	CF Sputum Birmingham Children's Hospital, Birmingham, UK	This study
Isolate 2			
Isolate 3			
Isolate 4			
Isolate 5			
Isolate 6			
Isolate 7			
Isolate 8			
pSB536	*E. coli* (bioreporter)	Prof Paul Williams, Molecular Microbiology, University of Nottingham	(Swift *et al.*, 1997) (Winson *et al.*, 1998)
pSB1142			

2.3.2 Microbiology media

S. aureus and *P. aeruginosa* cultures were routinely passaged on solid Luria Bertani (LB) agar plates and grown at 37 °C and 5% CO_2 for 48 h. Liquid medium cultures were routinely grown in LB broth Miller (granulated) supplemented with 1% (w/v) potassium nitrate, termed LBN broth, unless otherwise stated. For co-infection studies, *S. aureus* were grown on selective mannitol salt agar (MSA) plates, whilst *P. aeruginosa* was grown on selective pseudomonas isolation agar (PIA) with CN supplement to select specifically for the *P. aeruginosa* species. Selective agar was used to discriminate between the two bacterial species and facilitate the easier detection and enumeration of bacterial colonies. The microbiological agar and broth used in this work are listed in Table 4 below.

Table 4. Microbiology culture and assay media.

Medium	Constituents	Formulation g/L	Directions
LB Agar	LB agar (Miller, Pre-buffered capsules)	Tryptone: 10.0 Sodium chloride: 10.0 Yeast Extract: 5.0 Agar: 15.0 Tris/Tris HCL: 1.5	1 capsule dissolved in 500 mL of distilled water
LB Broth	LB Broth (Miller, Granulated)	Casein peptone: 10.0 Yeast extract: 5.0 Sodium chloride: 10.0	22.5 g of broth dissolved in 900 mL of distilled water
LBN broth	LB Broth (Miller, Granulated)	Casein peptone: 10.0 Yeast extract: 5.0 Sodium chloride: 10.0	22.5 g of broth dissolved in 900 mL of distilled water
	Potassium nitrate, 99+%		9 g of potassium nitrate dissolved in 900 mL of LB broth prior to autoclaving

Mannitol Salt Agar	Mannitol Salt Agar	'Lab-Lemco' powder: 1.0 Peptone: 10.0 Mannitol: 10.0 Sodium Chloride: 75.0 Phenol Red: 0.025 Agar: 15.0	99.9 g dissolved in 900 mL of distilled water
Pseudomonas Isolation Agar	Pseudomonas Isolation Agar	Gelatine peptone: 16.0 Casein hydrolysate: 10.0 Potassium sulphate: 10.0 Magnesium chloride: 1.4 Agar: 11.0	24.2 g dissolved in 500 mL of distilled water
	Glycerol		5 mL of glycerol added to the pseudomonas isolation agar prior to autoclaving
	Pseudomonas CN Selective supplement vial	Centrimide: 0.2 Sodium nalidixate: 0.015	Contents of 1 CN supplement vial rehydrated with 1 mL of sterile distilled water and 1 mL of ethanol Once the agar with glycerol had been autoclaved, the contents of 1 rehydrated vial was added and the bottle mixed

Skimmed Milk Agar	Nutrient Agar	Lab-Lemco' powder: 1.0 Yeast extract: 2.0 Peptone: 5.0 Sodium Chloride: 5.0 Agar: 15.0	25.2 g of nutrient agar powder dissolved in 900 mL of distilled water
	Instant dried skimmed milk powder		20.0 g of dried skimmed milk dissolved in 100 mL of distilled water and autoclaved separately Once both solution had been autoclaved and cooled to 50 °C, 100 mL of the sterilised skimmed milk solution was added to the 900 mL of nutrient agar and mixed
Swim Agar	Agar powder Potassium nitrate 99+%		Dissolve 1.5 g agar into 500 mL of distilled water Add 5 g of potassium nitrate into the agar solution prior to autoclaving
Swarm Agar	Agar powder Nutrient broth Glucose Potassium nitrate 99+%		Add 2.5 g agar into 500 mL of distilled water Add 4 g of nutrient broth to the agar solution Add 2.5 g of glucose to the agar solution Add 5 g of nitrate to the solution prior to autoclaving

All broth and solid agar granules/powders were autoclaved at 121 °C for 15 min to facilitate sterilisation.

2.3.3 Cultivation in broth

Single colonies from pure culture plates of *P. aeruginosa* or *S. aureus* were added to 10 mL of LB broth in sterile universals and incubated at 37 °C, 5% CO_2 for 16 h under static conditions. Other broth routinely used in this thesis included LB broth supplemented with 1% (w/v) potassium nitrate (LBN), to facilitate the anaerobic growth of *P. aeruginosa. E. coli* biosensor pSB536 was cultured in 10 mL of LB broth supplemented with 50 µg/mL ampicillin, whilst *E. coli* biosensor pSB1142 was grown in 10 mL of LB broth supplemented with 10 µg/mL tetracycline. Both bioreporter cultures were incubated at 37 °C for 16 h under static conditions.

For growth under anoxia, cultures were incubated under static conditions at 37 °C for 16 h in an anaerobic chamber containing 10% H_2, 10% CO_2, and 80% N_2. *P. aeruginosa* PAO1 was routinely streaked across the surface of a LB agar plate and used as a control. No growth on this plate demonstrated that the chamber was anaerobic.

2.3.4 Storage of isolates

Single colonies from a pure culture of *P. aeruginosa, S. aureus* or *E. coli* biosensor were grown in 10 mL of LB broth 37 °C and 5% CO_2 for 16 h under static conditions. Each overnight culture was vortexed for 20 s prior to 500 µL being transferred into a sterile 1.5 mL cryovial. 500 µL of sterile 50% (v/v) glycerol/water was subsequently added to the cryovial and the lid sealed. Vials were inverted 2-3 times prior to being stored at −80 °C.

2.3.5 Isolate resuscitation from frozen glycerol stocks

All bacterial strains were kept at −80 °C in 1.5 mL sterile cryovials. To resuscitate bacterial strains, frozen stocks were partially defrosted and a sterile inoculating loop was immersed into the glycerol-bacteria mixture and streaked across the surface of LB agar. Plates were incubated at 37 °C, 5% CO_2 for 48 h. This technique is illustrated in Figure 12.

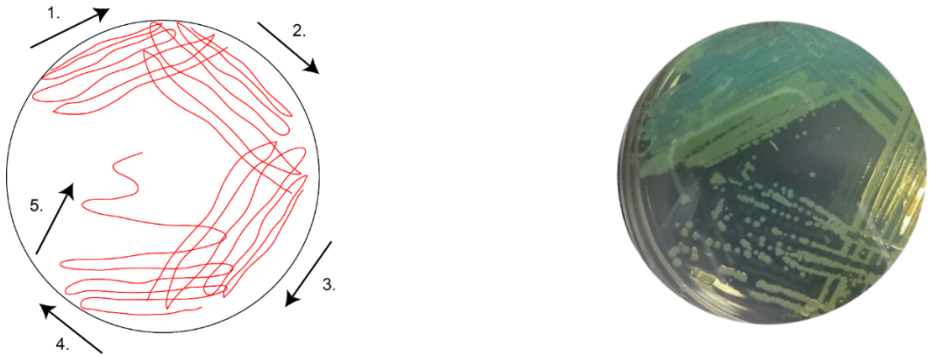


Figure 12. Quadrant streak plate method. The aim of this procedure is to isolate single bacterial colonies. A sterile inoculating loop containing a bacterial inoculum is spread across the area of quadrant one, from the edge of the plate, working inwards. Using one side of a new inoculating loop, the bacteria is streaked across the second quadrant of the plate in a zig-zag pattern, working from the outer edge inward. This process is repeated for quadrants 3, 4 and 5, each using a different side of the loop. Whilst quadrant 1 should have a dense bacterial inoculum following incubation, quadrant 5 should display scanty growth with well isolated single bacterial colonies (which are not touching).

2.3.6 Confirmation of isolate identity

Single colonies of *P. aeruginosa* PAO1 or CF clinical isolates grown on LB agar were streaked onto PIA plates containing Cetrimide, Nalidixic Acid (CN) supplement to confirm species identity. Plates were incubated at 37 °C, 5% CO_2 for 48 h prior to being examined. All *P. aeruginosa* isolates were identified by their growth upon PIA, a positive oxidase test, by their colony morphology and appearance as Gram-negative rods following Gram-staining.

A single colony of *S. aureus* following growth on LB agar was streaked across the surface of MSA plates. Plates were incubated at 37 °C, 5% CO_2 for 48 h. *S. aureus* was identified by growing as yellow colonies on MSA, surrounded by yellow zones. The ability to ferment mannitol causes the phenol red pH indicator in the agar to turn yellow. *S. aureus* grew as golden colonies upon LB agar, was oxidase-negative and appeared as Gram-positive cocci following Gram staining.

2.3.7 Standardisation of bacterial cultures

All assays were carried out using a standardised bacterial inoculum. Colonies of *S. aureus* or *P. aeruginosa* were inoculated into 10 mL of sterile LBN broth and were grown statically for 16 h at 37 °C under normoxia or anoxia. Bacterial cultures were subsequently centrifuged at 4,000 x *g* for 10 min at 4 °C to pellet the cells, before decanting the supernatant and resuspending the bacterial pellet in sterile LBN broth to an OD_{470} of 1.0 using a spectrophotometer, corresponding to approximately $1x10^8$ cells/mL. Bacteria were subsequently diluted accordingly to the desired OD for a given assay using sterile LBN broth. *E. coli* biosensor

strains were also standardised, however antibiotic supplemented LB broth was used (as mentioned in 2.3.3) and the cultures were grown under normoxia only.

2.3.8 Mucoid status

Single colonies of *P. aeruginosa* were spread onto the surface of PIA plates. Mucoidy status was determined by a slimy appearance of bacterial colonies following 48 h incubation at 37 °C and 5% CO_2.

2.3.9 Pyocyanin status

Single colonies of *P. aeruginosa* were cross-streaked across the surface of PIA plates, which enhances the production of pyocyanin due to the presence of magnesium chloride and potassium sulphate. Pyocyanin determination was assessed by visual inspection of the plates following a 48 h incubation at 37 °C and 5% CO_2. Pyocyanin-positive isolates exhibited a characteristic green pigmentation which diffuses into the growth medium surrounding the bacterial colonies.

2.3.10 Planktonic growth curves

Normalised overnight cultures of *P. aeruginosa* (OD_{470} of 1.0) grown statically under normoxia were diluted 1:10 and 100 µL was added to each well of a sterile 96-well plate. Plates were incubated at 37 °C, where growth kinetics were followed by measuring the OD_{470} at hourly intervals for 15 h.

2.3.11 Preparation of *S. aureus* and *P. aeruginosa* cell-free culture supernatants

Overnight cultures of *S. aureus* or *P. aeruginosa* grown under normoxia or anoxia in LBN broth were centrifuged at 4,000 x *g* for 10 min at 4 °C. Each supernatant was sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter and stored at −20 °C until use, with no more than one freeze-thaw cycle. To confirm sterility after each preparation, a small volume of the supernatant was streaked out onto a LB agar plate and incubated for approximately 18 h prior to reading.

For size exclusion experiments, 5 mL of the cell-free supernatant was added to a 3 kDa molecular weight cut off protein concentrator and centrifuged at 4,000 x *g* for 1 h. Apical and basal volumes were subsequently added to 2.0 mL sterile microcentrifuge tubes. Aliquots were either immediately used or stored at −20 °C. Where indicated, cell-free bacterial supernatants were placed in a heat block at 95 °C for 10 min, to heat-inactivate proteases and subsequently minimise airway epithelial cell cytotoxicity.

2.3.12 Skim milk agar protease activity

Total protease production and activity was determined using skimmed milk agar. 40 µL of cell-free supernatants from overnight cultures was loaded into wells in the agar plates and incubated at 37 °C for 24 h. Hydrolysis of the milk protein casein results in a clear zone surrounding the bacterial supernatant was evidence of protease production. The diameter of the clearance zones were measured in millimetres (mm) from the edge of the well. LBN broth alone was added to wells as the negative control. The protease activity of each sample was normalised to the densities (OD_{470}) of the cultures.

2.3.13 Staphylolytic activity

This method was adapted from (Kong *et al.*, 2005, Grande *et al.*, 2007) using *S. aureus* as a substrate for this assay. An overnight culture of *S. aureus* grown under static normoxia conditions was centrifuged at 4,000 x *g* for 10 min at 4 °C, prior to the pellet being resuspended in 250 µL of 25 mM diethanolamine buffer, pH 9.5. *S. aureus* was heated at 100 °C for 10 min and once cooled, were diluted to a final optimal density OD_{595} of 1.0. 400 µL of the adjusted heat-killed *S. aureus* were then added to each microtube. The cell-free supernatant from each *P. aeruginosa* isolate was diluted 1:10 with 25 mM diethanolamine buffer, prior to 100 µL being added to the heat-killed *S. aureus.* Staphylolytic activity was determined by measuring in the OD_{595} of heat-killed *S. aureus* after 60 min on a plate reader- where a decrease in OD indicates cell lysis. LBN broth alone was used as the negative control.

2.3.14 Quorum sensing

AHL production was determined using two *E. coli* biosensor strains, pSB536 and pSB1142, kindly provided by Professor Paul Williams (University of Nottingham). pSB536 was grown routinely in LB broth supplemented with 50 µg/mL ampicillin, whilst pSB1142 was grown in LB broth supplemented with 10 µg/mL tetracycline. Overnight cultures of each biosensor strain were normalised to an OD_{470} of 1.0 and diluted 1:100 into a sterile 96-well plate. 100 µL of cell-free supernatants from 24 h cultures of PAO1 or the CF isolates were added to the biosensor strain. LBN medium was added as the negative control. Plates were sealed with a Breathe-easy® membrane to facilitate gaseous exchange and minimise culture evaporation and incubated for 6 h at 37 °C. 100 µL aliquots were subsequently transferred to a white 96-well plate and the luminescence read, with luminescence values divided by the OD_{470} of the biosensor strain, to take into account differences in growth rates and final biosensor densities. Luminescence values were subsequently subtracted from the negative control (LBN broth only), to correct for background luminescence values.

2.3.15 Pyoverdine production in mono-culture

Overnight cultures of *P. aeruginosa* were centrifuged at 4,000 x *g* for 10 min and the supernatants passed through low-binding 0.22 µm polyethersulfone membrane filter. To measure pyoverdine production, 100 µL of the cell-free supernatant was added to a black 96-well plate and read at excitation and emission wavelengths $400/460_{nm}$ as performed previously (Andersen *et al.*, 2015, Krzyzanowska *et al.*, 2016) on Spectramax fluorescence plate reader. The background level of fluorescence was measured using 100 µL of LBN broth only. To take into account differences in final bacterial density, RFU values were normalised to the OD_{470} of each bacterial culture.

2.3.16 Pyoverdine production in mono-culture versus co-culture with *S. aureus*

Overnight cultures of *P. aeruginosa* and *S. aureus* grown under normoxia or anoxia were normalised to an OD_{470} of 1.0. 500 µL of each culture were then added to a 250 mL conical flask containing 50 mL of LBN broth (1:100 dilution). The flasks were incubated at 37 °C for 24 h, under static normoxia or anoxia. To measure pyoverdine production, 100 µL of the cell-free supernatant was added to a black 96-well plate and the fluorescence read at excitation and emission wavelengths $400/460_{nm}$, respectively. The background level of fluorescence was measured using 100 µL LBN broth only. To take into account differences in final bacterial density, fluorescence values were normalised to the OD_{470} of each bacterial culture.

2.3.17 Pyocyanin extraction and quantification in single and co-culture

Overnight cultures of *P. aeruginosa* and *S. aureus* grown separately under normoxia or anoxia were pelleted, resuspended in fresh medium and adjusted to an OD_{470} of 1.0. For single cultures, 500 µL of *S. aureus* or *P. aeruginosa* were added to a 250 mL conical flask containing 49.5 mL of LBN broth (1:100 dilution). For co-cultures, 500 µL of *S. aureus* and *P. aeruginosa* were then added in a 1:1 ratio, to a 250 mL conical flask containing 49 mL of LBN broth. The flasks were incubated at 37 °C for 24 h, under static normoxia or anoxia. After this period, samples were taken and serially diluted in 1x and plated onto PIA to determine the Log_{10} CFU/mL.

To quantify pyocyanin production, bacterial cells were pelleted by centrifugation at 4,000 x *g* for 25 min at 4 °C and the supernatant sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter. 7.5 mL of the sterile supernatant was added to 4.5 mL of chloroform and vortexed for ten, 2 sec intervals. The sample was centrifuged for 4,000 x *g* for 1 min at 4 °C, prior to 3 mL of the blue-green phase (chloroform phase) being aspirated into a new tube. 1.5 mL of 0.2 M hydrochloric acid was then added to the tube and vortexed again for ten, 2 second intervals, prior to centrifugation at 4,000 x *g* for 1 min at 4 °C. 100 µL of the

pink coloured phase was then transferred into a 96-well plate. 100 µL of the hydrochloric acid was added in triplicate as the control. The plate was then read at OD_{520} and multiplied by the extinction co-efficient 17.072 to determine the concentration of pyocyanin per mL of supernatant. This method was adapted from (Essar *et al.*, 1990, Wu *et al.*, 2014).

2.3.18 Drop collapse assay

Cell-free supernatants from overnight cultures of *P. aeruginosa* were serially diluted (1:1) in sterile distilled water containing 0.0005% (w/v) crystal violet for visualisation across a 96-well plate. A total of 20 µL of each dilution (including neat supernatant) was spotted onto the underside of a lid of a 96-well plate and the plate titled at a 90 ° angle. The assay works on the principle that if the droplet contains surfactants, the drops spread. However, as the quantity of surfactants decrease by dilution, the droplet eventually beads up due to an increase in surface tension. Surfactant scores are equal to the reciprocal of the greatest dilution at which there was surfactant activity (a collapsed drop). This method was performed as previously described (Price *et al.*, 2016, Deziel *et al.*, 2001).

2.3.19 Minimum Inhibitory Concentration (MIC)

Antibiotic efficacy was tested using tobramycin, amikacin and ciprofloxacin planktonic cultures of *P. aeruginosa* grown under normoxia and anoxia, using the microbroth dilution method. Overnight cultures of *P. aeruginosa* were diluted to an OD_{470} 0.5. 100 µL of each culture was added to wells of a sterile 96-well tissue culture treated plate. Each antibiotic was serially diluted in LBN broth: tobramycin (64-0.03 µg/mL), amikacin (256-0.15 µg/mL) and ciprofloxacin (32-0.002 µg/mL) and 100 µL added to each culture. Plates were sealed with a Breathe-easy® membrane and incubated for 24 h under normoxia or anoxia prior to being read. The MIC was determined as the lowest concentration of antibiotic which prevented visual bacterial turbidity.

2.3.20 Biofilm biomass determination using crystal violet staining

Normalised cultures of *P. aeruginosa* (OD_{470} of 1.0) grown under normoxia or anoxia were subsequently diluted tenfold, prior to 200 µL of each strain being added to a sterile 96-well tissue culture treated plate. The plate was sealed with a Breathe-easy® membrane, prior to it being incubated under static normoxia, at 37 °C for 24 h. To take into account differences in final bacterial densities, 100 µL of the planktonic fraction was added to a new 96-well plate and read at OD_{470}.

The remaining planktonic media in the original plate was decanted and the wells washed twice using sterile 1x PBS with 200 µL being loaded into each well. After allowing the inverted plate to dry overnight, 200 µL of 1% (w/v) crystal violet was added into each well for 10 min, prior to two further washes in a bucket of distilled water. Plates were dried overnight, prior to the stain being solubilised with 200 µL of 30% (v/v) acetic acid. The solubilised stain was then

transferred to a new 96-well plate and the density read at OD_{492}. The OD of the crystal violet staining was subsequently normalised to the bacterial density (OD_{470}) of each isolate.

2.3.21 *P. aeruginosa* swimming and swarming motility

Swimming motility of *P. aeruginosa* was investigated using 0.3% (w/v) nutrient agar plates supplemented with nutrient broth and 1% (w/v) potassium nitrate. Swarming of *P. aeruginosa* was determined using 0.5 % (w/v) of nutrient agar plates supplemented with nutrient broth, dextrose and 1% (w/v) nitrate. Overnight cultures of *P. aeruginosa* grown under normoxia or anoxia were adjusted to OD_{470} of 1.0 and a total of 5 µL of culture was added to the centre of each plate. To measure the effects of *S. aureus* on *P. aeruginosa* motility, a 1:100 diluted *S. aureus* cell-free culture supernatant (obtained from *S. aureus* grown under normoxia or anoxia) was added to the agar plate. Plates were incubated under static normoxia or anoxia for 24 h at 37 °C. The diameter of the zone travelled by *P. aeruginosa* was measured in mm.

2.3.22 Bacterial interaction on solid agar

Overnight cultures of *P. aeruginosa* and *S. aureus* grown under normoxia or anoxia were pelleted, resuspended in fresh broth and adjusted to an OD_{470} of 1.0. A sterile cotton swab was inoculated into a given *P. aeruginosa* normalised culture and streaked horizontally across the surface of an LB agar plate containing 1% (w/v) potassium nitrate. After air drying for 20 min, a sterile cotton swab was immersed into the normalised *S. aureus* culture and cross-streaked vertically across the surface of the agar. Plates were incubated either under normoxia or anoxia at 37 °C for 18 h, prior to being visually inspected for growth inhibition.

2.3.23 Planktonic mono- and co-culture

All growth curve experiments were conducted in 250 mL conical flasks containing 50 mL of LBN broth at 37 °C under static conditions. Overnight cultures of *S. aureus* and three of the *P. aeruginosa* CF isolates grown under normoxia or anoxia were pelleted, resuspended in fresh medium and adjusted to an OD_{470} of 1.0. For co-culture growth curves, the bacteria were inoculated at an equal ratio (1:1 *S. aureus*: *P. aeruginosa*) and incubated under static conditions at 37 °C for 24 h. Samples were taken at regular intervals, serially diluted in 1x PBS and 20 µL spots plated onto PIA and MSA, to allow differentiation between the two species. The plates were incubated for 18 h at 37°C and 5% CO_2, prior to enumerating the colony forming units (CFU/mL).

The competition index (CI) and Relative Increase Ratio (RIR) were calculated. The RIR was calculated on the single growth curve data using the *P. aeruginosa-S. aureus* ratio at a given time point, divided by the same ratio at time point 0 h (inoculum). The same ratio was used to calculate the CI, although this used data from the mixed culture. A CI that differs statistically

from the RIR indicates competition between the two organisms. This method was adapted from (Losa *et al.*, 2014).

2.3.24 Interaction in mixed species biofilms

Overnight cultures of *S. aureus* and *P. aeruginosa* grown under normoxia or anoxia were centrifuged and adjusted to OD_{470} 1.0. Cultures were diluted tenfold and 100 µL added to wells of a sterile 96-well tissue culture treated flat bottom plate either individually or in a 1:1 ratio for an hour under static conditions, at 37 °C. An equal volume of broth was added to the individual culture to compensate for any dilution effect. After 60 min, the well contents were aspirated and replaced with fresh LBN broth. Plates were incubated for a further 24 h at 37 °C under static conditions. Following this, biofilms were washed twice using 200 µL of 1x PBS, detached using 100 µL of trypsin-EDTA (0.25%), collected, vortexed for 70 sec, serially diluted and plated onto PIA and MSA. The plates were incubated for 18 h, prior to the enumerating the colony forming units (CFU/mL). Biofilm biomass was determined as described in 2.3.20.

2.3.25 *S. aureus* biofilm inhibition and disruption

S. aureus was grown statically under normoxia or anoxia overnight, prior to being diluted to an OD_{470} of 1.0. The culture was diluted tenfold and a total of 100µL was added into wells of a sterile 96-well plate. For the biofilm inhibition experiment 100µL of *P. aeruginosa* cell-free supernatants (following culture under normoxia or anoxia) were added to the wells, with 100µL of LBN broth being added to *S. aureus* as the negative control. The plates were sealed with a Breathe-easy® membrane and incubated statically at 37 °C for 24 h under normoxia or anoxia.

For the biofilm disruption experiment, 100µL of *P. aeruginosa* cell-free supernatants (following culture under normoxia or anoxia) were added to the plate following its 24 h incubation. The supernatants were added for 5 h, with 100µL of LBN broth being added to *S. aureus* as the negative control. In both experiments, biofilm production was visualised by crystal violet staining as described previously.

2.4 Mass Spectrometry

2.4.1 Protein gel electrophoresis solutions

25% (w/v) Trichloroacetic acid (TCA) dissolved in distilled water

Protein pellet solubilising solution: 50 mM pH 7.4 Tris-HCl containing 2 mM CHAPS, 7 M urea and 7 M thiourea

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Running buffer (10x): 30 g Tris, 144 g Glycine and 10 g SDS in 1 L of distilled water, pH 8.

Coomassie blue stain: 0.5% Coomassie blue stain in 40% aqueous methanol with 5% glacial acetic acid

Destaining solution: 10% ethanol and 7.5% glacial acetic acid

2.4.2 Solubilisation of secreted proteins

Five cultures for each *P. aeruginosa* isolate were set up and incubated at 37 °C under either normoxia or anoxia for 24 h. Following this, the culture was centrifuged at 4,000 x *g* for 30 min, prior to being filtered through a 0.22 µm filter to remove contaminating bacterial cells. Supernatants were concentrated using 3kDa cut-off filters prior to precipitation with 25% (w/v) TCA for 15 min on ice. Proteins were pelleted at 14,000 x *g* for 10 min, and pellets washed with acetone. Protein pellets were solubilised in 2 mM CHAPS, 7 M urea and 7 M thiourea in 50 mM pH 7.4 Tris-HCl containing using an ultrasonicating probe (30 s sonication per cycle, 65% full power, 2 cycles). Finally, solubilised proteins were collected after centrifugation at 14000 x *g* for 10 min and remaining pellets, if present, were discarded. Protein concentration was estimated against BSA calibration curve in the Bradford assay.

2.4.3 SDS PAGE

After quantifying the protein concentrations of the bacterial cell-free culture supernatants, SDS-PAGE was used to separate the secretome. SDS was used to denature the proteins and provide a net-negative charge, facilitating protein separation based on molecular mass, rather than mass-to-charge ratio. SDS-PAGE was performed on a 10 cm long 10% polyacrylamide resolving gel and 4% stacking gel as detailed in Table 5 below.

Table 5. Materials for casting SDS-polyacrylamide gels to separate *P. aeruginosa* cell-free secretomes.

10% Resolving Gel	**3 gels**
Distilled water	11.63 mL
40% Acrylamide:bis-acrylamide (37.5:1)	6.38 mL
1.5 M Tris pH 8.8	6.5 mL
10% SDS	0.25 mL
10% APS	0.25 mL
TEMED	25 µL
4% Stacking Gel	**3 gels**
Distilled water	7.86 mL
40% Acrylamide (37.5:1)	1.25 mL
0.5 M Tris pH 6.8	3.125 mL
10% SDS	0.125 mL
10% APS	0.125 mL
TEMED	12.5 µL

The resolving gel was left to polymerise for 30 min with isopropanol added to the top of the gel to prevent oxygen inhibiting polymerisation. When polymerisation was complete, the isopropanol was removed and washed off with distilled water. The stacking gel was then added onto the top of the resolving gel. After allowing the stacking gel to polymerise for an additional 20 min, the gel and plates were placed into the gel tank and immersed in 1 x SDS running buffer.

2.4.4 Sample preparation

Samples (30 µg where applicable) were reduced in 5x Laemmli buffer and heated at 65 °C for 15 min. Well combs were removed from the stacking gel and the wells loaded as follows: 1 x SDS-PAGE running buffer and pre-stained protein ladder in lane one and the denatured bacterial secretome samples in the remaining wells. The gel was run at 120 V for 5 min allowing the samples to pass through the stacking gel, prior to increasing the voltage to 200 V for 45-55 min until the dye front reached the bottom of the resolving gel. The protein bands were visualised by staining (overnight at 4 °C) with Coomassie G250 blue (0.5% in 40% aqueous methanol with 5% glacial acetic acid) for 1 h. Following this, the gels were immersed in

destaining solution (10% ethanol and 7.5% glacial acetic acid) and kept in the destaining solution at 4 °C overnight, prior to in-gel digestion.

2.4.5 Protein in-gel digestion

Protein in-gel digestion was performed following the method of Schevchenko *et al.* (Shevchenko *et al.*, 2006), with minor modifications. Gels were placed onto a clean glass slide and each sample lane was divided into 5 bands (of approximately the same size) and the stained bands were excised and diced in a clean polypropylene tube using a sterile scalpel. Gel pieces were subsequently destained with 50% acetonitrile in 50 mM ammonium bicarbonate, dehydrated with pure acetonitrile until they turned opaque and vacuum dried for 30 min. Proteins were in-gel digested using MS sequencing grade trypsin (trypsin-to-protein ratio 1:25) in 3 mM ammonium bicarbonate, coupled with shaking at 550 rpm at 37 °C overnight. Peptides were extracted for 15 min in an ultrasonic bath initially using pure acetonitrile equivalent to 50% of sample volume. The whole extract was transferred into a new tube, leaving only gel pieces for a further extraction with 150 µL of 50% acetonitrile in 50 mM ammonium bicarbonate. The liquid extract was collected into a corresponding tube, and the last extraction step was repeated once more for 15 min. Finally, 400 µL of pure acetonitrile was used to fully dehydrate the gel pieces and maximise peptide extraction. Thus, complete peptide extracts from a single sample lane was collected in separate polypropylene tubes, vacuum dried and stored at −20 °C prior to analysis.

2.4.6 Mass Spectrometry Analysis

Samples were reconstituted in 50 µL of 3%-aqueous acetonitrile and 0.1% formic acid for liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) analysis. Peptides were separated and analysed using an nLC system (Dionex 3000, ThermoScientific, UK) coupled to 5600 TripleTof (AB Sciex, UK) operating in information dependent (IDA) acquisition mode. Peptide solution (10 µL) was injected onto a trap column (PepMapTM, C18, 5 µm, 100 Å, 300 µm x 1 mm, ThermoScientific, UK) using 2% of eluent B (98% acetonitrile in aqueous 0.1 % formic acid) at a flow rate of 30 µL/min. Peptides were subsequently separated on an analytical column (AcclaimTM, PepMapTM C18, 3 µm, 100 Å, 75 µm x 150 mm, ThermoScientific, UK) with the following gradient: 0-3 min 2% B, 3-48 min 2-45% B, 48-52 min 45-90% B, 52-55 min 90% B, 55-70 min 2% B) at a flow rate of 300 nL/min. Electrospray was formed by spraying the nLC eluate at 2500 V using a PicoTipTM emitter (New Objective, Germany). The 10 most intense ions from each MS survey scan were selected for MS/MS, while acquired ions were temporarily excluded from MS/MS acquisition for 30 s. The mass spectrometer was calibrated prior to acquisition to ensure a high mass accuracy (<10 ppm) on both MS and tandem mass spectrometry (MS/MS) levels.

2.4.7 Data analysis

Relative quantification was done using QI for proteomics software (version 4, Nonlinear Dynamics, UK). MS/MS data were searched using MasconDeamon (ver 2.5) against the SwissProt database, with the following search restriction parameters: mass tolerance of 0.1 Da for MS and 0.6 Da for MS/MS spectra, a maximum of 2 trypsin miscleavages, *Pseudomonas aeruginosa* taxonomy, variable modifications of methionine oxidation and cysteine carbamidomethylation.

2.5 Cell Culture

2.5.1 Cell Lines

IB3-1 bronchial epithelial cell line was used in this research to construct the CF airways. Isolated from a CF patient and immortalised using the adeno-12-SV40 virus in 1992 (Zeitlin *et al.*, 1991, Flotte *et al.*, 1993),this cell line is a heterozygote, containing a class II (Phe508del) and a class III mutation (W1282X). Cells are deficient in cyclic-AMP (cAMP) mediated protein kinase A activation of chloride conductance (Flotte *et al.*, 1993).

C38 bronchial epithelial cell line was used to construct non-CF airways. It is derived from the IB3-1 cell line, which has been corrected using wildtype adeno-associated viral CFTR (AAVCFTR). The cell line expresses functional CFTR (Zeitlin *et al.*, 1991, Flotte *et al.*, 1993).

MRC-5 pulmonary fibroblast cell line was used to construct the sub-epithelial fibroblast population in both the CF and non-CF airway models. The cell line is derived from a 14 week old Caucasian male foetus (Jacobs *et al.*, 1970).

2.5.2 Cell culture media

IB3-1 and C38 cell lines were routinely grown in DMEM:F12 (1:1 mix of Dulbecco Modified Essential Medium with Ham's-12; Lonza), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and 1x antibiotic-antimycotic.

MRC-5 were maintained in EMEM (Eagle's Minimum Essential Medium; Lonza) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine and 1x antibiotic-antimycotic.

Low serum medium: DMEM:F12 supplemented with 1% (v/v) heat-inactivated FBS and 1x antibiotic-antimycotic.

Infection medium: DMEM:F12 supplemented with 10% (v/v) heat-inactivated FBS without antibiotic-antimycotic.

Freezing medium for IB3-1 and C38: 7mL DMEM:F12, 2mL of heat-inactivated FBS and 1 mL of DMSO.

Freezing medium for MRC-5: 9.5 mL of EMEM and 0.5 mL of DMSO.

2.5.3 Culturing cells from frozen

Frozen cells contained within a cryovial were removed from liquid nitrogen storage and placed into a bead bath for 5 min at 37 °C with regular agitation to facilitate thawing. Once thawed, the cryovial was transferred into a Class II safety cabinet and the 1mL cell suspension was resuspended and added into a labelled 75 cm^2 tissue culture flask, drop by drop, containing 15 mL of pre-warmed cell culture medium and placed in the incubator at 37 °C and 5% CO_2 for 6 h to allow the cells to attach. After this period, the medium containing the cryopreservant dimethyl sulfoxide (DMSO) was removed and fresh medium was added, prior to placing the flask back into the incubator at 37 °C and 5% CO_2. MRC-5 were the only exception to this procedure, where they were grown in 10 mL of EMEM in 25 cm^2 tissue culture flasks. Cell culture medium was changed every 2-3 days until the cells reached 80-90% confluency.

2.5.4 Passaging of cells

Once cells had reached 80-90 % confluency, the culture medium was removed from each tissue culture flask and cells washed with 3 mL (T75) or 1 mL (T25) of 1x PBS without calcium/magnesium, to remove any traces of heat-inactivated FBS which would inactivate the trypsin. 3 mL (T75) or 1 mL (T25) of trypsin-EDTA (0.25%) was then added to the flask and the flasks returned to the incubator at 37 °C and 5% CO_2 for 5 min to detach adherent cells. After this period, 3 mL of cell culture medium was added to neutralise the trypsin and the flask gently tapped to aid cell detachment. The flask wall was washed 3-4 times to detach any remaining cells and to break up any cell clumps. The cell suspension was then added to a sterile 50 mL centrifuge tube and centrifuged at 1,000 x *g* for 5 min. Following this, the supernatant was discarded and the cell pellet was resuspended in fresh medium. For every T75 flask, the cell pellet was resuspended in 2 mL of fresh medium. For every T25 flask, the cell pellet was resuspended in 0.5 mL of fresh medium. A 1 mL pipette tip was used to aid cell resuspension and to break any cell clumps.

For routine passaging, an appropriate volume of the cell suspension (dependent upon the split ratio) was added to each flask containing pre-warmed cell culture medium. A T75 flask contained 15 mL of cell culture medium, whilst a T25 flask contained 10 mL of cell culture medium.

2.5.5 Cell counting using trypan blue exclusion

To enumerate the number of cells within the cell suspension and adjust the density to those required for an experiment, following trypsinisation, centrifugation and resuspension, 25 µL of the cell suspension was added to 75 µL 0.2% trypan-blue in a sterile microcentrifuge tube. Trypan blue allowed only viable cells (clear) to be counted, where dead permeable cells would

take up the diazo dye and appear blue under the microscope. 10 µL of the cell suspension was added to an improved Neubauers haemocytometer. Unstained cells were counted in each of the four quadrants and totalled together to give the overall cell count, which was then multiplied by 10^4 to give the final number in 'cells/ mL'. This was then used to adjust the cell density for the experiment required.

2.5.6 Cryopreservation of cells

Epithelial cells from one 75 cm^2 tissue culture flask at 80-90% confluence were trypsinised, centrifuged (as described in 2.5.4) and resuspended in 3 mL of the IB3-1/C38 freezing medium and 1 mL was added to three labelled cryovials. MRC-5 cells to be frozen from a 25 cm^2 were resuspended in 1 mL of the MRC-5 freezing medium and added to a single cryovial. Each cryovial was labelled with the cell line, date and passage number and subsequently placed into a 'Mr Frosty' containing 250 mL of isopropanol to facilitate gradual freezing. The Mr Frosty was placed in the −80 °C freezer for 24 h, prior to cells being transferred to liquid nitrogen for indefinite storage.

2.5.7 Photography of submerged cells

Light microscopy was used to visually inspect the effects of *P. aeruginosa* supernatants upon submerged cultures of IB3-1 and C38 cell lines. Images were taken at a x 20 magnification on an inverted light microscope.

2.5.8 IB3-1 and C38 stimulation with *S. aureus* and *P. aeruginosa* filtrates

Overnight cultures of *S. aureus* and *P. aeruginosa* grown under static conditions and normoxia at 37 °C were diluted 1:00 into 50 mL of LB broth in 250 mL conical flasks. The cultures were subsequently grown at 37 °C under static normoxia for 24 h. Following this, 1 mL each culture was added to a sterile 1 mL UV/vis disposable cuvette and read at OD_{495} to measure bacterial density. The 50 mL culture was subsequently centrifuged at 4,000 x *g* and 4 °C for 30 min to pellet the bacteria. Bacterial supernatants were passed through 0.22 µm sterile filters and the cell-free filtrates stored at −20 °C.

IB3-1 and C38 cells were trypsinised and counted. Each cell line was plated separately into sterile 24-well plates at a density of $2.5x10^5$ cells/mL, with a 1mL volume being added to each well. Following an overnight incubation at 37 °C and 5% CO_2, the cells were visually inspected for confluence, prior to aspirating the medium and replacing it with low serum DMEM-F12 (containing 1% (v/v) FBS). This change of medium helped to prevent serum-dependent MAPK activation (which could elevate the baseline secretion of inflammatory mediators and interfere with experimental readings), prior to stimulation with bacterial cell-free filtrates. The bacterial

filtrates were thawed at room temperature and heat treated (as previously described) to minimise airway epithelial cell toxicity. The sterile filtrates were added to each cell line at a 10% (v/v) concentration, with LB broth being added as a negative control. Plates were incubated for a further 24 h prior to the airway epithelial cell supernatants being removed and clarified following centrifugation at 13,000 x *g* for 10 min and stored at −20 °C until further analysis.

2.5.9 Detection of Interleukin-8 (IL-8), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) in clarified airway epithelial cell culture supernatants

The concentration of human IL-8, IL-6 and IL-10 in the cell culture supernatants of IB3-1 and C38 cells following bacterial challenge were determined using ELISA. All reagents were included in each ELISA kit unless otherwise stated and were prepared according to the manufacturer's instructions. ELISA plates were coated with the capture antibody and sealed with multiwell plate sealing films, prior to being incubated at 4 °C overnight. The following day, the plates were washed three times (300 µL/well) with ELISA wash; 1x PBS with 0.05% (v/v)Tween®-20. The plates were then blocked for an hour with 200 µL 1x diluent and incubated at 25 °C for 1 h.

The ELISA standards (IL-8, IL-6 or IL-10) were reconstituted according to the manufacturer's protocol and added to the wells in duplicate (100 µL/well). 100 µL/well of each sample was also added to the appropriate wells on the plate in triplicate. The plate was subsequently incubated for 2 h at 25 °C. The wells were then washed three times, prior to 100 µL of detection antibody being added to each well for a further 1 h. After decanting the excess antibody, 100 µL avidin-HRP enzyme solution was added to each well and incubated at 37 °C for 30 min at 25 °C, prior to the plate being washed five times with wash buffer. 100 µL of TMB solution was then added to each well and the plate incubated for 15 min at room temperature in the dark. Following this, 50 µL of 2M H_2SO_4 was added to each well to stop the reaction. The absorbance of each well was then read on a plate reader at OD 490_{nm}. The IL-8, IL-6 and IL-10 concentrations in each sample were determined using the standard curve.

2.5.10 Human Placental Collagen Type IV

Human placental collagen type IV was dissolved in sterile filtered 3% (v/v) acetic acid to a stock concentration of 1 mg/mL and regularly agitated for 15 min to ensure it was fully dissolved. Transwell® inserts were coated at a working concentration of 10 µg/cm^2 and incubated at room temperature for 1 h within a Class II safety cabinet. After this period, excess collagen was removed and the transwells® washed in sterile DMEM:F12 cell culture medium

three times to neutralise the acidic pH. If they were not to be used immediately, collagen coated transwell® inserts were stored at 4 °C for up to a month.

2.5.11 Cell culture on Transwell® Inserts

Sterile cell culture transwell® inserts had a polyethylene terephthalate (PET) membrane, with a growth area of 0.3 cm^2 and pore size of 0.4 µm. A total of 6 transwells® were added to the central wells of a 24-well transwell® companion plate using sterile forceps. Each transwell® was then coated with human placental collagen type IV as described in 2.5.10.

Transwell® inserts were seeded apically with fibroblasts at a cell density of $3x10^4$ cells/well in a total volume of 300 µL of EMEM medium. A total of 600 µL of EMEM was added to the basolateral compartment. Four days later, apical medium was removed from the transwell® and IB3-1 or C38 cells were seeded on top of the fibroblasts at $5x10^4$ cells/well in DMEM:F12 medium. The basolateral medium was also removed and replaced with DMEM:F12. The co-cultures were then left under submerged conditions for four days, allowing the epithelial cells to form confluent monolayers on top of the fibroblasts. On day 4, the apical cell culture medium was removed and not refreshed, introducing the cells to an ALI and inducing cell differentiation. Medium in the basolateral compartment was refreshed every 3-4 days for a minimum of 14 days from the introduction of ALI.

2.5.12 Assessment of cell viability – CellTiter-Blue®

CellTiter-Blue® (CTB) is an endpoint assay based on fluorometry, which monitors cell viability. Metabolically active viable cells are able to convert resazurin (blue) reagent to its highly fluorescent product resorufin (pink). CTB was added to the cell culture medium using 20 µL for each 100 µL of cell culture medium. The reagent was incubated for 2 h at 37 °C (as per manufacturer's instructions). After incubation, supernatants were removed to a 96-well plate and the fluorescence read on a fluorescent plate reader using Ex560 and $Em590_{nm}$ wavelengths.

2.5.13 Bacterial adhesion to IB31- and C38 submerged monolayers

IB3-1 and C38 cells were plated separately into sterile 24-well plates at a density of $1.5x10^5$ cells/mL, with a 1 mL being added to each well. Cells were grown to confluence overnight in antibiotic-free DMEM-F12 media, at 37 °C and 5% CO_2. An overnight culture of *S. aureus* or *P. aeruginosa* grown statically at 37°C and 5% CO_2 was pelleted following centrifugation at 4,000 x *g* for 10 min and adjusted to an OD_{470} of 1.0 using infection medium. The standardised culture was further diluted in infection medium to a multiplicity of infection (MOI) of 5 (5 bacteria to 1 epithelial cell). The of bacterial suspension in a total of 1 mL of infection medium was added to each well. The plates were incubated for 2 h at 37 °C prior to the bacterial suspension being aspirated and each well being washed twice with 1 mL of 1x PBS to remove any non-

adherent bacteria. 100 µL of 0.25 % trypsin-EDTA was then added to detach the monolayer and left to incubate for 15 min at 37 °C and 5% CO_2. 900 µL of infection media was added to inactivate the trypsin. A 100 µL aliquot was taken from each well and serially diluted in sterile 1x PBS. A 20 µL aliquot of each dilution was plated onto LB agar and left to incubate for 24 h at 37 °C. The following day, the dilution containing between 2-20 colonies was counted and multiplied up to give the CFU/mL for bacterial adhesion.

2.5.14 Bacterial adhesion to IB3-1 and C38 ALI co-culture transwells®

24 h prior to infection, transwell® inserts grown at ALI were transferred to a new sterile companion plate containing 600 µL of infection medium within the basolateral compartment of each well. The apical cell surface was washed once with 100 µL of 1x PBS. Prior to infecting the transwells®, the basolateral medium was refreshed again with pre-warmed infection medium. Any apical medium was removed.

Overnight cultures of *S. aureus* and/or *P. aeruginosa* grown under static conditions at 37 °C and 5% CO_2 were centrifuged at 4,000 x *g* for 15 min at 4 °C and the pellet washed in 10 mL of sterile 1x PBS. This process was repeated three times. After the final wash, the bacterial pellet was resuspended in infection medium and adjusted to OD_{470} of 1.0. This inoculum was subsequently diluted to give a final MOI of approximately 10. 100 µL of the infection inoculum was added apically to each transwell® and incubated for 2 h at 37 °C and 5% CO_2. Following this, the apical bacterial inoculum was aspirated and the wells washed twice with 200 µL of 1x PBS to remove any non-adherent bacteria. For co-infections, the second bacterial species (*S. aureus* or *P. aeruginosa*) was then added and the plate incubated for a further 2 h at 37 °C and 5% CO_2.

After washing the transwells® once in 1x PBS following mono- or co-infection, 200 µL of ice-cold 0.25% (v/v) Triton X-100 was added to each apical well to lyse the airway epithelia-fibroblast co-cultures. The companion plates were left on ice for 30 min. 100 µL of the lysed suspension was then vortexed for 2 min, serially diluted (1:10) in 1x PBS and 20 µl inoculated onto the surface of MSA or PIA. Plates were incubated at 37 °C for 18-20 h, prior to the CFU/mL being enumerated.

2.6 Statistical Analysis

All results unless otherwise specified are expressed as mean ±S.E.M., with data for each experiment being collected from three independent repeats (N=3), each replicate performed in triplicate. All statistical analyses were performed using GraphPad Prism 6 software (Graphpad, La Jolla, CA, USA) with significance being set to P<0.05. The specific statistical tests and *post-hoc* tests used for each experiment are described in the figure legends.

3 Phenotypic characterisation of *P. aeruginosa* CF clinical isolates

3.1 Introduction

Initial infection of CF airways by a single environmental strain of *P. aeruginosa* typically occurs during early infancy (Johansen and Hoiby, 1992, Burns *et al.*, 2001). Whilst aggressive antibiotic regimens serve to eradicate the low numbers of bacteria present within the lungs, reservoirs of *P. aeruginosa* within the upper respiratory tract or acquisition of a different environmental strain lead to reinfection (Mainz *et al.*, 2012, Hansen *et al.*, 2012). Thus, infections are typically intermittent in nature, occurring over many months and years (Jelsbak *et al.*, 2007). Despite this inability to completely eradicate *P. aeruginosa,* the administration of narrow spectrum antibiotics to treat *P. aeruginosa* is effective in preventing the development of chronic *P. aeruginosa* infections during childhood (Hansen *et al.*, 2008).

As discussed in the introduction, *P. aeruginosa* possesses an arsenal of cell-associated and secreted virulence factors which facilitate CF airway colonisation, including flagella, pyocyanin and proteases. Whilst flagella facilitate swimming motility and pyocyanin induces host cell apoptosis and impairs mucociliary clearance (Usher *et al.*, 2002, Kanthakumar *et al.*, 1993), proteases degrade components of host immunity (Doring *et al.*, 1985, Mariencheck *et al.*, 2003, Horvat and Parmely, 1988, Kharazmi *et al.*, 1984), and the extracellular matrix (ECM) (Heck *et al.*, 1986). Secretion of these virulence factors is governed by QS, predominantly composed of the Las and the RhI systems (Van Delden and Iglewski, 1998) and their respective signal molecules, 3-oxo-C_{12}-HSL and C_4-HSL. Whereas the las system regulates the rhI system (Pesci *et al.*, 1997), it can also be activated independently by a third QS pathway, PQS (Diggle *et al.*, 2003). Mutations in QS genes are associated with a reduction in bacterial virulence in lung and wound infections (Pearson *et al.*, 2000, Wu *et al.*, 2001, Rumbaugh *et al.*, 1999).

Whilst variations in *P. aeruginosa* colony morphology are detected as early as within the first five years of life (Agarwal *et al.*, 2002, Agarwal *et al.*, 2005), most *P. aeruginosa* CF isolates during early infection continue to display a classic environmental phenotype, where isolates are non-mucoid and exhibit a susceptibility to anti-pseudomonal antibiotics (Burns *et al.*, 2001). Longitudinal analysis during the course of infection however, has demonstrated that isolates naturally accumulate several genetic aberrations overtime, including point mutations, chromosomal translocations and rearrangements within their genome (Darch *et al.*, 2015, Rodriguez-Rojas *et al.*, 2009, Marvig *et al.*, 2013, Folkesson *et al.*, 2012). Smith *et al.* (2006) reported 68 mutations in a *P. aeruginosa* CF isolate during 8 years of infection (Smith *et al.*,

2006a). *P. aeruginosa* CF isolates have also exhibited reductions in the size of their genome during adaptation to the CF lung through deletion of large genomic regions and limited DNA uptake (Rau *et al.*, 2012). Coupled with environmental pressures such as those exerted by antibiotics, extensive 'within-lung' and 'across-lung' variation in the *P. aeruginosa* phenotype is commonplace (Clark *et al.*, 2015a, O'Brien *et al.*, 2017, Mariencheck *et al.*, 2003, Williams *et al.*, 2015). During the course of chronic infection, *P. aeruginosa* CF isolates typically exhibit losses in virulence factors associated with acute infection, such as bacterial motility (Mahenthiralingam *et al.*, 1994), pyocyanin, pyoverdine and protease production (Smith *et al.*, 2006a), in favour of becoming resistant to antibiotics and overproducing the polysaccharide alginate (Govan and Deretic, 1996, Fothergill *et al.*, 2010). This adaptation is illustrated in Figure 13.

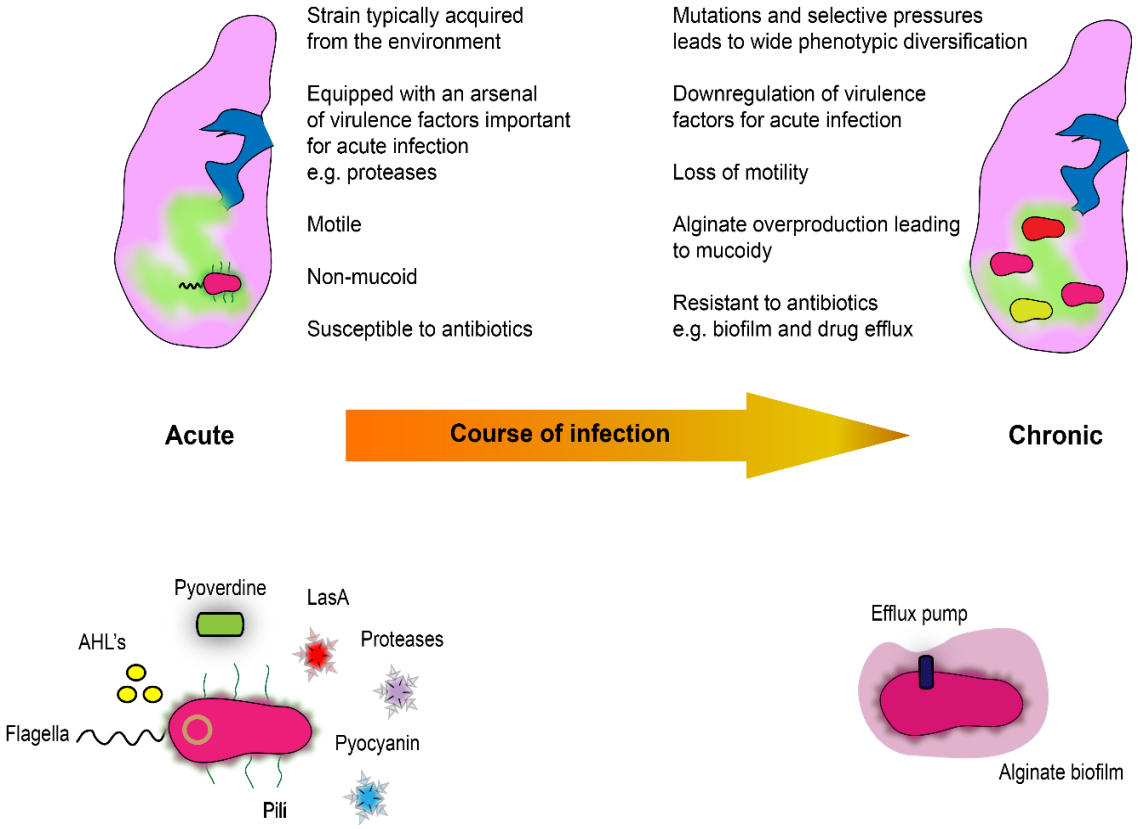


Figure 13. *P. aeruginosa* adapts to the CF lung environment during chronic infection. During early infection of CF airways, *P. aeruginosa* (shown in pink) expresses a potent arsenal of cell-associated and secreted virulence factors which facilitate airway colonisation. This includes surface pili for adhesion, flagella-mediated swimming motility and the secretion of pyocyanin and proteases. Over time, *P. aeruginosa* accumulates genetic mutations which under selective pressures and geographical isolation within the airways leads to phenotypic diversification. Chronically infecting CF isolates of *P. aeruginosa* (shown in pink, red and yellow) typically exhibit losses in virulence factor expression such as pyocyanin and proteases, are non-motile due to losses in flagella expression, secrete a thick alginate matrix and exhibit resistance to clinically relevant antibiotics due to the overexpression of drug efflux pumps. Adapted from (Sousa and Pereira, 2014).

Such changes are often reflected in bacterial morphology too, where cultured CF sputa illustrate increases in the frequency of small colony, mucoid, colourless, or quorum-signalling variants (Smith *et al.*, 2006a, Wilder *et al.*, 2009, Workentine *et al.*, 2013, Goodman *et al.*, 2004). These characteristic chronic phenotypes of *P. aeruginosa* are highly prevalent amongst the CF community, suggesting that common selective pressures operate within the CF lung. As many of these virulence factors which are lost are highly immunogenic, changes in the *P. aeruginosa* phenotype during the course of chronic infection is likely to be a host-restrictive adaptation to evade the host's immune response, thus promoting persistence and provide a survival advantage. Such genotypic and phenotypic changes over time are less of an abolition of virulence, but rather a transformation of virulence (Jelsbak *et al.*, 2007, Smith *et al.*, 2006a, D'Argenio *et al.*, 2007, Mathee *et al.*, 1999).

3.2 Aims

Phenotypic characterisation of eight *P. aeruginosa* CF clinical isolates acquired from Birmingham Children's Hospital was undertaken, alongside the reference laboratory strain PAO1. The battery of phenotypic characterisations focused upon those important for survival and persistence within the CF lung and thus included analysis of: colony morphology, pyocyanin production, growth curve profile, protease secretion, staphylolytic ability, siderophore production, swimming and swarming motilities, biofilm production and susceptibility to antibiotics.

3.3 Methods

Routine culture and colony morphology determination. Frozen stocks of *P. aeruginosa* were partially thawed and streaked onto LB agar plates and incubated at 37 °C and 5% CO_2 for 48 h. Pigmentation and colony morphology was visually inspected. For liquid cultures, *S. aureus* and *P. aeruginosa* were inoculated into 10 mL of LBN broth. PAO1 acted as a positive control for extracellular virulence factor secretion, motility and biofilm formation, whilst acting as a negative control for mucoidy.

Isolate confirmation testing. A single colony of each *P. aeruginosa* CF isolate was streaked onto PIA plates, whilst *S. aureus* was streaked onto MSA plates. Plates were incubated at 37 °C and 5% CO_2 for 48 h prior to inspection.

Oxidase. A drop of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride was added to a sterile cotton swab and pressed onto an individual colony of *P. aeruginosa* or *S. aureus.* Positive results were scored as the development of a dark purple colour (indophenol) on the tip of the swab within 10 s.

Gram staining. A single colony of each CF isolate of *P. aeruginosa* grown on LB agar was added to 5 drops of sterile PBS and emulsified onto a sterile glass microscope slide. Slides were then passed through a flame to heat fix, prior to staining using traditional Gram staining techniques with crystal violet, iodine and safranin. Slides were then viewed using light microscopy to confirm the presence of Gram-negative rods.

Mucoidy status. A single colony of *P. aeruginosa* was spread onto the surface of a PIA plate using a sterile cotton swab. Mucoidy status was determined as a slimy appearance following a 48 h incubation at 37 °C.

Pyocyanin status. A single colony of *P. aeruginosa* was streaked across the surface of a PIA plate. Pyocyanin determination was assessed following a 48 h incubation at 37 °C and 5% CO_2.

Planktonic growth. A single colony of *P. aeruginosa* was inoculated into 10 mL of LBN broth and incubated at 37 °C for 16 h. Cultures were centrifuged at 4,000 x *g* for 10 min at 4 °C and normalised to an OD_{470} of 1.0, diluted 1:10 in fresh LBN broth and 100 µL added to wells of a sterile 96-well plate. Plates were incubated at 37 °C, where growth kinetics were followed by measuring the OD_{470} at hourly intervals for 15 h.

Preparation of *P. aeruginosa* cell-free culture supernatant. Overnight cultures of *P. aeruginosa* were centrifuged at 4,000 x *g* for 10 min at 4 °C. Each supernatant was sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter and stored at −20 °C until

use. To confirm sterility after each preparation, a small volume of the supernatant was streaked onto LB agar plates and incubated for approximately 20 h at 37 °C and 5% CO_2 prior to reading.

Protease production. Protease production was determined using skimmed milk agar plates. Cell free supernatants (40 µL) from overnight cultures were loaded into wells of agar plates and incubated at 37 °C for 24 h. Hydrolysis of the milk protein casein results in a clear zone surrounding the bacterial supernatant, indicating protease production. LBN medium was also loaded as a negative control. The diameters of clearance zones were measured in mm.

Anti-staphylococcal activity. *S. aureus* was used as the substrate in this assay. An overnight culture of *S. aureus* grown under static normoxia conditions was centrifuged at 4,000 x *g* for 10 min at 4 °C, prior to the pellet being resuspended in 250 µL of 25 mM diethanolamine buffer, pH 9.5. The bacteria were heated at 100 °C for 10 min, before being diluted to a final optical density OD_{595} of 1.0. 400 µL of the adjusted heat-killed *S. aureus* were added to each microtube. The cell-free supernatant from each *P. aeruginosa* isolate was diluted 1:10 with diethanolamine buffer, prior to 100 µL being added to the heat-killed *S. aureus.* The OD of heat killed *S. aureus* after 60 min was read on a MultiSkan Go plate reader, with decreases in the OD being evidence of *S. aureus* lysis. LBN broth alone was used as a control. This method was adapted from Andrejko *et al.* (2013).

Pyoverdine production. Overnight cultures of *P. aeruginosa* and *S. aureus* were pelleted, resuspended in fresh medium and adjusted to an OD_{470} of 1.0. 500 µL of each culture was then added to a 250 mL conical flask containing 50 mL of LBN broth. The flasks were then incubated at 37 °C for 24 h, under static normoxia or anoxia. To measure pyoverdine production, 100 µL of the cell-free supernatant was added in triplicate to a black 96-well plate and the fluorescence read at excitation and emission wavelengths 400/460 nm, respectively. The background level of fluorescence was measured using 100 µL LBN broth only. To take into account differences in final bacterial density, RFU values were normalised to the OD_{470} of each bacterial culture.

Biofilm biomass. Overnight cultures of *S. aureus* and *P. aeruginosa* were centrifuged and adjusted to OD_{470} 1.0. Cultures were diluted tenfold and 100 µL added to the central wells of a 96-well tissue culture treated flat bottom plate for 1 h under static conditions, at 37 °C. After 60 min, the well contents were aspirated and replaced with fresh LBN broth. Plates were incubated for a further 24 h at 37 °C under static conditions. Following this, biofilms were washed twice using 200 µL of PBS and air dried overnight. To determine biofilm biomass, attached biofilms were stained with 200 µL 1% (w/v) crystal violet for 10 min, prior to two further washes in deionised water. Plates were dried overnight, prior to the stain being solubilised with 200 µL of 30% (v/v) acetic acid. The solubilised stain was then transferred to a new 96-well plate and read at OD_{570}. The greater OD, the greater the biofilm biomass.

Bacterial motility. Swimming motility of *P. aeruginosa* was investigated using 0.3 % (w/v) nutrient agar plates supplemented with nutrient broth and 1% (w/v) potassium nitrate. Swarming of *P. aeruginosa* was determined using 0.5 % (w/v) of nutrient agar plates supplemented with nutrient broth, dextrose and 1% (w/v) potassium nitrate. Overnight cultures of *P. aeruginosa* were adjusted to OD_{470} of 1.0 and a total of 5 µL of culture was added to the centre of each plate. Plates were incubated for 24 h at 37 °C. The diameter of the zone travelled by *P. aeruginosa* was then measured in mm using a ruler.

Minimum inhibitory concentration (MIC) determination. Overnight cultures of *P. aeruginosa* were normalised to an OD_{470} of 1.0, diluted to 10^6 CFU/mL and 100 µL added to serially diluted concentrations of tobramycin (64-0.25 µg/mL), ciprofloxacin (128-0.5 µg/mL) or amikacin (128-0.5 µg/mL). Plates were sealed with a Breathe-easy® membrane and incubated statically for 24 h at 37 °C under normoxia or anoxia. The MIC was determined by visual inspection of the 96-well plates.

Statistical Analysis. All results, unless otherwise specified, are expressed as mean ±S.E.M., with data for each experiment being collected from three independent experiments (N=3), each performed in triplicate. All statistical analyses were performed using GraphPad Prism 6 software (Graphpad, La Jolla, CA, USA), with significance being set to P<0.05. The specific tests and *post-hoc* used for each experiment are described in the figure legends.

3.4 Results

3.4.1 *P. aeruginosa* phenotype using LB culture medium

Phenotypic investigations were carried out on eight CF clinical isolates of *P. aeruginosa* isolated from CF sputum samples of patients attending Birmingham Children's Hospital between 1991-1999. The laboratory reference strain *P. aeruginosa* PAO1 was also used. To initially characterise these isolates, frozen cultures were streaked onto LB agar plates and visually inspected following 48 h incubation under normoxia at 37 °C. A single colony from each plate was subsequently inoculated into 10 mL of sterile LB broth and visually inspected following 48 h incubation under normoxia, at 37 °C.

From Figure 14 it is evident that laboratory strain PAO1 and 6 of the CF isolates produce an array of diffusible water-soluble pigments typically associated with *P. aeruginosa* following growth on solid LB agar and in liquid LB broth. Whilst PAO1 and CF clinical isolates 1, 3, 4, 5 and 6 produce green hues characteristic of pyocyanin, isolate 8 produces a brown-red pigment, characteristic of pyomelanin. CF isolates 2 and 7 lacked characteristic *P. aeruginosa* pigmentation following growth on LB agar and in LB broth and were both yellow in colour. Additionally, all isolates were identified as being oxidase positive, Gram-negative by crystal violet staining and exhibited a characteristic grape-like "fruity" odour.

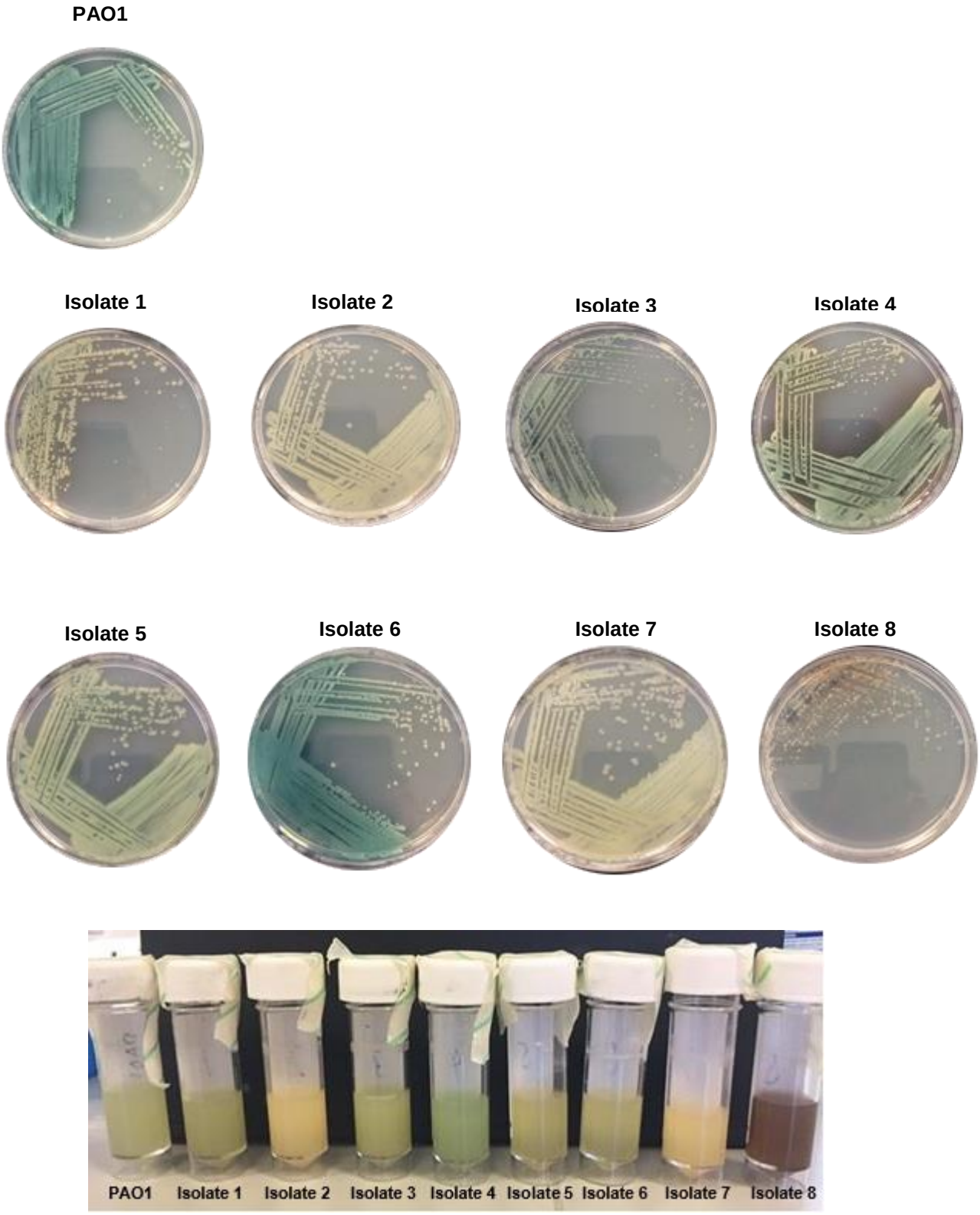


Figure 14. Pigmentation varies across *P. aeruginosa* isolates. Bacteria were incubated under static aerobic conditions either on solid LB agar plates (top) or in 10 mL of LB broth (bottom) for 48 h at 37 °C. Images are representative of numerous bacterial subcultures performed throughout this study**.**

3.4.2 Pyocyanin status

PAO1 and the eight CF isolates were streaked onto PIA plates (containing magnesium chloride and potassium sulphate), which enhances the production of pyocyanin. The presence of cetrimide also served to confirm species identity. As shown in Figure 15, PAO1 and CF isolates 1, 4, 5 and 6 all produced detectable levels of pyocyanin, with PAO1 and CF isolate 6 producing the greatest amount of this green phenazine, as depicted by the intense green hue. CF isolate 8 was the only isolate to produce the dark brown pigment pyomelanin.

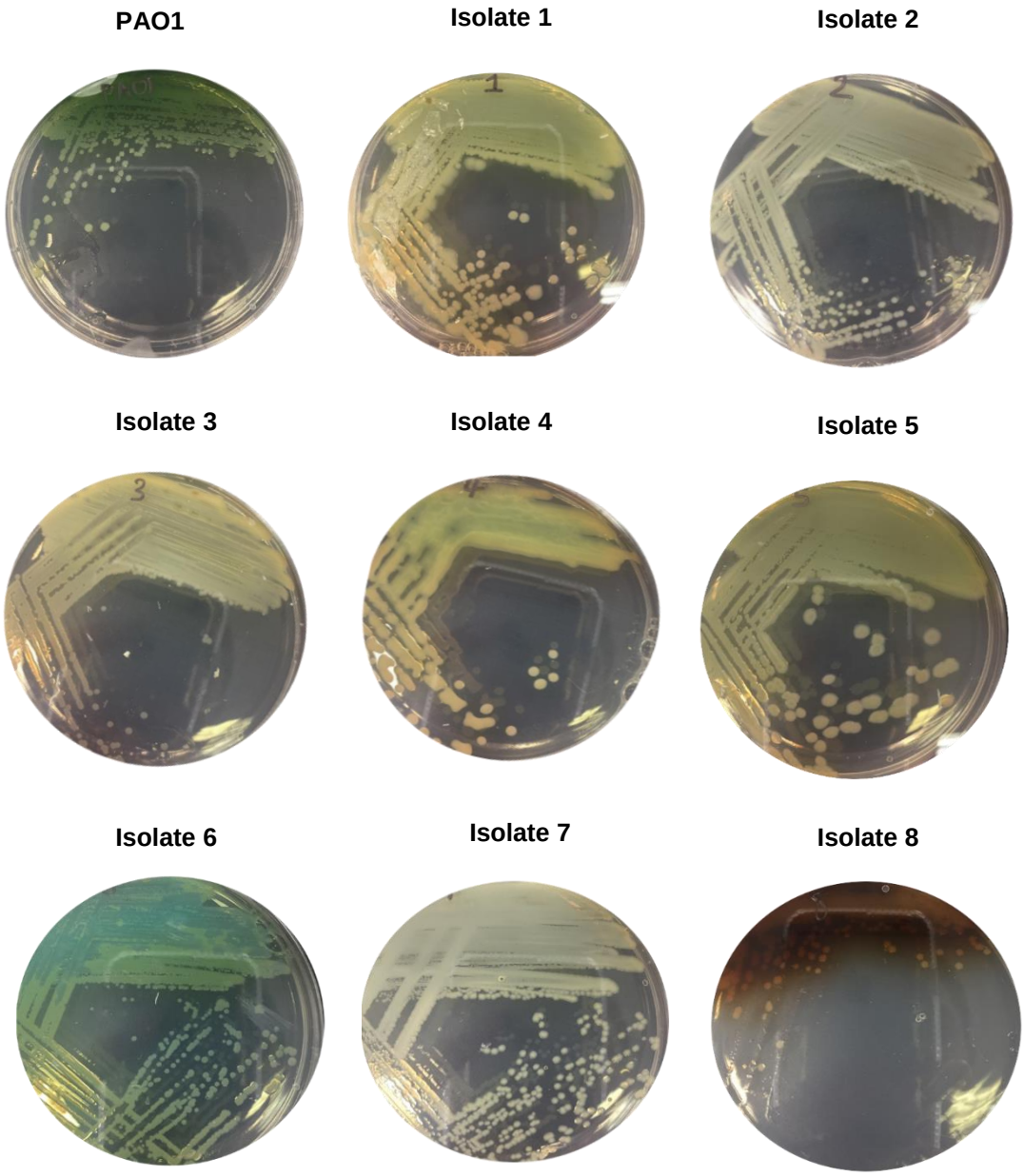


Figure 15. Pyocyanin production by *P. aeruginosa*. PAO1 and the eight CF isolates were streaked from frozen stocks onto PIA agar and incubated for 48 h at 37 °C prior to visual inspection. The phenazine pyocyanin diffuses into the agar and gives rise to a characteristic green hue. Images shown are representative of three independent experiments, each performed in duplicate.

3.4.3 Colony Morphology

P. aeruginosa colony morphology was assessed following growth on LB agar plates and incubation at 37 °C for 48 h. As shown in Table 6, eight morphological characteristics were studied, ranging from colony pigmentation and mucoidy status to colony surface texture.

Table 6. Colony morphology of *P. aeruginosa* laboratory strain PAO1 and clinical CF isolates upon LB agar plates. The production of four colony pigmentations were studied. Colonies were positive for mucoidy status if they exhibited a slimy appearance. Colony form assessed the basic shape of the colonies. Autolysis assessed whether the bacteria grew as colonies that were lysed in their centres, whilst margin determined the edge of the colonies.

Isolate	**Pigmentation**				**Colony Size**		**Mucoidy status**		**Form**		**Optical Property**	**Autolysis**	**Surface Texture**		**Margin**
	Brown	Green	White	Opaque	Small	Large	Mucoid	Non-	Circular	Irregular	Opaque		Rough	Smooth	Entire
PAO1		**+**				**+**		**+**	**+**		**+**	**-**		**+**	**+**
1			**+**	**+**		**+**		**+**	**+**		**+**	**-**		**+**	**+**
2				**+**		**+**		**+**	**+**		**+**	**-**		**+**	**+**
3			**+**			**+**		**+**	**+**		**+**	**-**		**+**	**+**
4	**+**	**+**				**+**	**+**		**+**		**+**	**-**		**+**	**+**
5		**+**				**+**		**+**	**+**		**+**	**-**		**+**	**+**
6		**+**				**+**		**+**	**+**		**+**	**-**		**+**	**+**
7			**+**			**+**		**+**	**+**		**+**	**-**		**+**	**+**
8	**+**					**+**		**+**	**+**		**+**	**-**		**+**	**+**

Based upon eight morphological features studied, six distinct colony morphotypes were detected across PAO1 and the CF clinical isolates of *P. aeruginosa.*

3.4.4 Mucoidy status

To confirm the mucoidy status of CF isolates, overnight cultures of *P. aeruginosa* were adjusted to OD_{470} 1.0 and streaked onto PIA and incubated at 37 °C for 48 h, prior to visual inspection.

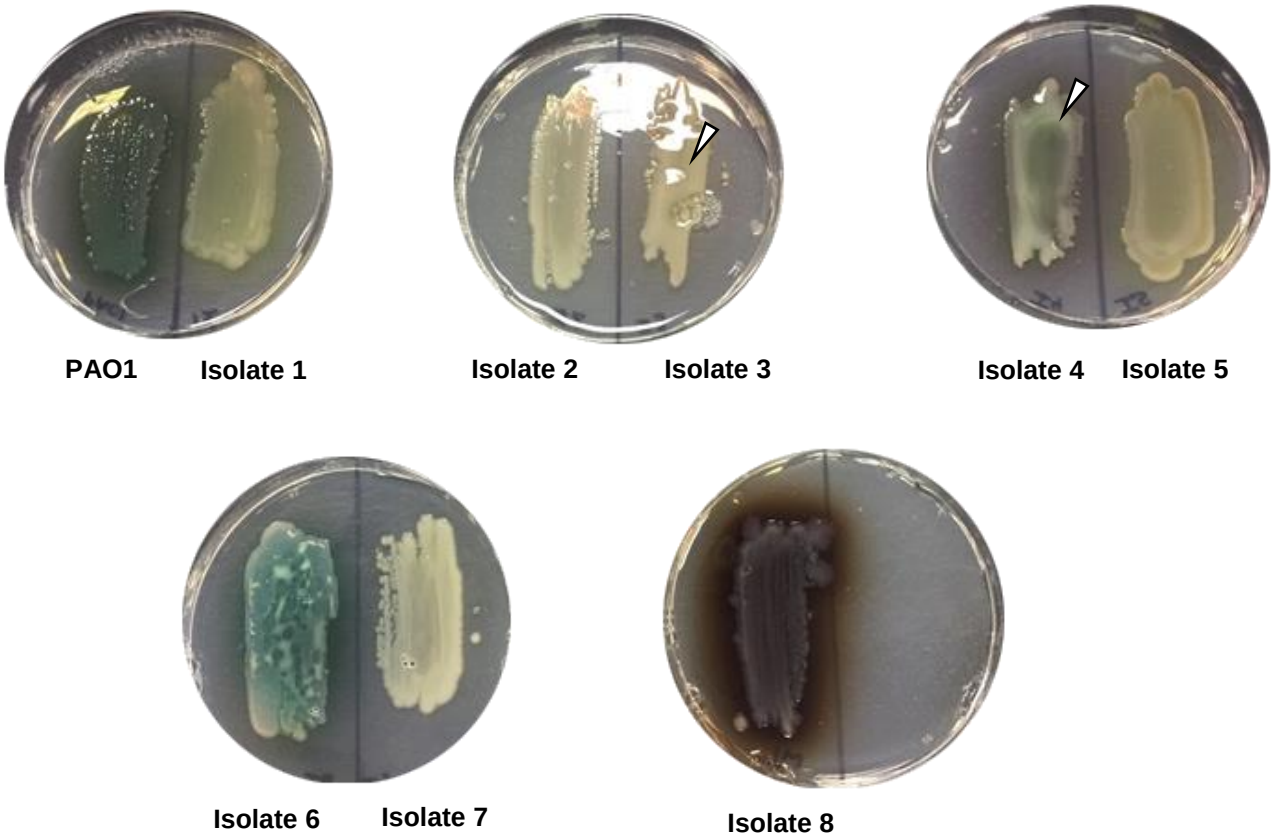


Figure 16. Assessing *P. aeruginosa* CF isolate mucoidy. CF isolates of *P. aeruginosa* were streaked onto PIA plates and incubated under normoxia for 48 h at 37 °C. Images shown are representative of three independent experiments, each performed in duplicate. Mucoid isolates are indicated by the white arrow heads.

In Figure 16 above, only *P. aeruginosa* CF isolates 3 and 4 exhibited a mucoid phenotype following growth on PIA, evident by their slimy appearance (white arrow heads). All remaining CF isolates and laboratory strain PAO1 were non-mucoid.

3.4.5 Growth rates in planktonic culture

Growth curves were constructed to identify whether the CF isolates exhibited differences in their rates of planktonic growth compared to one another and in comparison to the non-CF laboratory strain PAO1. Once overnight cultures were normalised, diluted in fresh LBN broth and added to a sterile 96-well plate, changes in OD were measured over a 15 h period, at hourly intervals.

As shown in Figure 17, *P. aeruginosa* CF isolates displayed considerable heterogeneity in their growth kinetics. *P. aeruginosa* CF isolate 1 reached the highest OD of all the isolates tested at 15 h, which was approximately 36% higher than PAO1, with CF isolate 6 being the only isolate to grow to a lower final OD than PAO1 (approximately 7% lower). There was no correlation seen between colony morphotype and growth rate. Mucoid CF isolates 3 and 4 grew to similar final OD as the non-mucoid isolates, such as CF isolates 2 and 5. Non-mucoid PAO1 and CF isolates 5 and 6 all produced pyocyanin and yet PAO1 grew to a lower final OD. CF isolates 3 and 7 grew as white non-mucoid isolates and grew to a similar final OD as CF isolates 5 and 6 which exhibited a green colony morphotype. The bacterial OD was measured at 470 nm as this wavelength minimises the effect and influence of *P. aeruginosa* pigments which could interfere with the OD readings of the bacterial density.

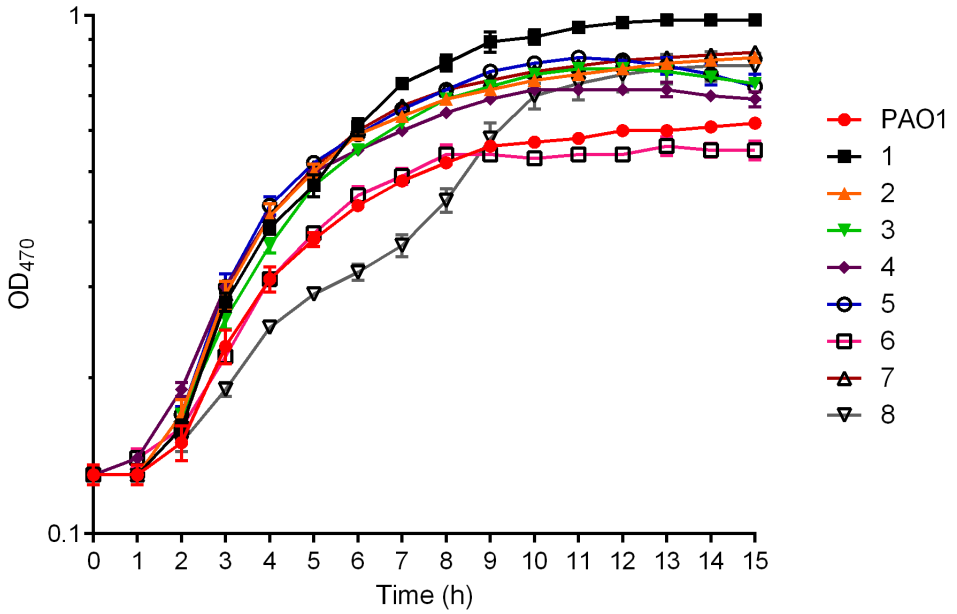


Figure 17. Planktonic growth curves of *P. aeruginosa* PAO1 and CF clinical isolates. Overnight cultures of *P. aeruginosa* were standardised, diluted and incubated at 37°C for 15 h, with OD_{470} readings being taken every hour. The results are expressed as the mean ± S.E.M of three independent experiments (*N*=3), each performed in triplicate.

The doubling times during the exponential phase of planktonic growth for PAO1 and the eight CF isolates were determined from data presented in Figure 17. Table 7 illustrates that all CF isolates (except CF isolates 6 and 8) have a shorter doubling time than laboratory strain PAO1 (approximately 138 min), with CF isolate 7 having the shortest doubling time of all the isolates tested (approximately 109 min). Conversely, CF isolate 8 exhibited the longest exponential phase, with an approximate doubling time of approximately 184 min.

Table 7. Doubling times for *P. aeruginosa* PAO1 and the eight CF clinical isolates. Doubling times were calculated from data obtained in Figure 17.

***P. aeruginosa* isolate**	**Approximate doubling time (min)**
PAO1	138
CF Isolate 1	116
CF Isolate 2	116
CF Isolate 3	123
CF Isolate 4	132
CF Isolate 5	112
CF Isolate 6	142
CF Isolate 7	109
CF Isolate 8	184

3.4.6 Protease production

Given that proteases are an essential virulence factor used by *P. aeruginosa* to promote colonisation of the CF lung (Smith *et al.*, 2006b), their production was assessed across the eight clinical isolates. As shown in Figure 18, PAO1 and six of the eight CF isolates tested secreted detectable levels of protease. There was also no correlation between protease production and mucoidy status. Both mucoid CF isolates 3 and 4 secreted detectable levels of protease, along with non-mucoid isolates 1, 2, 5 and 7. Protease production for two of the non-mucoid CF isolates (6 and 8) was below the limit of detection for this assay, which was also the two CF isolates which exhibited the longest doubling time (Figure 17).

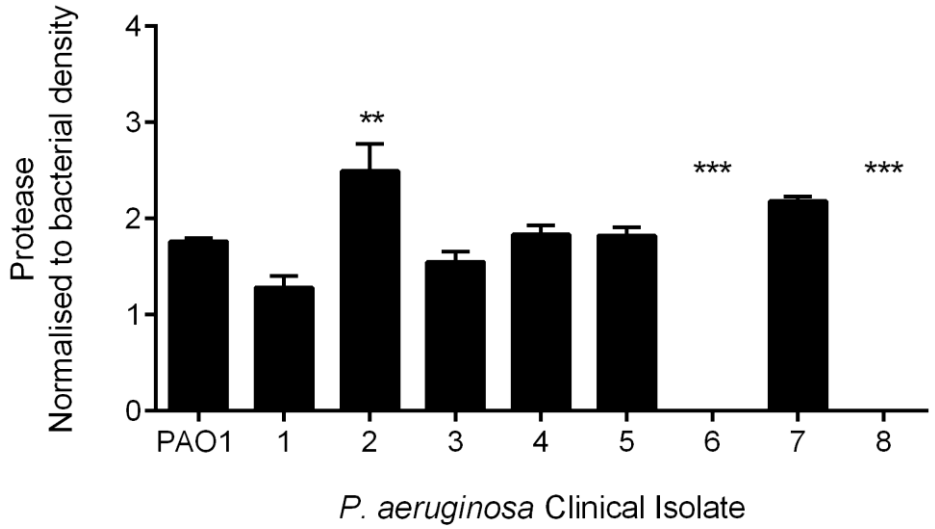


Figure 18. Protease production varies across *P. aeruginosa* CF clinical isolates. Cell-free supernatants from overnight cultures of *P. aeruginosa* (40 µL) were added to wells in the milk agar and the diameter of zones of clearance were measured following 24 h incubation. Data shown are the mean ± S.E.M. of three independent experiments (*N*=3), each performed in triplicate. Data has been normalised to account for differences in final bacterial cell density. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* test (vs. PAO1). ***P*<0.01 and ****P*<0.001.

3.4.7 Staphylolytic activity

As *P. aeruginosa* can co-exist with *S. aureus* and compete for nutrients within CF airways (Limoli *et al.*, 2016), the ability of *P. aeruginosa* CF clinical isolates to lyse *S. aureus* was also determined. Cell-free supernatants from overnight cultures of *P. aeruginosa* were added to suspensions of heat-killed *S. aureus* and changes in OD were measured after an hour. Any detectable decreases in optical density were evidence of *S. aureus* lysis.

As shown in Figure 19, PAO1 and six of the eight CF isolates were able to significantly reduce the OD of heat-killed *S. aureus* after an hour compared to the negative control (heat-killed *S. aureus* with LBN broth). Conversely, CF isolates 6 and 8 which exhibited the longest doubling time and failed to produce detectable proteases, were unable to lyse heat-killed *S. aureus.*

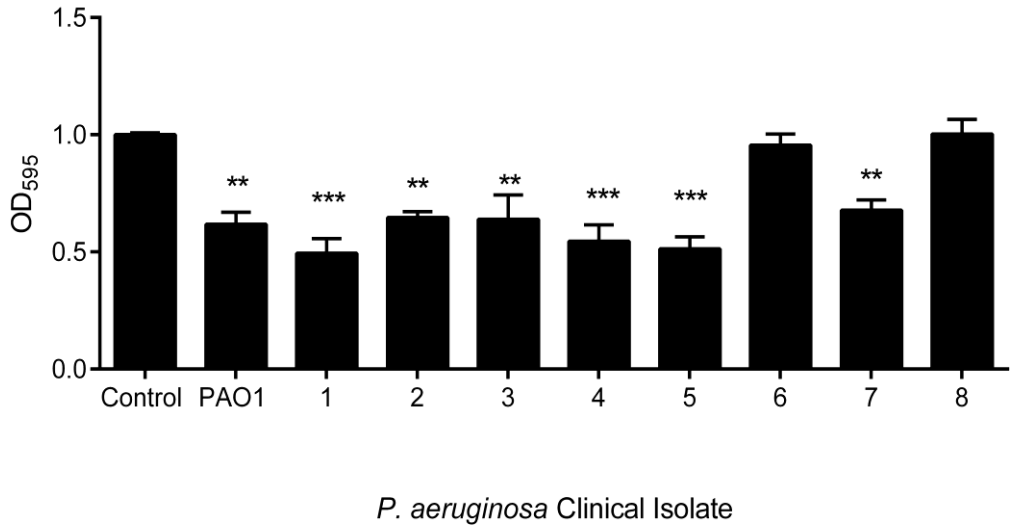


Figure 19. Staphylolytic ability of *P. aeruginosa* CF clinical isolates. Reference strain PAO1 and eight CF isolates were grown statically overnight under normoxia. Cell-free supernatants were added to heat-killed *S. aureus* and staphylolytic ability was determined by measuring changes in the OD_{595} after 60 min. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in quadruplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* (vs. control). Control consisted of LBN broth added to heat-killed *S. aureus*. ***P*<0.01 and ****P*<0.001.

3.4.8 Pyoverdine production

Iron is an essential micronutrient required by *P. aeruginosa* for a range of proteins including catalases and cytochromes (Konings *et al.*, 2013). The production of the major iron binding siderophore pyoverdine was determined across PAO1 and the panel of CF isolates. Due to its ability to fluoresce, the relative fluorescence units (RFU) of cell-free culture supernatants were recorded and normalised to the bacterial cell density (OD_{470}). As shown in Figure 20, all isolates produced detectable levels of pyoverdine, with seven isolates producing the phenazine at levels similar to laboratory strain PAO1, except CF isolate 6 which produced pyoverdine at significantly lower levels (*P*<0.05).

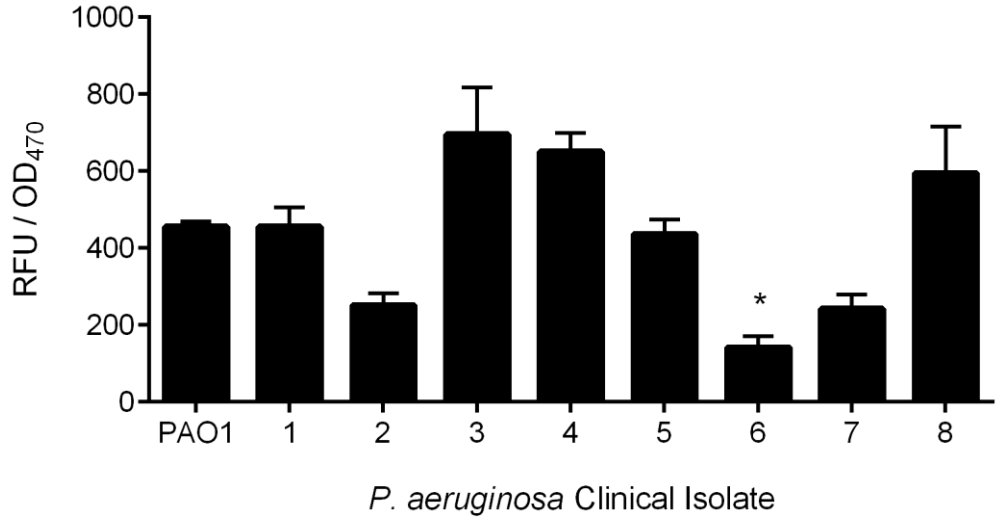


Figure 20. *P. aeruginosa* pyoverdine production. The amount of pyoverdine within *P. aeruginosa* cell-free supernatants was quantified by exploiting the fluorescent nature of this main siderophore at 400 nm excitation and 460 nm emission wavelengths. To take into account differences within the final cell density, the RFU was normalised to the bacterial culture OD. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* (vs. PAO1). **P*<0.05.

3.4.9 Biofilm Biomass

The production of an alginate-predominant biofilm by *P. aeruginosa* within CF airways provides a survival advantage to the bacterium, facilitating resistance against neutrophil-mediated phagocytosis and increasing tolerance to antibiotics (Hoiby *et al.*, 2010). *P. aeruginosa* isolates were grown in sterile 96-well plates, with the biofilm biomass being determined after 24 h using crystal violet staining. Biofilm staining intensity was normalised to the OD_{470} of the 24 h bacterial culture.

In Figure 21 below, CF isolates of *P. aeruginosa* show varying degrees of biofilm formation. Mucoid CF isolates 3 and 4 did not produce the greatest amount of biofilm biomass. CF isolate 6 was the weakest biofilm former (*P*<0.001) compared to laboratory strain PAO1, whilst mucoid isolate 8 exhibited the greatest biofilm biomass (*P*<0.01). Interestingly, as mentioned previously, these two CF isolates exhibited the longest doubling time (Figure 17). CF isolates 1, 3, 4 and 7 all secreted a biofilm biomass similar to that of reference strain PAO1.

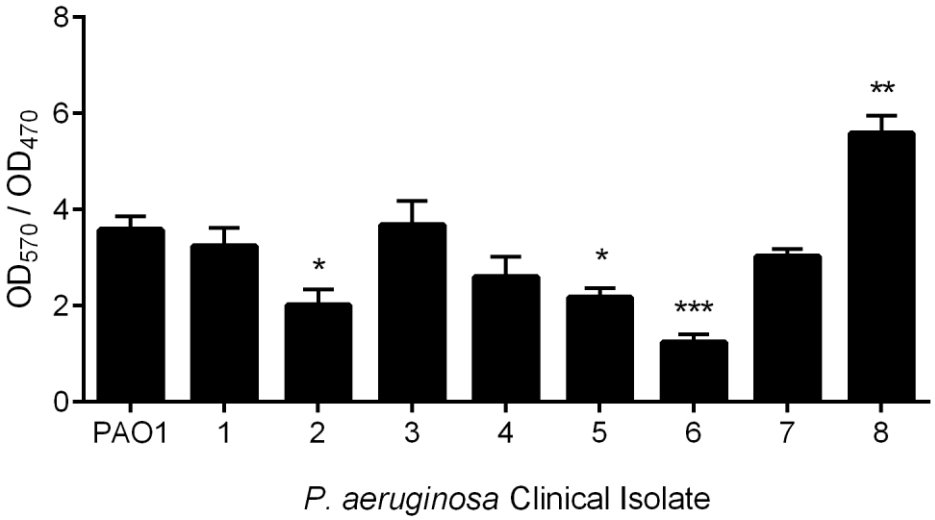


Figure 21. Biofilm biomass of CF clinical isolates of *P. aeruginosa*. Biofilms from 24 h cultures of *P. aeruginosa* were stained with 1% (w/v) crystal violet and solubilised with 30% (v/v) acetic acid, prior to being read at OD570. Data shown are the mean ± S.E.M. of three independent experiments (*N*=3), each performed in triplicate. Data has been normalised to account for differences in bacterial cell density at OD470. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* test (vs. PAO1). **P*<0.05, ***P*<0.01 and ****P*<0.001.

3.4.10 Motility – swimming and swarming

P. aeruginosa motility plays an important role in surface colonisation, as well as in the formation of biofilms. Swimming and swarming motility were determined using both swim and swarm agar plates. As shown in Figure 22, PAO1 and the CF isolates displayed varying degrees of swimming motility. Whilst CF isolates 1 and 8 exhibited smaller swim zones, CF isolate 5 demonstrated the highest degree of swimming motility compared to PAO1.

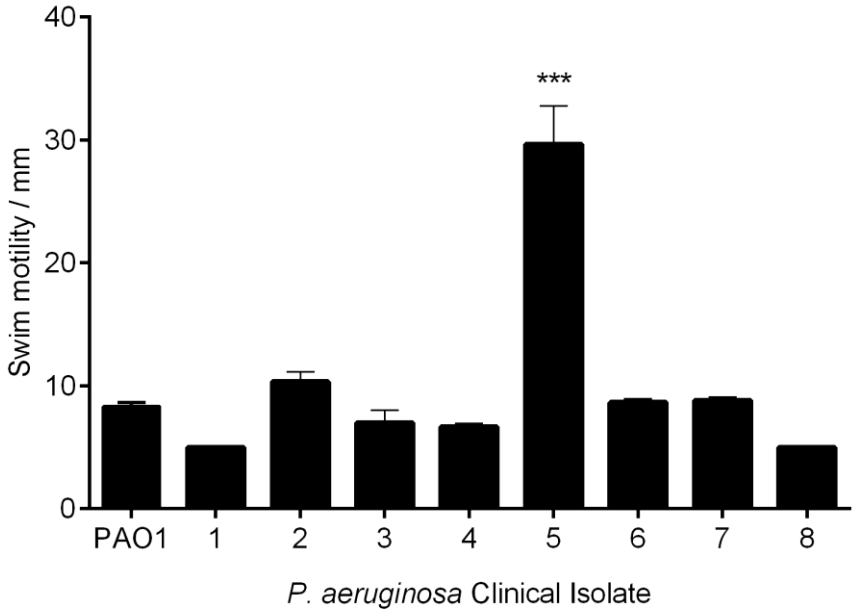


Figure 22. *P. aeruginosa* swimming motility. Overnight cultures of *P. aeruginosa* were normalised, prior to 5 µL of culture being inoculated into the agar of swim plates. Plates were incubated for 24 h at 37 °C prior to being read. Data shown are the mean ± S.E.M. of three independent experiments (*N*=3), each performed in duplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* test (vs. PAO1). ****P*<0.001.

As shown in Figure 23, CF isolates 1, 3, 4, 5 and 8 displayed varying degrees of swarming motility, with CF isolate 1 exhibiting the greatest swarming motility, with characteristic finger-like projections protruding out from the point of inoculation. PAO1 and CF isolates 2, 6 and 7 exhibited minimal swarming motility.

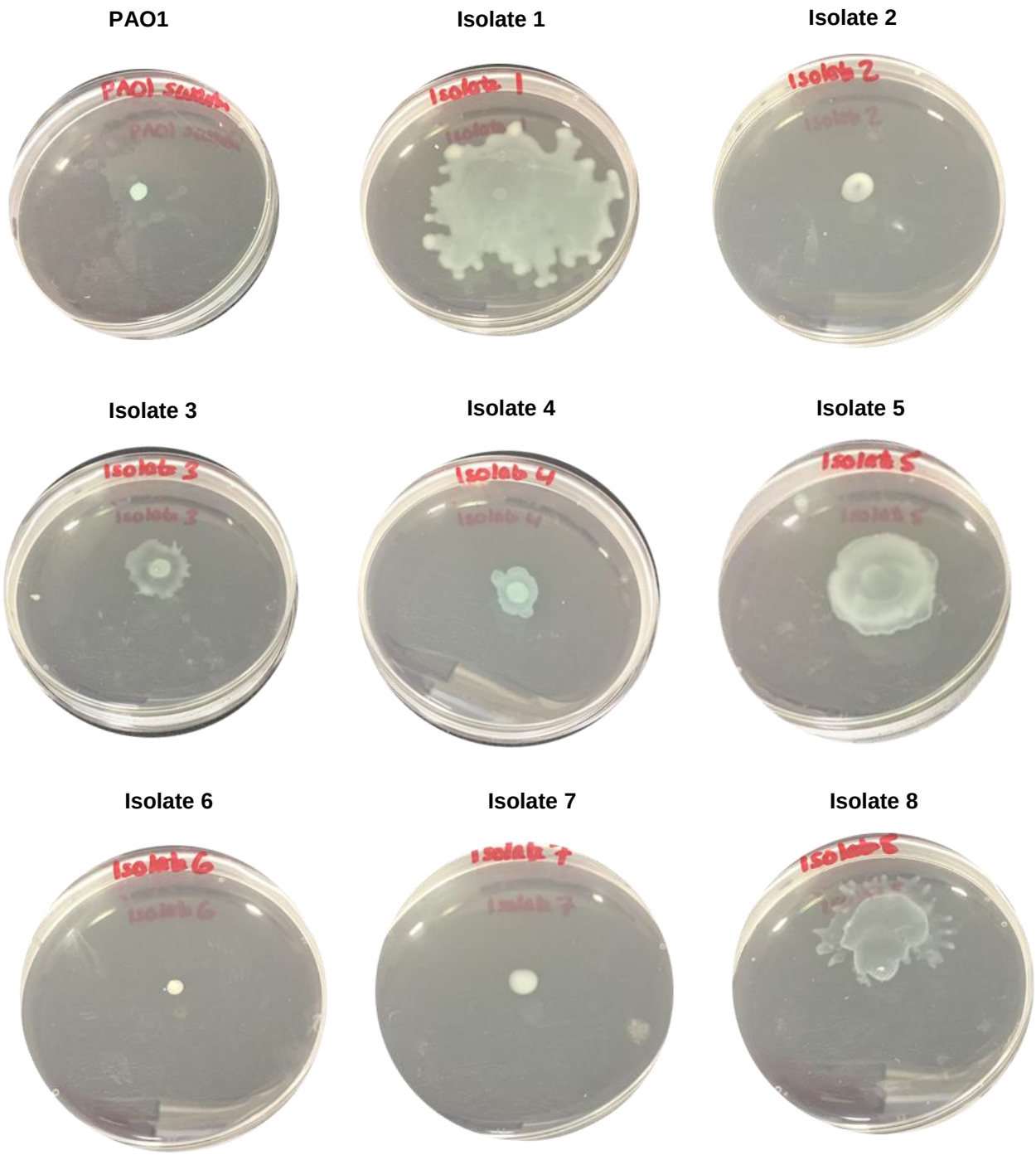


Figure 23. *P. aeruginosa* swarming motility. The cell densities of overnight cultures of *P. aeruginosa* were normalised, prior to 5 µL of culture being inoculated onto the surface of swarm agar. Plates were incubated for 24 h at 37 °C prior to being read. Images shown are representative of three independent experiments (*N*=3), each performed in duplicate.

3.4.11 Minimum inhibitory concentration (MIC) determination – amikacin, ciprofloxacin and tobramycin

CF sputa which are culture positive for *P. aeruginosa* require the patient to undergo an aggressive antibiotic regimen in an attempt to eradicate the organism from the airways. Employing the use of three anti-pseudomonal antibiotics, the MIC for PAO1 and the eight CF clinical isolates using the microbroth dilution method was determined.

As shown in Figure 24, the susceptibility of *P. aeruginosa* isolates to the three antibiotics varied widely. CF isolates 2, 6 and 7 exhibited the greatest resistance to the aminoglycoside amikacin (64 µg/mL), whilst PAO1 and CF isolate 5 were the most susceptible (16 µg/mL). CF isolate 6 also displayed the greatest resistance to fluoroquinolone ciprofloxacin and the aminoglycoside tobramycin, with a MIC of 4 µg/mL and 16 µg/mL respectively. PAO1 and the remaining CF isolates all exhibited the same susceptibility to ciprofloxacin (1 µg/mL), with PAO1 and CF isolate 4 displaying the greatest susceptibility to tobramycin (2 µg/mL).

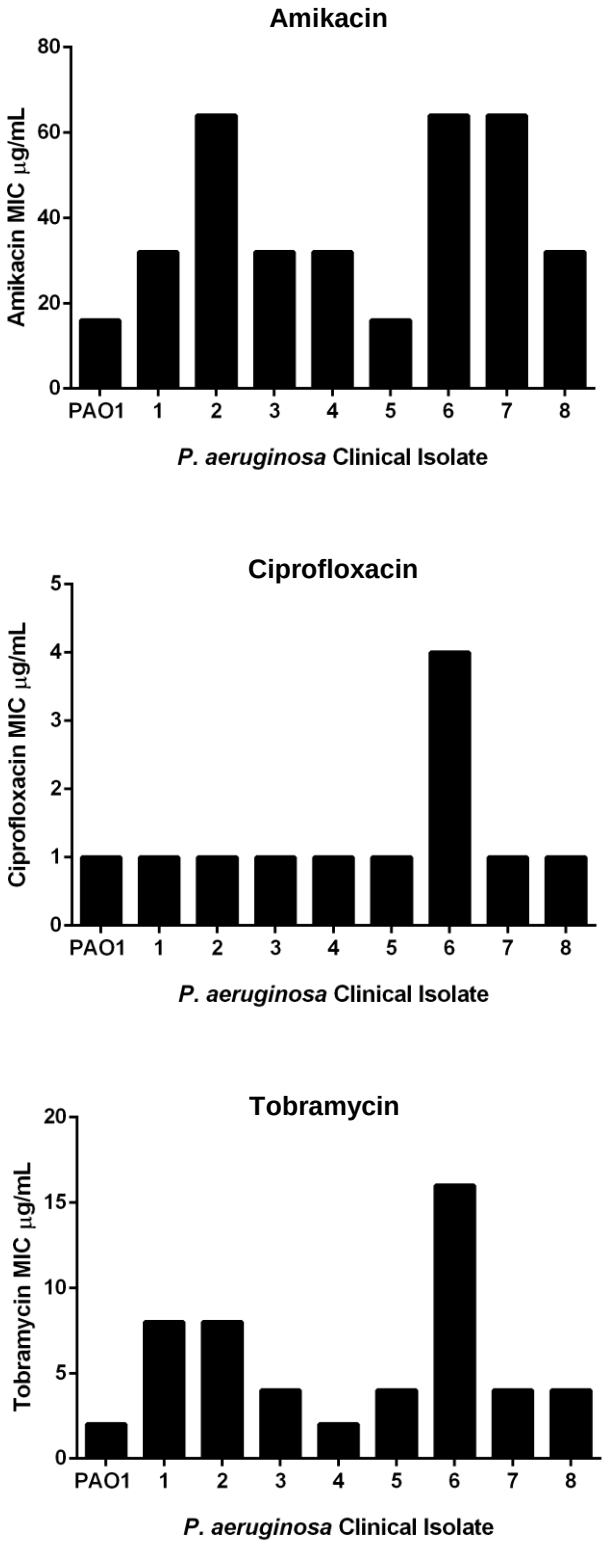


Figure 24. *P. aeruginosa* MIC to three clinically relevant antibiotics. *P. aeruginosa* CF isolates were grown in the presence of serially diluted concentrations amikacin, ciprofloxacin and tobramycin. Plates were incubated statically for 24 h at 37 °C under normoxia. The minimum inhibitory concentration (MIC) was determined by visual inspection. Data is obtained from three independent experiments (*N*=3), each performed in duplicate.

3.4.12 *S. aureus* characterisation

The *S. aureus* laboratory strain used in this research (ATCC 6538) displayed the characteristic golden morphology (from which the organism derives its species name) following growth upon LB agar. To ensure culture purity, *S. aureus* was streaked onto the selective MSA and grew as characteristic yellow colonies (Figure 25). The yellow zones surrounding the colonies are due to the ability of *S. aureus* to ferment mannitol, causing a colour change in the phenol red pH indicator within the agar.

As a quality assurance check, MSA was used to discriminate between *S. aureus* and the bacterium *Staphylococcus epidermidis (S. epidermidis)*, which has a similar colony morphology. As *S. epidermidis* cannot ferment mannitol, it produces red colonies, with no colour change around them. This quality check was performed during each subculture of the bacterium. Moreover, as *P. aeruginosa* failed to grow upon MSA, it would be used as a selective agar in future co-culture experiments to discriminate between colonies of *S. aureus* and *P. aeruginosa.*

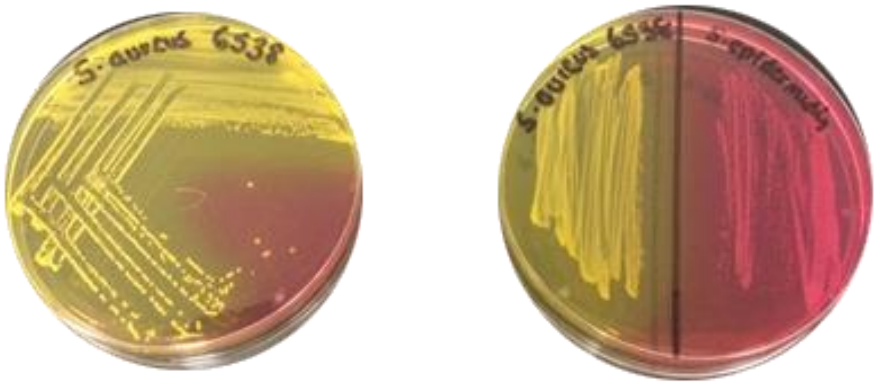


Figure 25. *S. aureus* grown on the selective and differential media MSA. As depicted in the left-hand image, when streaked onto MSA, *S. aureus* grows as distinct yellow colonies with yellow zones, due to its ability to ferment mannitol. However, as shown in the right-hand image, other staphylococci such as *S. epidermidis* appear as red colonies with red zones.

3.5 Discussion

Although phenotypic heterogeneity of *P. aeruginosa* CF isolates within and across patients has been extensively reported (Mowat *et al.*, 2011, Clark *et al.*, 2015b, Tingpej *et al.*, 2007, Winstanley *et al.*, 2016, Mayer-Hamblett *et al.*, 2014b, Workentine *et al.*, 2013, Ashish *et al.*, 2013), this chapter aimed to determine the phenotypic properties of eight novel CF clinical isolates of *P. aeruginosa* obtained from Birmingham Children's Hospital. This would serve to not only characterise the isolates, particularly in regards to their virulence, but also allowed informed decisions to be made as to which isolates were to be carried forward in downstream experiments. Although the results for the CF isolates are compared to one another, as well as to the literature, it is recognised that the sample size is small, particularly in the case of mucoid isolates - a phenotype only seen in two of those sampled. Furthermore, due to the isolates having been previously purified from CF sputum, the relative abundance of other *P. aeruginosa* morphotypes and presence of other bacterial pathogens such as *S. aureus* in the original CF sputum samples is unknown. Together this meant detailed statistical correlations could not be performed comparing different virulence factors across CF isolates. The lack of clinical data also meant that the phenotype could not be compared to the length of airway colonisation, exacerbation status and antibiotic history.

Initial characterisation supported the wide variations in *P. aeruginosa* colony morphology that have been previously reported in CF sputum, throat swabs and static cultures (Wahba and Darrell, 1965, Thomassen *et al.*, 1979, Clark *et al.*, 2015b, Foweraker *et al.*, 2005, Deziel *et al.*, 2001, Haussler *et al.*, 2003). Morphological analysis forms an important diagnostic role within the clinical microbiology laboratory, in selecting isolates for further analysis. Interpretation of colony morphologies was performed following a 48 h incubation at 37 °C as it is documented that *P. aeruginosa* morphologies are stable after this time period (Sousa *et al.*, 2013). The eight CF isolates of *P. aeruginosa* displayed extensive heterogeneity (Table 6), with the eight isolates exhibiting six distinct morphotypes.

As shown in Table 6, none of the isolates displayed an iridescent colony surface metallic sheen or central colony autolysis, both associated with mutations in *lasR* (D'Argenio *et al.*, 2007). Autolysis has been shown to arise due to the overproduction of the PQS (D'Argenio *et al.*, 2002), although PQS production was not assessed in this study. The production of pyocyanin has been linked to a switch from a rough to smooth morphotype (Dietrich *et al.*, 2008), although all pyocyanin producing isolates displayed a smooth colony morphotype macroscopically.

Whilst most studies culture *P. aeruginosa* under shaking conditions (Baldan *et al.*, 2014a, O'Brien *et al.*, 2017, Turner *et al.*, 2015), this study grew *P. aeruginosa* under static conditions, in an attempt to mimic more closely the static growth environment within CF airways. Previous studies have demonstrated that *P. aeruginosa* typically grows as a suspended microcolony

within CF sputum (Sriramulu *et al.*, 2005, Petrova *et al.*, 2012), where the static growth of the CF isolates caused them to grow as macroscopic visible clumps. Only two of the CF isolates exhibited mucoidy (Figure 16) following growth upon PIA, a method used previously to determine mucoidy status (Duong *et al.*, 2015). The mucoid morphotype in CF predominantly arises due to a mutation in *mucA*, leading to alginate operon overexpression and the overproduction of the anionic polysaccharide alginate (Mathee *et al.*, 1999, Martin *et al.*, 1993, Li *et al.*, 2005). Associated with a poor prognosis (Henry *et al.*, 1992), the phenotype is a hallmark of established biofilm mode of infection (May *et al.*, 1991) and provides phagocytic resistance against host neutrophils and macrophages which are unable to adhere to the bacteria (Meshulam *et al.*, 1982, Cabral *et al.*, 1987, Krieg *et al.*, 1988).

Non-mucoid isolates are also able to produce biofilm however, through the secretion of the polysaccharides psl and pel (Franklin *et al.*, 2011, Byrd *et al.*, 2009). Psl is a mannose-rich neutral polysaccharide which has been shown to be produced by the laboratory strain PAO1 (Jennings *et al.*, 2015, Colvin *et al.*, 2012), whilst Pel is a cationic polysaccharide and is the only polysaccharide produced by the laboratory strain PA14 (Colvin *et al.*, 2012, Jennings *et al.*, 2015). A study assessing how Pel, Psl and alginate contribute to *P. aeruginosa* biofilm formation demonstrated that Psl-negative mutants produced more Pel polysaccharide, whilst Pel-negative mutants displayed enhanced alginate production. It is likely that there is competition for metabolic precursors between the three polysaccharide biosynthesis pathways. Moreover, alginate overproduction was shown to decrease Psl production, suggesting that there is also an inverse regulation of both biosynthesis operons. Losses in alginate production also abolished the formation of a biofilm, suggesting that the absence of one polysaccharide impacts another warrants further study (Ghafoor *et al.*, 2011).

None of the *P. aeruginosa* isolates exhibited other characteristic morphotypes associated with CF, including rugose small colony variants (RSCV). These pin-head colonies have a wrinkled surface and are hyper-adherent and auto-aggregative when grown on agar (Starkey *et al.*, 2009, Haussler *et al.*, 2003). The phenotype has also been shown to exhibit enhanced resistance to hydrogen peroxide and the antimicrobial peptide LL-37 produced by host neutrophils (Pestrak *et al.*, 2018). Furthermore, none of the CF isolates grew as colonies with irregular edges, which has been associated a failure to eradicate following antibiotic treatment, as well as enhanced biofilm production (Mayer-Hamblett *et al.*, 2014a, D'Argenio *et al.*, 2007, Gupta and Schuster, 2012).

As shown in Figure 17, most of the *P. aeruginosa* CF isolates grew to a greater density than PAO1 at 15 h, with CF isolate 1 showing approximately 36% greater growth than PAO1, with the final density of CF isolate 6 being approximately 7% lower than the reference strain. This is in contrast to Fang *et al.* who showed that all four clinical isolates of *P. aeruginosa* tested grew to a lower density than PAO1 under normoxia (Fang *et al.*, 2013). However, it remains

unclear from the article as to whether these clinical isolates used were isolated from CF sputa or another anatomical site. Despite the use of LB broth, the doubling times of the CF isolates were comparable to the published literature of *P. aeruginosa* growth in CF sputum, which demonstrated an average doubling time of 115-154 min (Yang *et al.*, 2008). Furthermore, there were no significant differences between the growth rates of mucoid and non-mucoid isolates in this study, a finding also supported by Yang *et al.* (2008).

CF isolates were also grown on PIA as previously used to confirm species identity, as well as to determine pyocyanin production, due to it containing magnesium chloride and potassium sulphate which enhances the elaboration of this green phenazine. As shown in Figure 15**,** PAO1 and four of the CF isolates produced detectable levels of pyocyanin, with this being the greatest for PAO1 and CF isolate 6. This characteristic green hue is routinely seen on agar plates in diagnostic microbiology laboratories and in CF sputum (Reyes *et al.*, 1981, Wilson *et al.*, 1988). This metabolite is known to be multifunctional in the context of virulence, from impairing airway cilia beating (Kanthakumar *et al.*, 1993), inducing IL-8 release (Denning *et al.*, 1998b) and immunomodulating respiratory epithelia (Denning *et al.*, 2003), to facilitating neutrophil apoptosis (Usher *et al.*, 2002) and being involved in *P. aeruginosa* redox homeostasis (Price-Whelan *et al.*, 2007). Furthermore, pyocyanin has been shown to reduce CFTR expression in cultured nasal and lung epithelia (Kong *et al.*, 2006). The concentration of phenazines within CF airways has been negatively correlated with pulmonary function, as well as polymicrobial diversity (Hunter *et al.*, 2012).

Unlike mucoid isolate 4, mucoid isolate 3 did not produce detectable levels of pyocyanin on solid agar (Figure 15) or in liquid culture (Figure 14). Mucoid isolates of *P. aeruginosa* have also been shown to produce low levels of pyocyanin during early and late stationary growth phase compared to non-mucoid isolates, with mutations in *mucA22* repressing QS and virulence gene expression (Ryall *et al.*, 2014). Only three of the six non-mucoid isolates of *P. aeruginosa* produced detectable pyocyanin (Figure 15). Whilst the inability to detect pyocyanin by a number of CF isolates may be due to production being below the limit of detection, pyocyanin production is known to be lost during the course of adaptation to the CF lung (Hogardt and Heesemann, 2010). Pyocyanin-negative isolates of *P. aeruginosa* have been associated with the development of autoantibodies against the bactericidal/permeability increasing protein (BIP) present within neutrophils and is responsible for binding to LPS (Rotschild *et al.*, 2005, Schultz *et al.*, 2000). The detection of autoantibodies against BIP has been associated with worsened lung disease (Carlsson *et al.*, 2007). CF isolate 8 was the only isolate in the panel to secrete the water soluble brown-pigment pyomelanin, previously reported in CF isolates (Hunter and Newman, 2010) and believed to be linked with an increased persistence in the CF lung (Rodriguez-Rojas *et al.*, 2009).

Protease production by *P. aeruginosa* serves a number of roles in CF airways, from the degradation of tight junctions (Nomura *et al.*, 2014) and pulmonary surfactants (Malloy *et al.*, 2005, Kuang *et al.*, 2011), to the lysis of *S. aureus* (Kessler *et al.*, 1993b) and cleavage of host antibodies (Fick *et al.*, 1985). Losses in protease production during acute infection has been shown to attenuate virulence (Lore *et al.*, 2012), yet this phenotype has been reported in a third of all CF patients chronically infected with *P. aeruginosa* (Smith *et al.*, 2006a, Hoffman *et al.*, 2009). Whilst losses in protease production are typically associated with mutations in the lasR transcription factor (D'Argenio *et al.*, 2007, Hoffman *et al.*, 2009), other mutations also give rise to this phenotype, such as mutations in *lasB* and *mucA22* (Hamood *et al.*, 1996, Ryall *et al.*, 2014).

As with pyocyanin production, there was no correlation between mucoidy status and protease production. Both mucoid CF isolates 3 and 4 exhibited protease activity on skimmed milk agar, whilst two of the non-mucoid isolates failed to produce detectable protease activity (Figure 18). Such findings are supported by a previous study which demonstrated that both mucoid and non-mucoid CF isolates of *P. aeruginosa* produced proteases, although a higher percentage of non-mucoid isolates were proteolytic (Jagger *et al.*, 1983). Another study demonstrated that losses in *P. aeruginosa* protease activity prevented the degradation of host pro-inflammatory mediators secreted by respiratory epithelia (LaFayette *et al.*, 2015). Whilst this may amplify airway inflammation during chronic CF airway infection, loss of lasR which controls protease production is associated with a *P. aeruginosa* growth advantage under high nitrate conditions, along with growth in the presence of the amino acid phenylalanine, a carbon and energy source (D'Argenio *et al.*, 2007, Barth and Pitt, 1996, Jones *et al.*, 2014).

All of the isolates which produced detectable levels of protease on skimmed milk agar were also able to lyse heat-killed *S. aureus* (Figure 19). This finding is unsurprising as previous studies have demonstrated that the compound responsible for *S. aureus* lysis is the protease LasA (Kessler *et al.*, 1993b, Preston *et al.*, 1997, Mashburn *et al.*, 2005b). Whilst the two methodologies used for determining protease production and staphylolytic activity are commonplace in the published literature (Casilag *et al.*, 2015, Oldak and Trafny, 2005, O'Brien *et al.*, 2017, Park *et al.*, 2012, Lee *et al.*, 2005), future work would seek to employ immunoblotting to confirm the presence or absence of elastase A (Las A) in the *P. aeruginosa* CF isolate secretome.

A lack of correlation was seen across the isolates between mucoidy status (Figure 16) and production of the iron-binding siderophore pyoverdine (Figure 20), a finding reported previously (De Vos *et al.*, 2001). All isolates produced detectable levels of pyoverdine within cell-free supernatants following fluorometric determination, a routine method used to detect this siderophore (Granato and Kummerli, 2017, Lopez-Medina *et al.*, 2015, Kang and Kirienko, 2017). Mucoid isolates 3 and 4 produced high levels of pyoverdine, at levels similar to the non-

mucoid isolates (Figure 20). As with protease and pyocyanin production, chronic infection is typically associated with a loss in pyoverdine production (Smith *et al.*, 2006a). Despite this loss, clinical isolates of *P.aeruginosa* have been shown to retain their ability to take up pyoverdine (De Vos *et al.*, 2001).

As shown in Figure 21, PAO1 and all of the CF isolates tested exhibited a capacity to produce biofilms *in vitro* as determined by crystal violet staining. Non-mucoid isolates of *P. aeruginosa* are known to produce biofilm through secretion of the neutral polysacchairde psl and the secretion of the cationic polysaccharide pel, either in combination or independently (Franklin *et al.*, 2011, Byrd *et al.*, 2009), where visually mucoid isolates (such as CF isolates 3 and 4) are known to overproduce the anionic polysaccharide alginate (Mathee *et al.*, 1999, Martin *et al.*, 1993, Li *et al.*, 2005). A previous study has shown however, that even in spite of alginate overproduction, the formation of a biofilm is still dependent upon the production of Pel and Psl (Ghafoor *et al.*, 2011). The formation of biofilm serves several survival benefits to *P. aeruginosa* following growth in the CF lung including reducing the neutrophil respiratory burst (Jensen *et al.*, 1990), and provide resistance to antibiotics (Mah and O'Toole, 2001, Mah *et al.*, 2003). As mucin has been shown to partially disrupt and reduce *P. aeruginosa* biofilms (Haley *et al.*, 2014), variations in mucin production during excerbations (Henke *et al.*, 2007) is likely to influence this phenotype. In support of a previous study, there is no apparent link between motility and biofilm-forming potential (Head and Yu, 2004).

PAO1 and CF isolates 2, 3, 4, 5 and 6 all displayed varying degrees of swimming motility, with this being the greatest for CF isolate 5 (Figure 22). PAO1 is known to display swimming motility and was included as a positive control (Lindhout *et al.*, 2009, Rashid *et al.*, 2000). During early infection, flagella mediated swimming motility may facilitate amino-acid directed chemotaxis to the respiratory epithelium in CF airways (Schwarzer *et al.*, 2016). The presence of flagellin is known to induce an immune response in the host, from initiating airway inflammation, phagocytosis and the release of NETs, to the induction of antibodies (Hybiske *et al.*, 2004, Cobb *et al.*, 2004, Lovewell *et al.*, 2014, Floyd *et al.*, 2016, Anderson *et al.*, 1989). Losses in flagella mediated swimming motility has previously been documented in chronically infecting CF isolates of *P. aeruginosa* (Mahenthiralingam *et al.*, 1994) and may explain why CF isolates 1, 7 and 8 exhibited minimal swimming motility. Such losses may enhance persistence and survival in the CF lung through reductions in macrophage-mediated bacterial recognition and phagocytosis (Mahenthiralingam and Speert, 1995, Mahenthiralingam *et al.*, 1994, Luzar *et al.*, 1985), along with reducing TLR5 mediated airway inflammation (Hayashi *et al.*, 2001, Smith and Ozinsky, 2002).

The *P. aeruginosa* CF isolates all exhibited varying degrees of swarming motility (Figure 23). CF isolate 1 exhibited the greatest swarm zone, with finger-like projections radiating out from the central point of surface inoculation (Figure 23), with CF isolates 5 and 8 also producing large swarm zones. Conversely, CF isolates 2, 6 and 7 exhibited minimal swarming. Such variations may be a result of adaptations to the CF lung environment, where swarming motility has been suggested to facilitate *P. aeruginosa* movement through airway mucus (Fraser and Hughes, 1999), whilst decreases in this form of motility have been associated with the formation of aggregates, microcolonies and the development of structured biofilms (Shrout *et al.*, 2006).

In spite of this, *P. aeruginosa* swarming motility is also known to be highly variable and influenced by a number of laboratory conditions, from media composition, the drying time of swarm plates under laminar flow and the hardness of the agar surface (Kamatkar and Shrout, 2011, Tremblay and Deziel, 2008). Such factors have been shown to limit rhamnolipid production, with rhamnolipids serving to reduce the surface tension of the water surrounding the swarming cells (Caiazza *et al.*, 2005). Nitrogen availability within swarm agar is also known to influence *P. aeruginosa* swarming, where increased concentrations of nitrate impair this form of motility (Kohler *et al.*, 2000). Potassium nitrate was added to the swarm plates to allow comparisons to be made to *P. aeruginosa* swarming under normoxia and anoxia (discussed in chapter 4). As PAO1 has previously been shown previously to exhibit considerable swarming motility (Van Alst *et al.*, 2007, Overhage *et al.*, 2007), conditions such as the length of time the plates were allowed to dry under laminar flow and the addition of nitrate used in this study may have impaired the swarming of PAO1.

P. aeruginosa swarming is associated with protease expression (Overhage *et al.*, 2008). As shown in Figure 18**,** swarming CF isolates 1, 3, 4 and 5 all produced detectable levels of protease, although CF isolate 8 did not. Moreover, PAO1 and CF isolates 2, 6 and 7 exhibited minimal swarming, whilst PAO1 and CF isolates 2 and 7 all produced detectable levels of protease.

Wide phenotypic variation was also seen concerning susceptibility profiles of CF isolates to first line pseudomonal specific antibiotics: tobramycin, amikacin (both aminoglycosides) and ciprofloxacin (a fluoroquinolone). Whilst aminoglycosides bind to the 30S subunit of ribosome's inhibiting protein synthesis, the quinolones bind to the A subunit of DNA gyrase (Lambert, 2002). Two previous surveys identified that approximately 15% of *P. aeruginosa* clinical isolates exhibit resistance to aminoglycosides and fluoroquinolones (Williams *et al.*, 1984, Chen *et al.*, 1995). Furthermore, increases in treatment intensity has been associated with increased antibiotic resistance in CF isolates of *P. aeruginosa,* particularly multi-drug resistance (Smith *et al.*, 2016, Jansen *et al.*, 2016). As LBN broth was used instead of Mueller

Hinton broth to allow susceptibilities to be tested under anoxia (chapter 4), the MICs were not used to interpret whether isolates were sensitive or resistant using clinical breakpoints listed in the European Committee on Antimicrobial Susceptibility Testing guidelines for bacteria (European Committee on Antimicrobial Susceptibility Testing, 2018).

The transition to a mucoid phenotype is shown to be associated with an increase in antibiotic resistance, where negatively charged alginate polymers bind to cationic aminoglycosides (Nichols *et al.*, 1988). Despite this, visually mucoid CF isolates 3 and 4 did not display the greatest resistance to both tobramycin and amikacin (Figure 24). Interestingly, CF isolate 6 exhibited the highest MIC to the aminoglycoside tobramycin, as well as one of joint highest MIC to the aminoglycoside amikacin. Whilst *P. aeruginosa* exhibits a range of different resistance mechanisms to antibiotics, including drug efflux and enzymatic modification as summarised previously (Lambert, 2002), a lack of clinical data relating to the prescription of antibiotics and length of treatment, along with molecular analysis makes further comments difficult.

Whilst characterising *P. aeruginosa* from CF sputum has relied on sampling a few colonies from the most abundant morphotypes, the relevance of colony morphology within diagnostic microbiology laboratories is under challenge (Hill *et al.*, 2005). The advent of modern molecular techniques which detect bacterial species using PCR and mass spectrometry provide faster results and are often able to detect those species which are not easily recovered using traditional culture techniques, including obligate anaerobes such as *Prevotella* spp. (Bittar and Rolain, 2010, Salipante *et al.*, 2013, Weile and Knabbe, 2009). Furthermore, environmentally induced changes in *P. aeruginosa* CF isolate morphology can often make pathogen identification based upon traditional morphological characteristics difficult (Folkesson *et al.*, 2012). Different mucoid colonies obtained from the same CF sputum sample are shown to vary in their susceptibility to antibiotics, whilst mixing purified single colonies of the same morphotype also gives rise to different susceptibilities compared to when tested in isolation (Foweraker *et al.*, 2005). Furthermore, mixing four colonies of one morphotype has been shown to lead to different susceptibility profiles (Foweraker *et al.*, 2005). This may also explain why antimicrobial susceptibility results fail to provide benefits to CF patients in treating their pulmonary infections (Foweraker *et al.*, 2005, Foweraker *et al.*, 2009, Hurley *et al.*, 2012, Rosenfeld *et al.*, 2003).

Lastly, this chapter demonstrated MSA was a suitable medium to confirm the identity of *S. aureus* during routine passage, and provided an ideal selective medium to isolate *S. aureus* following co-culture with *P. aeruginosa.*

Despite using a small number of isolates, this study confirmed that colony morphotype is a poor predictor of other *P. aeruginosa* phenotypic traits (Clark *et al.*, 2015b). It also demonstrated a co-occurrence of phenotypes typically associated with markers of acute infection, including production of pyocyanin, protease and siderophores, along with markers of chronic infection, such as mucoidy (Hogardt and Heesemann, 2010, Carlsson *et al.*, 2011, Smith *et al.*, 2006a) (Mayer-Hamblett *et al.*, 2014b, Hogardt and Heesemann, 2010).

This study and others have shown that many phenotypes of *P. aeruginosa* can emerge and exist within CF airways, with mucoid and non-mucoid isolates, as well as motile and non-motile isolates being detected in relatively equal prevalence (Leone *et al.*, 2008, Fothergill *et al.*, 2010, Workentine *et al.*, 2013). Genetic indels, frameshift mutations and chromosomal inversions within the large genome of *P. aeruginosa*, encourage bacterial diversification (Darch *et al.*, 2015, Klockgether *et al.*, 2013) along with deletions and a reduction in its genome (Rau *et al.*, 2012). This is further driven by the accessory genome, where plasmids and genomic islands containing antibiotic resistance genes, can be transferred between strains, with the latter being integrated within chromosomal DNA (Juhas *et al.*, 2009). Comparisons of reference strains PAO1 and PA14 with CF isolates PA2192 (from a chronically infected individual with CF) and the Manchester epidemic strain C3719, has demonstrated that strain specific insertions of genetic information and deletions of specific chromosomal segments in other strains occurs at limited chromosomal loci (Mathee *et al.*, 2008).

Spatial segregation of bacterial communities has also been suggested to promote as well as maintain *P. aeruginosa* diversity (Winstanley *et al.*, 2016). Variations in CF mutation severity, nutrient availability, osmotic, oxidative and nitrosative stresses, mucus plugging, spatial distribution and bacterial competition are likely to exert numerous selective pressures upon *P. aeruginosa* (Zierdt and Schmidt, 1964, Markussen *et al.*, 2014, Hoffman *et al.*, 2010, Worlitzsch *et al.*, 2002). Additional pressures exerted by sub-inhibitory antibiotic concentrations and oxygen radicals as a result of the host's immune response can further drive *P. aeruginosa* diversity (Palmer *et al.*, 2005, Wright *et al.*, 2013, Boles *et al.*, 2004, Ciofu *et al.*, 2005). The growth of *P. aeruginosa* in fluoroquinolone supplemented medium for example has been shown to select for antibiotic resistance (Wong *et al.*, 2012). It is because of this, extensive within-lung and across-lung variation in the *P. aeruginosa* phenotype is commonplace (Clark *et al.*, 2015b, O'Brien *et al.*, 2017, Markussen *et al.*, 2014, Williams *et al.*, 2015). In turn, this will encourage adaptive radiation, where a bacterial population evolves to fill available niches (Kassen, 2009).

Social cheating is also likely to contribute to the wide phenotypic diversity that exists. Inactivation of the transcriptional activator *lasR* gene is a common phenomenon within CF and often precedes the switch to a mucoid phenotype (Hoffman *et al.*, 2009). It is possible that such mutants which do not provide detectable levels of different virulence factors such as

proteases, benefit from co-existing with isolates which are QS-proficient and which do secrete virulence factors. The emergence of such mutants are likely to provide fitness benefits, where LasR mutants have also been shown to exhibit enhanced growth in the presence of amino acids such as phenylalanine present within CF airway fluid, as well as exhibit resistance to β-lactam antibiotics (D'Argenio *et al.*, 2007).

3.6 Limitations

One of the limitations of this chapter is that additional phenotypic traits of *P. aeruginosa* were not studied, including type III secretion activity and auxotrophy which have been studied in *P. aeruginosa* CF isolates and are known to be altered during the course of chronic airway infection (Jain *et al.*, 2004, Jain *et al.*, 2008, Barth and Pitt, 1995). Sensitivity to other antibiotics used clinically such as azithromycin and colistin (Hoiby, 2011) were also not determined. Furthermore, other notable *P. aeruginosa* CF phenotypes were missing from the sample population, including RSCV's.

3.7 Future work

The aim of this chapter was to phenotypically characterise eight CF isolates of *P. aeruginosa* to inform future experiments in this thesis. However, future work would seek to include other notable *P. aeruginosa* phenotypes which are isolated from individuals with CF, including RSCV's which are associated with poor clinical outcomes (Haussler *et al.*, 1999). Moreover, whilst LBN broth was used as the growth medium, the use of sterile CF sputum or artificial CF sputum would allow phenotypic experiments to be conducted under more physiologically growth relevant conditions (Kirchner *et al.*, 2012).

Additional *P. aeruginosa* phenotypic traits known to be important in CF airway infection would also be studied, including type III secretion activity which is known to kill airway epithelia (Finck-Barbancon *et al.*, 1997, Fleiszig *et al.*, 1997). The production of these exoenzymes would be determined by immunoblotting. Elastase is known to degrade elastin within the airways, where its production across the isolates would be determined following the addition of *P. aeruginosa* cell-free culture supernatants to elastin congo red plates (Caballero *et al.*, 2001). Whilst swimming and swarming motility were studied, twitching motility is important in *P. aeruginosa* biofilm production (O'Toole and Kolter, 1998). This form of motility would be assessed following stab-inoculation of a *P. aeruginosa* colony into 1% (w/v) agar plates (Semmler *et al.*, 1999). The production of lipases known to degrade the lipid component of airway surfactants (Woo *et al.*, 2016) would also be determined following the addition of cell-free culture supernatants to egg yolk agar and to polyoxethylene sorbitans (Lonon *et al.*, 1988). A large number of *P. aeruginosa* CF isolates are known to display the auxotroph phenotype, with their growth being dependent upon the presence of specific amino acids (Barth and Pitt, 1995). Auxotrophy

across the eight CF isolates could be determined following their growth upon control plates (without amino acids) and a series of agar plates each supplemented different amino acids (Taylor *et al.*, 1992, Barth and Pitt, 1996). Compared to *P. aeruginosa*, the role of *S. aureus* within CF airway microbiology is poorly understood. Thus, phenotypic analysis of CF isolates of *S. aureus* would address this gap in the field.

3.8 Conclusion

This chapter employed classic phenotypic experiments to characterise eight CF isolates of *P. aeruginosa.* The results presented are supported by the published literature, including the wide morphological and phenotypic diversity known to exist across *P. aeruginosa* CF isolates. Despite the very small sample size, colony morphology appears to be a poor predictor of other phenotypic traits, including the production of exoproducts, as well as susceptibility to antibiotics. Furthermore, there is a lack of correlation between phenotypic traits produced by a single isolate.

4 Impact of anoxia upon the virulence properties of *P. aeruginosa* cystic fibrosis isolates and the interaction with *S. aureus* in co-culture

4.1 Chapter Transition

Characterising the CF clinical isolates of *P. aeruginosa* in chapter 3 supported previous evidence of the wide phenotypic diversity that exists across *P. aeruginosa*. Many factors are likely to impact upon bacterial phenotype, such as the selective pressures exerted by the host immune system and antimicrobial treatment strategies. Environmental factors unique to the CF lung are also likely to impact upon the phenotype of *P. aeruginosa* and their complex interactions with other common CF pathogens.

4.2 Introduction

The advent of culture-independent techniques has revealed the sheer diversity and abundance of the CF airway microbiome (Harris *et al.*, 2007, Rogers *et al.*, 2003, Rogers *et al.*, 2009, Bittar *et al.*, 2008, Stressmann *et al.*, 2011b). One study demonstrated that approximately 65% of pathogens detected by 16S rRNA sequencing were recovered using traditional microbiological culture, where increases in incubation time and culture under anoxia increased this cultivability to 84% (Sibley *et al.*, 2011). Molecular techniques also detect pathogens not typically seen in routine culture, such as anaerobic genera including *Prevotella* and *Gemella* (Mahboubi *et al.*, 2016), with obligate anaerobes reportedly making up nearly half of the CF microbial community in adults (Sibley *et al.*, 2011).

The microbial diversity of the CF lung is known to decrease as an individual approaches adulthood (Cox *et al.*, 2010, Frayman *et al.*, 2017), with the main driver having been associated with antibiotic use, rather than decreases in pulmonary function (Zhao *et al.*, 2012). Despite decreases in diversity, the bacteria *S. aureus* and *P. aeruginosa* remain the most prevalent CF pathogens (Filkins *et al.*, 2015, Cystic Fibrosis Trust, 2018). Whilst *S. aureus* colonises the lungs in the first few months of life and dominates during childhood, *P. aeruginosa* predominates in adolescence, with over 50% of individuals being colonised in adulthood (Cystic Fibrosis Trust, 2018).

This highly ordered and sequential nature of infection has been supported by numerous *in vitro* and *in vivo* studies demonstrating the ability of *P. aeruginosa* to outcompete *S. aureus* in co-culture (Filkins *et al.*, 2015, Baldan *et al.*, 2014a, Limoli *et al.*, 2016), including the hypervirulent MRSA strain USA 300 (Pastar *et al.*, 2013). *P. aeruginosa* can inhibit the growth of *S. aureus* through the secretion of numerous extracellular virulence factors, including the respiratory inhibitors pyocyanin, hydrogen cyanide and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO). It can also kill *S. aureus* through the secretion of the staphylolytic enzyme, LasA (Machan *et al.*, 1992, Hoffman *et al.*, 2006, Filkins *et al.*, 2015, Haba *et al.*, 2003, Mashburn *et al.*, 2005b, Korgaonkar *et al.*, 2013, Kim *et al.*, 2015). *P. aeruginosa* has also been shown to kill *S. aureus* indirectly. Laboratory strains and CF isolates of *P. aeruginosa* have been shown to upregulate the production of type-IIA-secreted phospholipase A2 by CF bronchial epithelia. Whilst this phospholipase enzyme exerts a minimal effect upon *P. aeruginosa*, it favours *S. aureus* killing (Pernet *et al.*, 2014).

Though *P. aeruginosa* is the most prevalent organism isolated from CF sputa later in life, *S. aureus* is detected in a third of those adults culture positive for *P. aeruginosa* and is present at higher rates than previously appreciated (Limoli *et al.*, 2016, Cystic Fibrosis Trust, 2018). Together, these two key opportunistic pathogens can co-exist as a community within CF airways and have been shown to occupy identical regional niches of the CF lung (Wakeman *et al.*, 2016, Hogan *et al.*, 2016). Co-colonisation of *S. aureus* and *P. aeruginosa* is also responsible for poor pulmonary function, increased exacerbations and mortality rates in CF, compared to mono-infection (Limoli *et al.*, 2016, Maliniak *et al.*, 2016, Hubert *et al.*, 2013).

Studies assessing *S. aureus-P. aeruginosa* interactions to date have been conducted under normoxia (21% environmental oxygen), with vigorous culture aeration (200-250 rpm) (Kessler *et al.*, 1993b, Baldan *et al.*, 2014a, Biswas *et al.*, 2009a, Fugere *et al.*, 2014a, Filkins *et al.*, 2015). However, *P. aeruginosa* has been shown to localise to hypoxic regions of thick static airway mucus within the lumen of CF airways (Worlitzsch *et al.*, 2002, Baltimore *et al.*, 1989). CF sputum has also been shown to contain an upper oxygenated zone and a lower anoxic zone (Cowley *et al.*, 2015).

The increased uptake by CF airway epithelia, intra-alveolar exudates, multiplying and respiring polymicrobial populations, extensive tissue damage and oxygen consumption by host phagocytes typically results in regions of the CF lung becoming anaerobic (Sanderson *et al.*, 2008, Worlitzsch *et al.*, 2002, Costerton, 2002, Werner *et al.*, 2004, Kolpen *et al.*, 2014, Stutts *et al.*, 1986). Furthermore, thick mucus plugs form a mix with neutrophils and other inflammatory products, leading to the formation of ‘mucopurulent material’ (Ribeiro *et al.*, 2005). This intraluminal gel is likely to influence oxygen exchange in the lower airways. The presence of obligate anaerobes within CF airways has also been shown to contribute to

disease severity and inflammation in CF airways (Sherrard *et al.*, 2016, Tunney *et al.*, 2008, Harris *et al.*, 2007).

Though *P. aeruginosa* is a facultative aerobe that preferably undergoes aerobic respiration, its ability to grow under anoxia is due to the presence of nitrate (Costerton, 2002, Schreiber *et al.*, 2007) which has been detected in both ASL and sputum of CF airways (Worlitzsch *et al.*, 2002, Yoon *et al.*, 2002, Hassett *et al.*, 2009). The presence of membrane bound nitrate reductase has been shown to facilitate the anaerobic growth of *P. aeruginosa* in CF sputum (Palmer *et al.*, 2007b), where nitrate ultimately undergoes a sequential eight-electron reduction to nitrogen (Yoon *et al.*, 2007). A microarray study of *P. aeruginosa* obtained from CF sputum detected genes essential for *P. aeruginosa* denitrification (Son *et al.*, 2007), whilst sera from individuals with CF have also been shown to contain antibodies to *P. aeruginosa* respiratory nitrate reductase 1 alpha chain (narg) and periplasmic nitrate reductase protein (napA) (Beckmann *et al.*, 2005).

Whilst anoxia has previously been shown to influence the *P. aeruginosa* phenotype including growth, biofilm production, virulence factor secretion and sensitivity to antibiotics (Fang *et al.*, 2013, Worlitzsch *et al.*, 2002, Lee *et al.*, 2011, Gaines *et al.*, 2005, Zimmermann *et al.*, 1991, Bragonzi *et al.*, 2005, Borriello *et al.*, 2004) its impact upon the interactions of *P. aeruginosa* with other CF pathogens has yet to be investigated. This chapter aimed to explore how the two more physiologically relevant conditions of static growth and anoxia found within regions of the CF lung, impact upon *P. aeruginosa* CF isolate virulence properties and their interaction with *S. aureus* in mixed planktonic co-culture and mixed species biofilm.

4.3 Aims

As *P. aeruginosa* is known to transition from free-swimming planktonic growth, to sessile biofilm growth, initial experiments aimed to determine the impact of oxygen availability upon the interspecies interactions between *S. aureus* and CF clinical isolates of *P. aeruginosa* in planktonic co-culture and mixed species biofilms *in vitro*.

Next, the impact of oxygen upon the production of a number of known anti-staphylococcal virulence properties were assessed. *P. aeruginosa* cell-free culture supernatants were also subjected to size fractionation and heat-treatment, in an attempt to decipher the identity of the *P. aeruginosa* virulence factor(s) which are likely to govern these complex interspecies interactions. Mass spectrometry was conducted to analyse the secretome of *P. aeruginosa* PAO1 and select CF isolates following growth under normoxia and anoxia, to strengthen the phenotypic data presented and provide insights into the effect of oxygen upon *P. aeruginosa* physiology and its ability to survive within CF airways. The impact of *P. aeruginosa* cell-free culture supernatants upon *S. aureus* biofilm disruption and inhibition was also determined under normoxia and anoxia, in addition to the impact of *S. aureus* cell-free supernatants upon *P. aeruginosa* growth and motility. Lastly, the influence of oxygen upon *P. aeruginosa* susceptibility to the anti-pseudomonal antibiotic tobramycin was determined.

4.4 Materials and Methods

Bacterial Culture. Single well isolated colonies of *S. aureus* or *P. aeruginosa* were inoculated into 10 mL of sterile LBN broth and grown at 37 °C under static conditions of normoxia or anoxia overnight (16 h).

Cross-Streak assay on solid agar. Overnight cultures of *S. aureus* and *P. aeruginosa* grown separately under normoxia or anoxia were pelleted, resuspended in fresh LBN broth and adjusted to an OD_{470} of 1.0. A sterile cotton swab was immersed in a given *P. aeruginosa* normalised culture and streaked horizontally across the surface of a LB agar plate. After air drying for 20 min, a sterile cotton swab was immersed in the normalised *S. aureus* culture and cross-streaked vertically across the surface of the agar plate. Plates were incubated either under normoxia or anoxia at 37 °C for 18 h, prior to being visually inspected for growth inhibition.

Mono-culture and co-culture planktonic growth curves. All growth curve experiments were conducted in 250 mL conical flasks containing 50 mL of LBN broth at 37 °C under static conditions. Overnight cultures of *S. aureus* and three of the *P. aeruginosa* CF isolates grown under normoxia or anoxia were pelleted, resuspended in fresh LBN broth and adjusted to an OD_{470} of 1.0. For co-culture growth curves, the bacteria were inoculated at a 1:1 ratio of *S. aureus*: *P. aeruginosa*, and incubated under static conditions at 37 °C for 24 h. Samples were taken at regular intervals, serially diluted in sterile 1x PBS (Fisher, UK) and 20 µL spots plated onto PIA and MSA, to allow differentiation between the two species. The plates were incubated for 18 h, prior to enumerating the colony forming units Log_{10}(CFU/mL).

The competition index (CI) and Relative Increase Ratio (RIR) were calculated. The RIR was calculated on single growth curve data using the *P. aeruginosa*:*S. aureus* ratio at a given time point, divided by the same ratio at the 0 h time point (inoculum). The same ratio was used to calculate the CI, although this used data from the mixed culture. A CI that differs statistically from the RIR indicates competition between the two organisms. This method was adapted from Macho *et al.,* (2007).

Mono-culture and co-culture biofilm formation. Overnight cultures of *S. aureus* and *P. aeruginosa* were centrifuged and adjusted to OD_{470} 1.0. Cultures were diluted ten-fold and 100 µL added to the central wells of a sterile 96-well plate either individually or in a 1:1 ratio for 1 h under static conditions, at 37 °C. An equal volume of broth was added to the individual culture to compensate for any dilution effect. After 60 min, the well contents were aspirated and replaced with fresh LBN broth. Plates were incubated for a further 24 h at 37 °C under static conditions. Following this, biofilms were washed twice using 200 µL of 1x PBS, detached using 100 µL of trypsin-EDTA (0.25%), collected, vortexed for 70 s, serially diluted and plated onto PIA and MSA. The plates were incubated for 18 h, prior to enumerating Log_{10}CFU/mL.

To determine biofilm biomass, biofilms were visualised by crystal violet staining, wells were washed twice with 200 µL of PBS and left to dry overnight. Wells were then stained with 200 µL 1% (w/v) crystal violet for 10 min, prior to two further washes in deionised water. Plates were dried overnight, prior to the stain being solubilised with 200 µL of 30% (v/v) acetic acid. The solubilised stain was then transferred to a new 96-well plate and the OD read at OD_{492}.

Preparation of *P. aeruginosa* cell-free culture supernatant. Overnight cultures of *P. aeruginosa* were centrifuged at 4,000 x *g* for 10 min at 4 °C. Each supernatant was sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter and stored at −20°C until use. To confirm sterility after each preparation, a small volume of the supernatant was streaked onto LB agar plates and incubated for 20 h prior to reading. For size exclusion experiments, 10 mL of cell-free supernatant was added to a 3 kDa molecular weight cut off protein concentrator and centrifuged at 4,000 x *g* for 1 h. Apical and basal volumes were subsequently added to 2 mL sterile microcentrifuge tubes. For heat treated fractions, microcentrifuge tubes containing cell-free supernatant were added to a heat block and boiled at 95 °C for 10 min, prior to cooling.

Determination of total protease production. Protease production was determined using skimmed milk agar. Cell-free supernatants (40 µL) from overnight cultures of *P. aeruginosa* grown under normoxia or anoxia were loaded into wells in agar plates and incubated at 37 °C for 24 h. Hydrolysis of the milk protein casein results in a clear zone surrounding the bacterial supernatant and would show evidence of protease production. LBN medium was also loaded as a negative control. The diameter of the clearance zones was measured in mm.

Staphylolytic activity. This method was adapted from (Kong *et al.*, 2005, Grande *et al.*, 2007). An overnight culture of *S. aureus* grown under static normoxia conditions was centrifuged at 4,000 x *g* for 10 min at 4 °C, prior to the pellet being resuspended in 250 µL of 25 mM diethanolamine buffer, pH 9.5. The bacteria were heated at 100 °C for 10 min, before being diluted to a final optimal density OD_{595} of 1.0. 400 µL of adjusted heat-killed *S. aureus* was then added to each microtube. The cell-free supernatant from each *P. aeruginosa* isolate was diluted 1:10 with diethanolamine buffer, prior to 100 µL being added to the heat-killed *S. aureus.* The plates were read after 60 min, with lysis of heat-killed *S. aureus* shown by absorbance decreases in $OD_{595.}$ The addition of LBN broth to heat-killed *S. aureus* was used as the negative control, whilst PAO1 cell-free supernatant is known to lyse heat-killed *S. aureus* and served as a positive control (Mashburn *et al.*, 2005b, Radlinski *et al.*, 2017).

Pyocyanin extraction and quantification in single and co-culture. This method was adapted from (Essar *et al.*, 1990, Wu *et al.*, 2014). Overnight cultures of *P. aeruginosa* and *S. aureus* grown separately under normoxia or anoxia were pelleted, resuspended in fresh medium and adjusted to an OD_{470} of 1.0. For single cultures, 500 µL of *S. aureus* or *P.*

aeruginosa were added to a 250 mL conical flask containing 49.5 mL of LBN broth (1:100 dilution). For co-cultures, 500 µL of *S. aureus* and *P. aeruginosa* were added in a 1:1 ratio, to a 250 mL conical flask containing 49 mL of LBN broth. The flasks were incubated at 37 °C for 24 h, under static normoxia or anoxia. After this period, samples were taken and serially diluted in PBS and plated onto PIA to determine the Log_{10} CFU/mL.

To quantify pyocyanin production, bacterial cells were pelleted by centrifugation at 4,000 x *g* for 25 min at 4 °C and the supernatant sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter. 7.5 mL of the sterile supernatant was added to 4.5 mL of chloroform and vortexed for ten, 2 s intervals. The sample was centrifuged at 4,000 x *g* for 1 min at 4 °C, prior to 3 mL of the blue-green phase (chloroform phase) being aspirated into a new tube. 1.5 mL of 0.2 M hydrochloric acid was then added to the tube and vortexed again for ten, 2 s intervals, prior to centrifugation at 4,000 x *g* for 1 min at 4 °C. 100 µL of the pink coloured phase was transferred into a 96-well plate. 100 µL of hydrochloric acid was added as a blank control. The plate was read at OD_{520} and multiplied by the extinction co-efficient 17.072 to determine the concentration of pyocyanin per mL of supernatant (Essar *et al.*, 1990).

Drop collapse assay. Cell-free supernatants from overnight cultures of *P. aeruginosa* grown under normoxia or anoxia were serially diluted (1:1) in sterile dH_2O containing 0.0005% (w/v) crystal violet for visualisation across a 96-well plate. A total of 20 µL of each dilution (including neat supernatant) was spotted onto the underside of a 96-well plate lid and the plate titled at a 90° angle. The assay works on the principle that if the droplet contains surfactants, the drops spread. However, as the quantity of surfactants decrease by dilution, the droplet eventually beads up due to an increase in surface tension. Surfactant scores are equal to the reciprocal of the greatest dilution at which there was surfactant activity (a collapsed drop).

***P. aeruginosa* pyoverdine production.** Overnight cultures of *P. aeruginosa* and *S. aureus* grown under normoxia or anoxia were pelleted, resuspended in fresh medium and adjusted to an OD_{470} of 1.0. 500 µL of each culture was added to a 250 mL conical flask containing 49.5 mL of LBN broth for mono-culture, or to 49 mL of LBN broth for co-culture. The flasks were incubated at 37 °C for 24 h, under static normoxia or anoxias. To measure pyoverdine production, 100 µL of the cell-free supernatant was added to a black 96-well plate and the fluorescence read at excitation and emission wavelengths 400/460 nm as performed previously (Andersen *et al.*, 2015, Krzyzanowska *et al.*, 2016). The background level of fluorescence was measured using 100 µL LBN broth only.

Semi-quantification of AHLs. AHL production was determined using two *E. coli* biosensor strains, pSB536 and pSB1142, kindly provided by Professor Paul Williams (University of Nottingham). pSB536 was grown routinely in LB broth supplemented with 50 µg/mL ampicillin, whilst pSB1142 was grown in LB broth supplemented with 10 µg/mL tetracycline. Both biosensor strains were grown overnight (16 h) at 37 °C. Each given biosensor strain was normalised to an OD_{470} of 1.0 and diluted 1:100 into a sterile 96-well plate. 100 µL of cell-free supernatants from 24 h cultures of PAO1 or CF isolates were added to the biosensor strain. LBN broth only was added to each biosensor as the negative control. Plates were sealed with a Breathe-easy® membrane and incubated for 6 h at 37 °C. Bioluminesence was read and divided by the OD_{470} of the biosensor strain, to take into account differences in growth rates and final biosensor densities. Values were subsequently subtracted from the negative control (LBN broth with biosensor only), to correct for background luminescence values.

Bacterial motility. Swimming motility of *P. aeruginosa* was investigated using 0.3% (w/v) nutrient agar plates supplemented with nutrient broth and 1% (w/v) potassium nitrate. Swarming of *P. aeruginosa* was determined using 0.5% (w/v) of nutrient agar plates supplemented with nutrient broth, dextrose and 1% (w/v) potassium nitrate. Overnight cultures of *P. aeruginosa* were adjusted to OD_{470} of 1.0 and a total of 5 µL of culture inoculum was added to the centre of each plate. To measure the effects of *S. aureus* exoproducts upon *P. aeruginosa* motility, a 1:100 diluted *S. aureus* cell-free culture supernatant was added to the agar plate. Plates were incubated under static normoxia or anoxia for 24 h at 37 °C. The diameter of the motility zone travelled by *P. aeruginosa* was measured in mm.

***S. aureus* biofilm inhibition and disruption.** *S. aureus* was grown statically overnight under normoxia, prior to being diluted to an OD_{470} of 1.0. A 1:10 dilution was made and a total of 100 µL was added into the central wells of a sterile 96-well plate. For the biofilm inhibition experiment, 100 µL of *P. aeruginosa* cell-free supernatants (from overnight cultures grown under normoxia or anoxia) were added to the wells, with 100 µL of LBN broth being added to *S. aureus* as a negative control. The plates were sealed with a Breathe-easy® membrane and incubated statically at 37 °C for 24 h under normoxia. For the biofilm disruption experiment, 100µL of *P. aeruginosa* cell-free supernatants (from cultures grown under normoxia or anoxia) were added to the plate following 24 h incubation. The supernatants were added for 5 h, with 100 µL of LBN broth to *S. aureus* was used as the control. In both experiments, biofilm production was visualised by crystal violet staining.

Solubilisation of secreted proteins for MS. Five pooled overnight cultures of *P. aeruginosa* and grown under normoxia or anoxia were pelleted following centrifugation at 4,000 x *g* for 30 min at 4 °C. The supernatant containing extracellular proteins was sterile filtered through a 0.22 µm polyethersulfone membrane filter. Supernatants were concentrated using Amicon 3kDa cut-off filters prior to precipitation with 25% (w/v) trichloroacetic acid for 15 min on ice.

Proteins were pelleted following centrifugation at 14,000 x *g* for 10 min and the pellets washed with acetone. Protein pelleted were solubilised in 50 mM pH 7.4 Tris-HCl containing 2 mM CHAPS, 7 M urea and 7 M thiourea using an ultrasonicating probe (30 s sonication per cycle, 65% full power, 2 cycles) and quantified against a BSA calibration curve using the Bradford assay.

SDS-PAGE of bacterial secretomes. Proteins (30 µg) were reduced in Laemmli buffer for 15 min at 65 °C and separated by SDS-PAGE on a 10% polyacrylamide gel. Separated proteins were stained with Coomassie G250 blue (0.5% in 40% aqueous methanol and 10% glacial acetic acid) for 1h and destained in an aqueous solution of 10% ethanol and 7.5% glacial acetic acid.

Protein in-gel digestion. Protein in-gel digestion was performed following the protocol of Schevchenko *et al.* (Shevchenko *et al.*, 2006), with minor modifications. Each sample lane was divided into 5 bands (approximately equal size), gel bands were excised and diced in a clean polypropylene tube using a sterile scalpel. Gel pieces were subsequently destained with 50% acetonitrile in 50 mM ammonium bicarbonate, dehydrated with acetonitrile and vacuum dried for 30 min. Proteins were in-gel digested using trypsin in 3 mM ammonium bicarbonate (25:1 protein to trypsin ratio; Trypsin Gold, sequencing grade) coupled with shaking at 550 rpm at 37 °C overnight.

Extraction of peptides. Peptides were extracted for 15 min in an ultrasonic bath initially using pure acetonitrile equivalent to 50% of sample volume. Further extractions were performed with 150 µL of 50% acetonitrile in 50 mM ammonium bicarbonate. Finally, 400 µL of pure acetonitrile was used to fully dehydrated the gel pieces and maximise peptide extraction. Each time the complete peptide extract was collected in a sterile polypropylene tube. Finally, peptide extracts were vacuum dried and stored at −20 °C prior to analysis.

Mass spectrometry. Samples were reconstituted in 50 µL of 3% aqueous acetonitrile and 0.1% formic acid for liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) analysis. Peptides were separated and analysed using an nLC system (Dionex 3000, ThermoScientific, UK) coupled to 5600 TripleTof (AB Sciex, UK) operating in information dependent (IDA) mode. Peptide solution (10 µL) was injected onto a trap column (PepMapTM, C18, 5 µm, 100 Å, 300 µm x 1 mm, ThermoScientific, UK) using 2% of eluent B (98% acetonitrile in aqueous 0.1 % formic acid) at a flow rate of 30 µL/min. Peptides were subsequently separated on an analytical column (AcclaimTM, PepMapTM C18, 3 µm, 100 Å, 75 µm x 150 mm, ThermoScientific, UK) with the following gradient: 0-3 min 2% B, 3-48 min 2-45% B, 48-52 min 45-90% B, 52-55 min 90% B, 55-70 min 2% B). Electrospray was formed by spraying the nLC eluate at 2500 V using a PicoTipTM emitter (New Objective, Germany). The 10 most intense ions from each MS survey scan were selected for MS/MS, while acquired

ions were temporarily excluded from MS/MS acquisition for 30 s. The mass spectrometer was calibrated prior to acquisition to ensure a high mass accuracy (<10 ppm) on both MS and tandem mass spectrometry (MS/MS) levels.

Data analysis. Relative quantification was done using QI for proteomics software (version 4, Nonlinear Dynamics, UK). MS/MS data were searched using MasconDeamon (ver 2.5) against the SwissProt database, with the following search restriction parameters: mass tolerance of 0.1 Da for MS and 0.6 Da for MS/MS spectra, a maximum of 2 trypsin miscleavages, *Pseudomonas aeruginosa* taxonomy, variable modifications of methionine oxidation and cysteine carbamidomethylation.

Antibiotic susceptibility assay to tobramycin. Overnight cultures of *P. aeruginosa* grown under normoxia or anoxia were normalised to an OD_{470} of 1.0 and diluted to 10^6 CFU/mL and 100 µL added to 100 µL of serially diluted concentrations of tobramycin (64-0.125 µg/mL). Plates were sealed with a Breathe-easy® membrane and incubated statically for 24 h at 37 °C under normoxia or anoxia. The MIC was determined by visual inspection.

Statistical Analysis. All results unless otherwise specified are expressed as mean ±S.E.M., with data for each experiment being collected from three independent replicates (N=3), each replicate performed in triplicate. All statistical analyses were performed using GraphPad Prism 6 software (Graphpad, La Jolla, CA, USA) with significance being set to P<0.05. The specific tests and *post-hoc* tests used for each experiment are described in the figure legends.

4.5 Results

4.5.1 Inhibition of *S. aureus* growth by *P. aeruginosa* CF clinical isolates

Laboratory strain PAO1 and the eight *P. aeruginosa* CF clinical isolates were tested for growth inhibition of *S. aureus* under normoxia, using an agar plate based cross-streak assay. As shown in Figure 26, following 24 h incubation, PAO1 and CF isolates 1, 2, 4, 5 and 7 all inhibited *S. aureus* growth in co-culture (dashed white box). Conversely, CF isolates 3, 6 and 8 exerted no effect upon *S. aureus* growth.

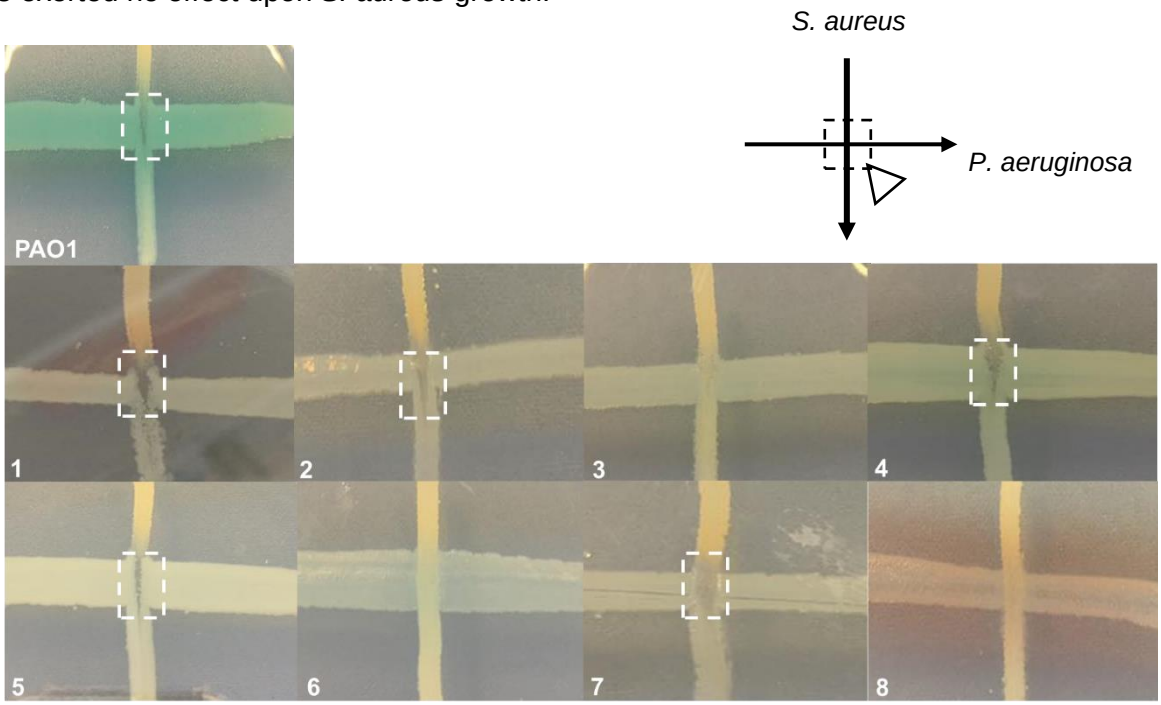


Figure 26. Cross-streak growth inhibition assay between CF clinical isolates of *P. aeruginosa* with *S. aureus* on solid LB agar plates. Overnight bacterial cultures of *S. aureus* and *P. aeruginosa* grown under normoxia were normalised to an OD_{470} of 1.0. *P. aeruginosa* was inoculated horizontally across the centre of the agar plate and allowed to dry. *S. aureus* was subsequently streaked vertically. Plates were incubated overnight, prior to being read. Zones of growth inhibition are marked by white dashed boxes. Images are representative of three individual experiments (*N*=3), each performed in duplicate.

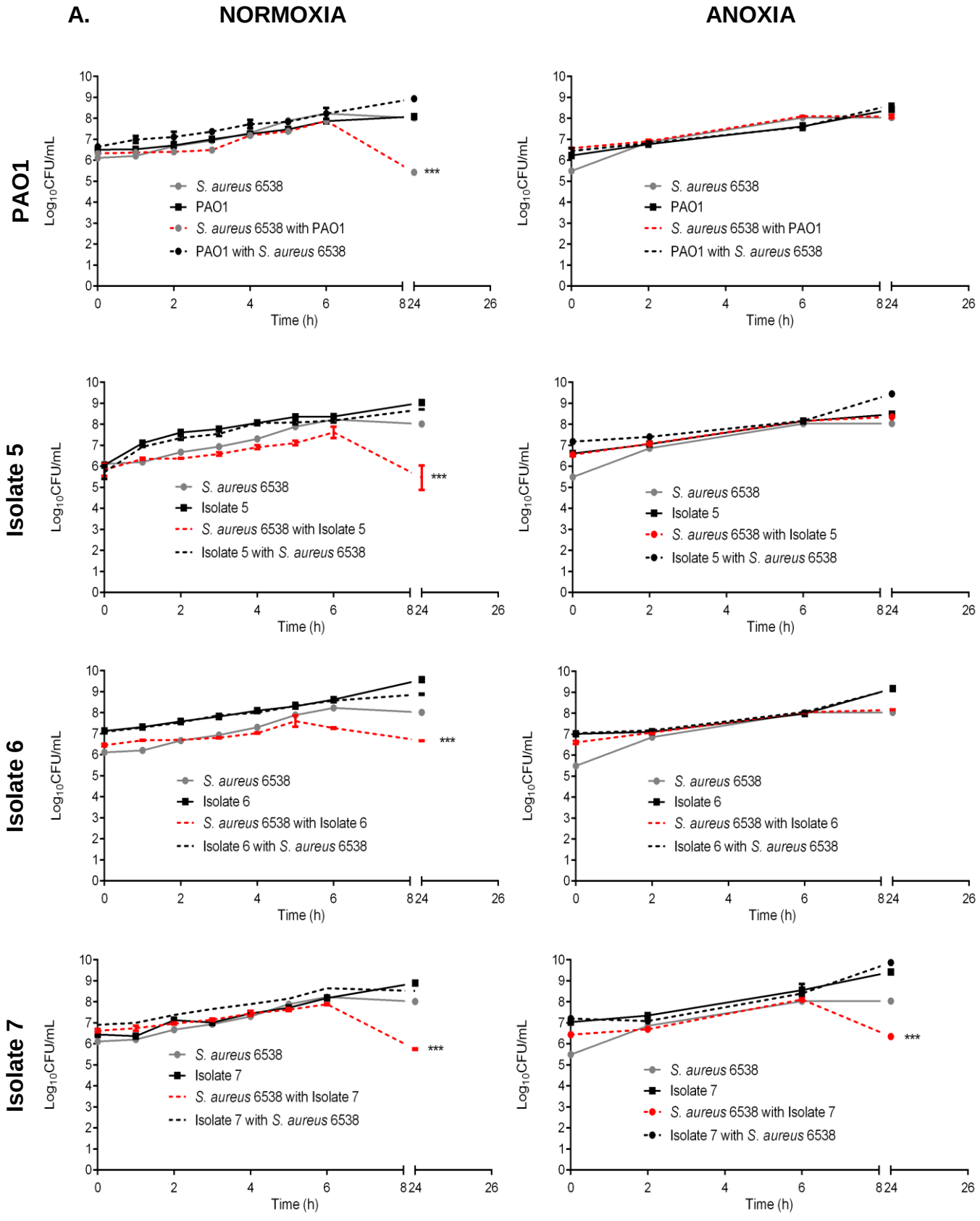
4.5.2 Influence of anoxia upon competition between *S. aureus and P. aeruginosa* in planktonic co-culture

From the phenotypic characterisation performed in chapter 3 and the results obtained in Figure 26, PAO1 and three CF clinical isolates of *P. aeruginosa* were carefully selected to determine whether changes in oxygen availability influenced planktonic (free-swimming) interactions overtime. CF isolates 5 and 7 were both non-mucoid and exhibited protease and staphylolytic activity. Yet whilst CF isolate 5 produced pyocyanin and exhibited swimming and swarming motility, CF isolate 7 did not. CF isolate 6 was also non-mucoid but didn't produce detectable levels of protease or staphylolytic activity. CF isolate 6 also exhibited smaller swim zones and no swarming motility, in addition to the lowest biofilm biomass.

Density matched *S. aureus* and *P. aeruginosa* overnight cultures were inoculated at 1:1 ratio, with planktonic growth being assessed over 24 h, comparing the colony counts of the bacteria grown in pure culture, to those grown in co-culture. To clearly compare the differences of growth in monoculture with co-culture, the CI and RIR were calculated. The CI compared differences in the growth of mixed cultures, whilst the RIR compared the growth of the two species in monoculture. Both the CI and RIR were used to determine statistical significance.

As shown in Figure 27 (panel A), PAO1 and all the CF isolates tested were able to outcompete *S. aureus* under normoxia. PAO1 caused an approximate 2.5 log reduction in *S. aureus* CFU/mL when co-cultured at 24 h (P<0.001), whilst maintaining the same growth rate as it did in pure culture. CF isolate 5 also caused an approximate 2.5 log reduction in *S. aureus* CFU/mL at 24 h (P <0.001), whilst CF isolate 6 also induced a significant reduction in *S. aureus* at 24 h, but this was to a lesser extent (an approximate 1.5 log reduction). CF isolate 7 also induced an approximate 2.5 log reduction in *S. aureus* at 24 h (P<0.001). PAO1 was a unique isolate under normoxia however. Whilst the CI for all the CF isolates was significantly higher than the RIR at the 6 and 24 h time points only (panel B), PAO1 was also able to outcompete *S. aureus* at 1, 2, 5, 6 and 24 h. In all co-cultures under normoxia, *S. aureus* killing was incomplete, with *S. aureus* still being detected at high bacterial counts.

Upon repeating the experiments under anoxia, as shown in Figure 27 (panel A), *S. aureus* growth was unaffected by the presence of *P. aeruginosa* PAO1 and CF isolates 5 and 6 at all the time points tested. Interestingly, CF isolate 7 was the only isolate to retain its ability to dominate at 24 h, causing an approximate 1 log reduction in the growth of *S. aureus* (P<0.001) compared to monoculture.


A.
NORMOXIA
ANOXIA
PAO1
Isolate 5
Isolate 6
Isolate 7
Log10CFU/mL
Time (h)
S. aureus 6538
PAO1
S. aureus 6538 with PAO1
PAO1 with S. aureus 6538
Isolate 5
S. aureus 6538 with Isolate 5
Isolate 5 with S. aureus 6538
Isolate 6
S. aureus 6538 with Isolate 6
Isolate 6 with S. aureus 6538
Isolate 7
S. aureus 6538 with Isolate 7
Isolate 7 with S. aureus 6538
***

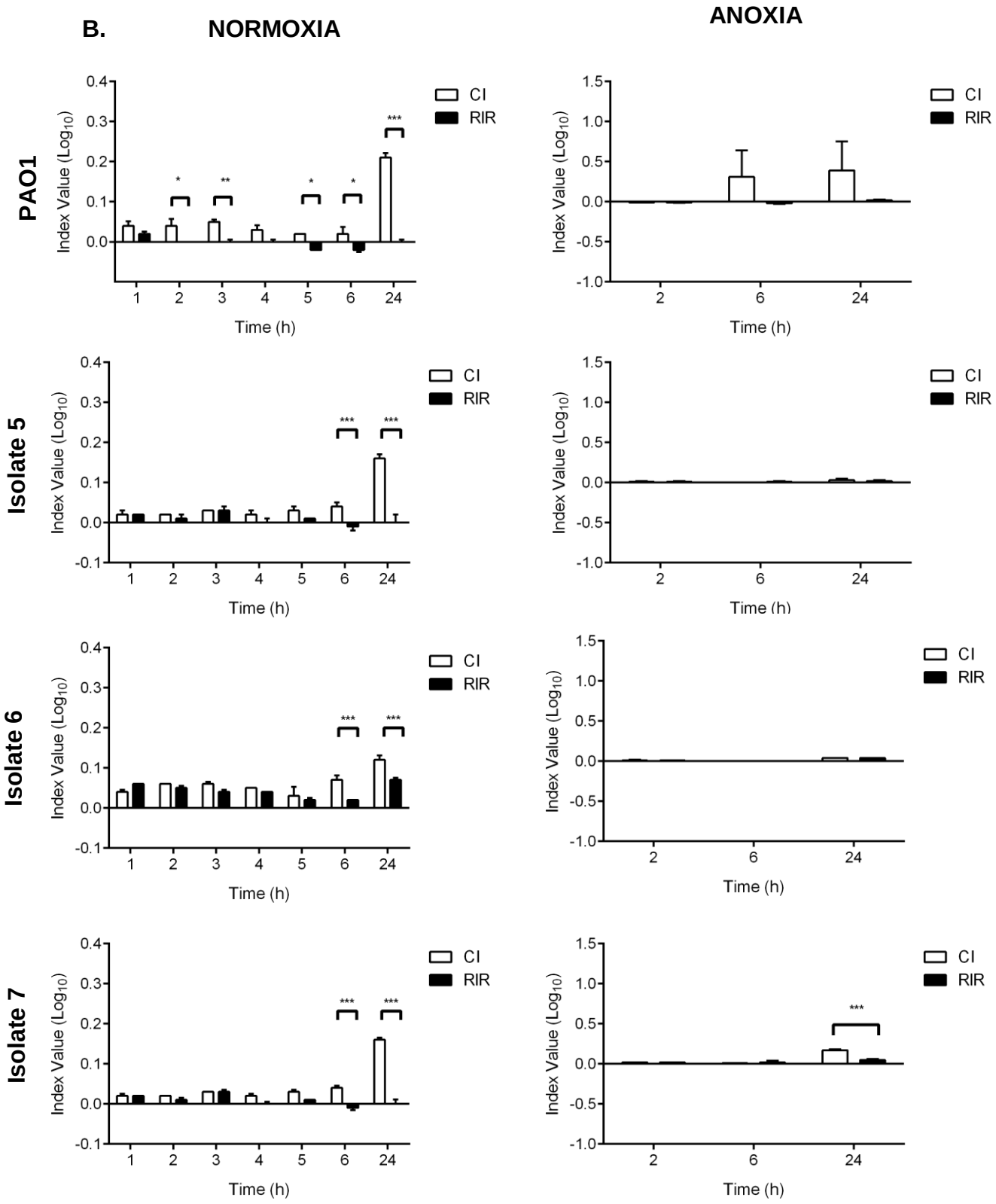


Figure 27. *S. aureus* and *P. aeruginosa* mono and co-culture growth curves under normoxia and anoxia. Panel A: *S. aureus* and *P. aeruginosa* were grown statically at 37 °C for 24 h in either single or dual culture (1:1 ratio), under normoxia or anoxia. At regular intervals, aliquots were taken and plated onto PIA and MSA. Each data point represents the mean ± S.E.M of three independent experiments (*N*=3), each performed in triplicate. Panel B: Each value represents the mean of the CI and RIR ± S.E.M of three independent experiments (*N*=3), each performed in triplicate. Statistical differences were determined using two-way ANOVA comparing the CI to the RIR, with Bonferroni's multiple comparisons test. **P*<0.05, ***P*<0.01 and ****P*<0.001.

4.5.3 Composition of mixed *S. aureus*-*P. aeruginosa* biofilms

Although *S. aureus* and *P. aeruginosa* interact in planktonic culture, both bacteria typically grow in mixed species biofilms within CF airways. The number of viable sessile bacteria within single and mixed (1:1) cultures was subsequently determined, along with the effect of these interspecies interactions upon biofilm biomass.

As shown in Figure 28 (panel A), under normoxia *S. aureus* viability was reduced in the presence of PAO1 (P<0.01) and CF isolates 5 (P <0.001) and 7 (P <0.001), causing a significant decrease in viable *S. aureus* compared to monoculture. Unlike planktonic culture (Figure 27), CF isolate 6 was unable to outcompete *S. aureus* in mixed species biofilm. Conversely as shown in Figure 28 (panel B), *S. aureus* was unable to exert a detrimental effect upon any of the *P. aeruginosa* isolates tested, with CF isolates of *P. aeruginosa* being recovered from mixed species biofilms in numbers comparable to those isolated in single species biofilm. CF isolate 7 however, demonstrated a significant increase in viability in mixed species biofilm compared to single culture (P<0.05).

Under anoxia, PAO1 and CF isolates 5 and 6 were no longer able to outcompete *S. aureus* in mixed species biofilm (Figure 28) whilst CF isolate 7 retained its ability to predominate. This finding supports the planktonic co-culture data shown in Figure 27. Once more, all CF *P. aeruginosa* isolates tested were unaffected by the presence of *S. aureus* under anoxia (Figure 28), producing biofilm at levels similar to those in single species biofilms.

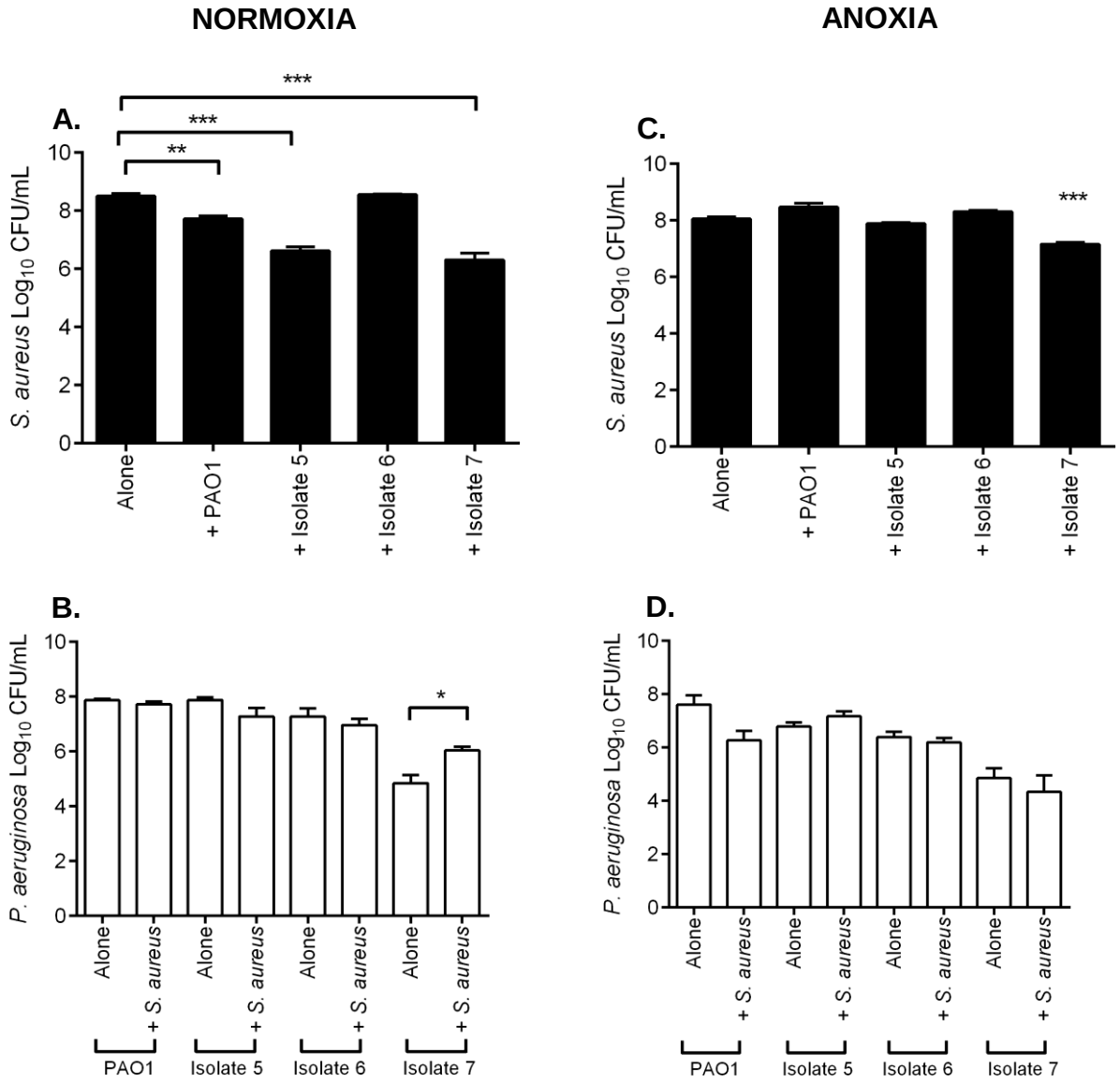


Figure 28. *S. aureus* and *P. aeruginosa* mono and co-culture biofilms under normoxia and anoxia. *S. aureus* and *P. aeruginosa* were grown in a 96-well plate either individually or in a 1:1 ratio for 24 h at 37 °C under static conditions of normoxia or anoxia. Biofilms were washed using PBS, detached, serially diluted and plated onto PIA and MSA for quantification. Bars represent the mean ± S.E.M of three individual experiments (*N*=3), each performed in triplicate. Statistical differences were determined using one-way ANOVA with Tukey's *post-hoc*. **P*<0.05, ***P*<0.01, ****P*<0.001.

As shown in Figure 29, quantification of single and mixed species biofilms showed that biomass production for PAO1 and CF isolates 5 and 7 were unaffected by the presence of *S. aureus* under normoxia. An increase in biofilm biomass was seen for CF isolate 6 however, when co-cultured with *S. aureus* (P<0.05). Under anoxia, the same trend was seen, with an increase in biomass also being detected in the mixed species biofilm of *S. aureus* and CF isolate 6 (P <0.05).

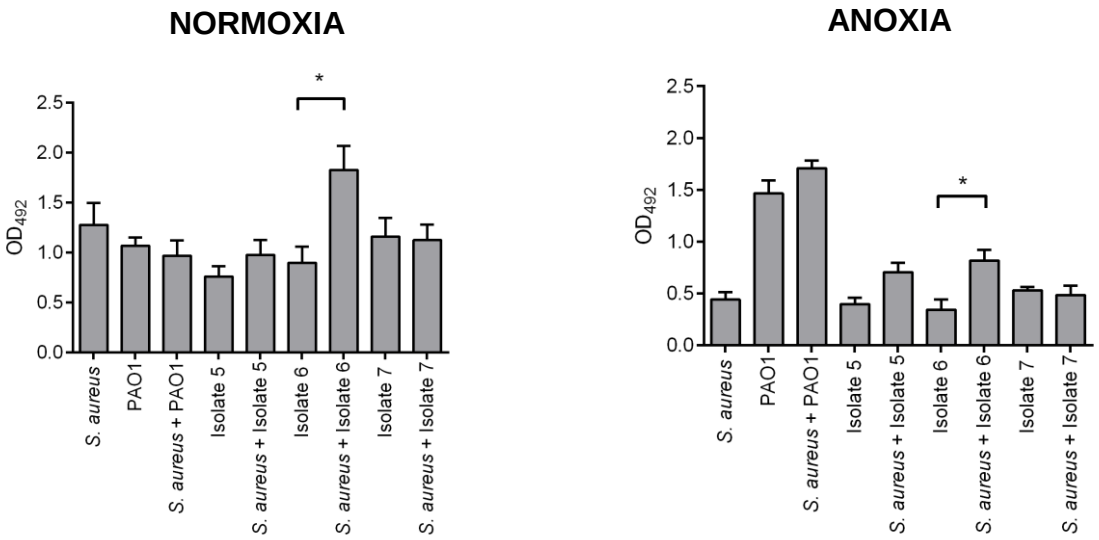


Figure 29. *S. aureus* and *P. aeruginosa* mono and co-culture biofilm biomass under normoxia and anoxia Bacteria were grown in a 96-well plate either individually or in a 1:1 for 24 h at 37 °C under normoxia or anoxia. Biofilm was quantified by crystal violet staining and acetic acid solubilisation, prior to measuring the absorbance at OD_{492}. Bars represent the mean ± S.E.M of three individual experiments (N=3), each performed in triplicate. Statistical differences between single and mixed species biofilms were determined using one-way ANOVA with Tukey's *post-hoc*. *P<0.05.

4.5.4 Influence of oxygen availability upon *P. aeruginosa* protease production and staphylolysis

To greater understand the mechanisms governing these interspecies interactions, the production of a number of known anti-staphylococcal compounds across the *P. aeruginosa* CF isolates were determined following culture under anoxia and comparing these to results obtained under normoxia.

Cell-free supernatants obtained from overnight cultures of *P. aeruginosa* grown under anoxia were added to wells of skimmed milk agar plates and incubated for 24 h to determine protease activity. The diameter of the zone of clearance was measured from the edge of the wells in mm. The clearance zones were normalised to the bacterial densities of the cultures grown.

The data obtained for protease production under normoxia was taken from Figure 18 to allow comparisons to be made to anoxia. Under anoxia (Figure 30), laboratory strain PAO1 and CF isolates 1 and 3 lost their ability to produce detectable levels of protease compared to normoxia (P<0.001). *P. aeruginosa* CF isolates 6 and 8 failed to produce any detectable protease under both normoxia and anoxia, whilst CF isolates 4, 5 and 7 produced significantly more protease under anoxia compared to normoxia (P<0.01, P<0.05 and P<0.01 respectively).

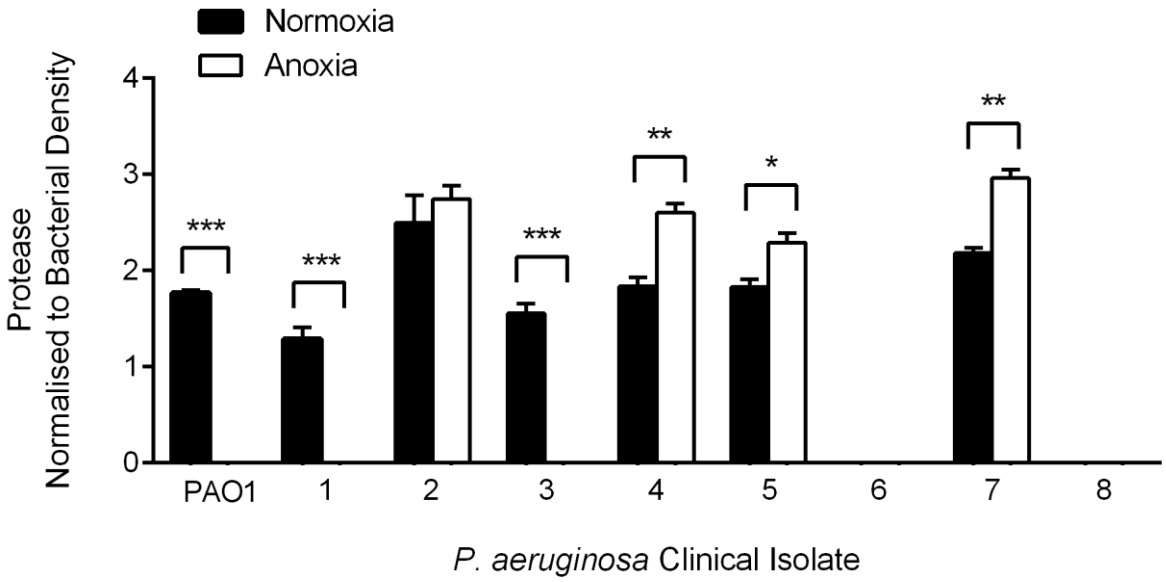


Figure 30. Influence of oxygen availability upon protease production by *P. aeruginosa* CF clinical isolates. Cell-free supernatants obtained from cultures of *P. aeruginosa* grown under normoxia oranoxia were added to skimmed milk agar plates and incubated for 24 h, prior to the diameter of the zones of clearance being measured. Data shown are the mean ± S.E.M. of three independent experiments (N=3), each performed in triplicate. Zones of clearance were normalised to account for differences in final bacterial cell density. Statistical differences were determined using an unpaired two-tailed *t*-test comparing each isolate under normoxia and anoxia. *P<0.05, **P<0.01 and ***P<0.001.

Next, the effect of anoxia upon the ability of *P. aeruginosa* cell-free supernatants to lyse *S. aureus* was determined. Cell-free supernatants from overnight cultures of *P. aeruginosa* grown under anoxia were added to suspensions of heat-killed *S. aureus* and incubated for 1 h, prior to plate being read at OD_{595}. The control bar represents the negative control, consisting of heat-killed *S. aureus* with sterile LBN broth. Any detectable decreases in OD after 1 h were evidence of *S. aureus* lysis. This established method was adapted from those described in previous studies (Grande *et al.*, 2007, Kessler *et al.*, 1993b, Kong *et al.*, 2005).

The data obtained for the lysis of heat-killed *S. aureus* under normoxia was taken from Figure 19 to allow comparisons to be made to staphylolytic activity under anoxia. As shown in Figure 31 below, CF isolates 2, 4, 5 and 7 retained their staphylolytic ability under anoxia (white bars), causing significant reductions in the OD_{595} of heat-killed *S. aureus* (*P*<0.05, *P*<0.05, *P*<0.001, *P*<0.01 respectively). Culture supernatants obtained from CF isolates 6 and 8 were unable to lyse *S. aureus* under both normoxia and anoxia.

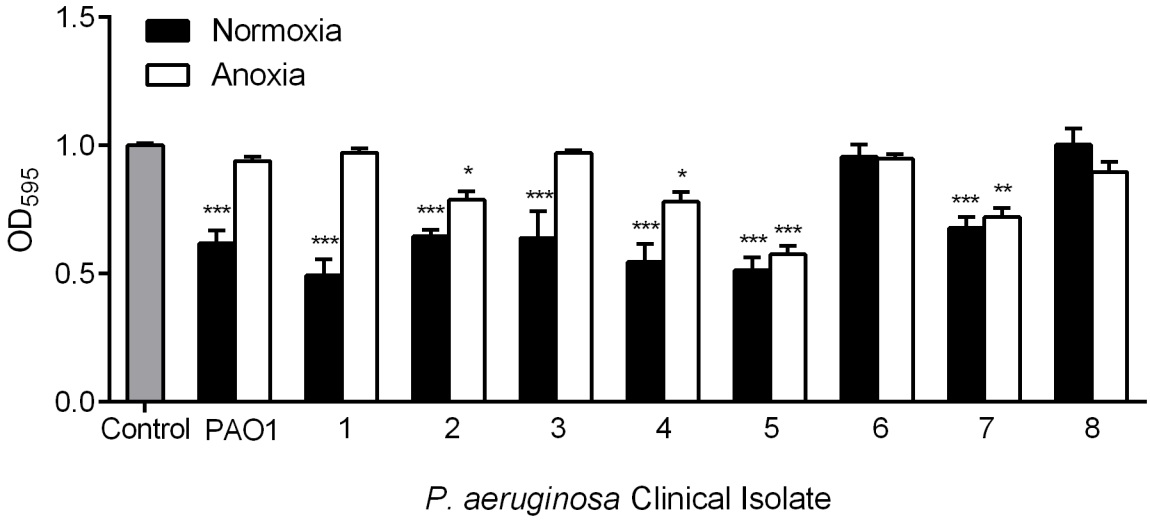


Figure 31. Influence of oxygen availability upon the staphylolytic activity of *P. aeruginosa* CF clinical isolates. Cell-free supernatants obtained from *P. aeruginosa* CF isolates grown under normoxia or anoxia were added to heat-killed *S. aureus* and incubated for 1 h prior to the OD being read at 595 nm. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in quadruplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc* (vs. control). Control consisted of heat-killed *S. aureus* with the addition of LBN broth only. **P*<0.05, ***P*<0.01 and ****P*<0.001.

4.5.5 The impact of oxygen upon *P. aeruginosa* pyoverdine production

The presence of the major iron-binding siderophore pyoverdine was determined in cell-free supernatants of PAO1 and the three selected CF isolates of *P. aeruginosa*. As pyoverdine is fluorescent, the amount produced was determined by measuring the RFU following excitation at 460 nm and emission at 490 nm. Experiments compared pyoverdine production by *P. aeruginosa* under monoculture conditions, as well as following co-culture with *S. aureus*. Additionally, experiments conducted under normoxia were compared to experiments repeated under anoxia.

Under normoxia (Figure 32), as expected *S. aureus* did not produce any detectable pyoverdine. PAO1 and CF isolate 5 both secreted high amounts of pyoverdine, compared to CF isolates 6 and 7. The presence of *S. aureus* in the co-culture enhanced the production of pyoverdine by PAO1 only (*P*<0.001) and not for CF isolates 5, 6 and 7.

Under anoxia, PAO1 secreted significantly reduced levels of pyoverdine compared to normoxia, in both monoculture (*P*<0.01) and in co-culture with *S. aureus* (*P*<0.001). CF isolate 5 also produced significantly lower levels of pyoverdine under anoxia in monoculture (*P*<0.05) and in co-culture with *S. aureus* (*P*<0.01). CF isolates 6 and 7 again produced minimal levels of pyoverdine under anoxia, in both monoculture and co-culture which were significantly lower compared to CF. Under anoxia, the presence of *S. aureus* enhanced pyoverdine production in both PAO1 and CF isolate 5.

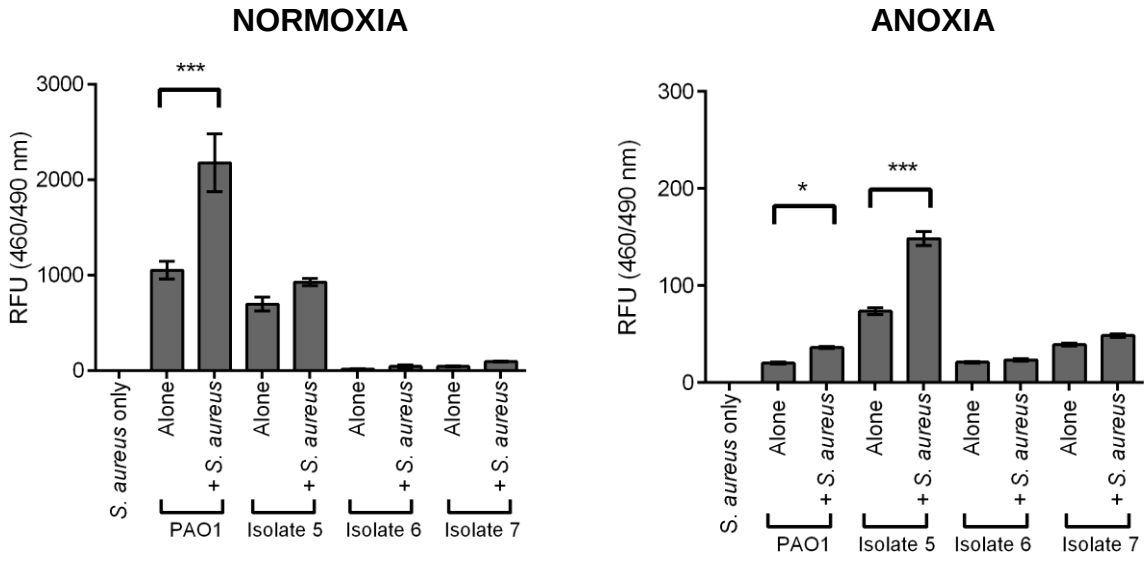


Figure 32. RFU as a measure of pyoverdine production. RFU of cell-free supernatants obtained following growth of PAO1 and *P. aeruginosa* CF isolates 5, 6 and 7 grown in the presence or absence of *S. aureus* was measured at 460/490 nm. Bars represent the mean ± S.E.M of three individual experiments (*N*=3), each performed in triplicate. Statistical differences between single and co-cultures were determined using one-way ANOVA with Tukey's *post-hoc*. **P*<0.05, ****P*<0.001.

4.5.6 Effect of oxygen and co-culture with *S. aureus* upon pyocyanin production by *P. aeruginosa*

The production of the green phenazine pyocyanin by *P. aeruginosa* was determined under both normoxia and anoxia, including after co-culture with *S. aureus*. As expected, no pyocyanin was detected for *S. aureus.* No significant differences (*P*>0.05) were observed for *P. aeruginosa* PAO1 pyocyanin production between growth in monoculture and co-culture with *S. aureus,* a finding also seen in CF isolates 5 and 6 (

Figure **33**). CF isolate 7 failed to secrete any detectable pyocyanin. As shown in

Figure **33**, no pyocyanin was detected for any of the *P. aeruginosa* isolates grown under anoxia (supported by panels B and C), suggesting that its production or detection is dependent upon the presence of oxygen. PAO1 and CF isolates 5 and 6 both produced pyocyanin following culture under normoxia (Figure 15).

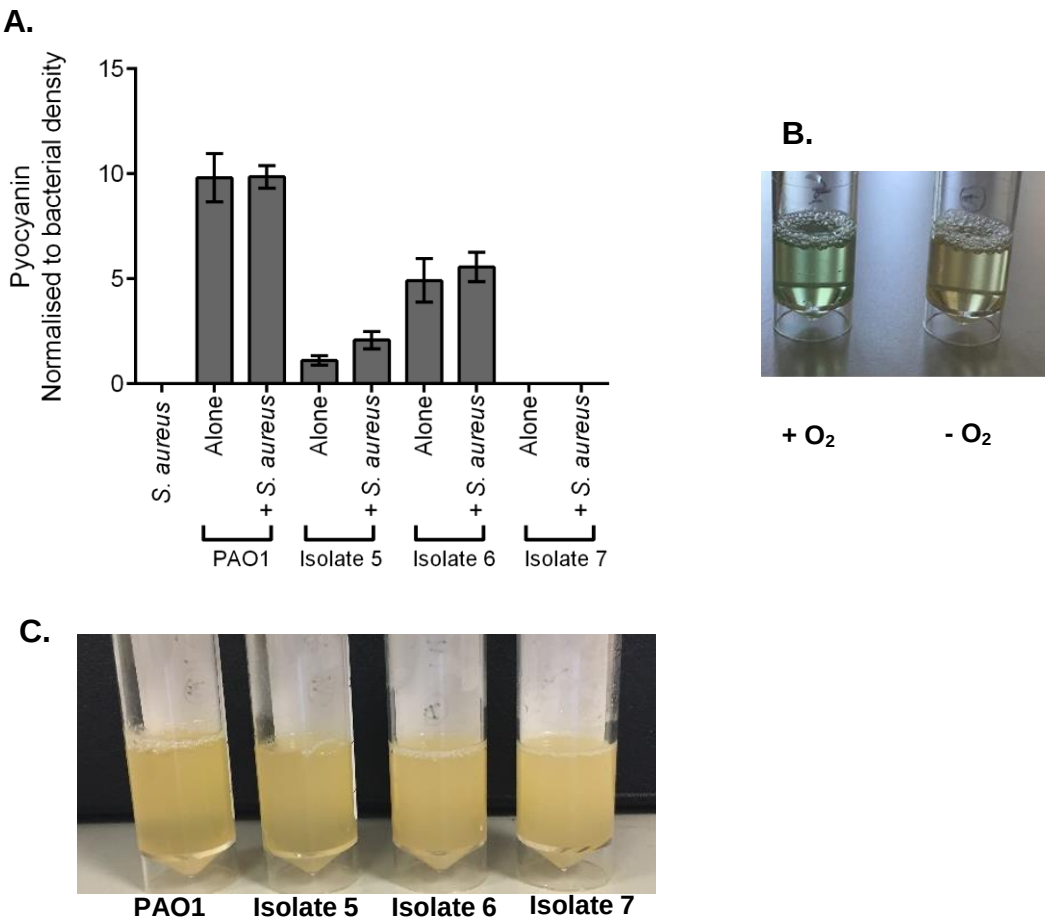


Figure 33. *P. aeruginosa* pyocyanin production in the presence and absence of *S. aureus*, following growth under normoxia and anoxia. *P. aeruginosa* CF isolates were grown in the presence or absence of *S. aureus*, following growth under normoxia or anoxia. (A) Pyocyanin was extracted by phenol-chloroform extraction and normalised to the bacterial density. Bars represent data ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. Statistical differences between single and co-cultures were determined using one-way ANOVA with Tukey's *post-hoc*. (B) PAO1 cell-free supernatants obtained after cultures grown overnight under normoxia (+O2) and anoxia (-O2). (C) P. aeruginosa PAO1 and CF isolates 5, 6 and 7 demonstrate a lack of detectable pyocyanin following 16 h growth under anoxia.

4.5.7 Effect of oxygen upon *P. aeruginosa* surfactant activity

The surfactant activity of *P. aeruginosa* PAO1 and the three CF isolates was determined using a well-established drop collapse assay. Surfactant scores were determined as the reciprocal of the greatest dilution to which surfactant activity was quantifiable. As shown in Figure 34, CF isolate 7 demonstrated the greatest surfactant activity under normoxia, whilst CF isolate 6 exhibited the lowest surfactant activity. Under anoxia, CF isolate 7 again exhibited the highest surfactant activity, whilst CF isolate 6 exhibited the lowest activity. The surfactant activity of PAO1 and CF isolate 5 was halved following growth under anoxia.

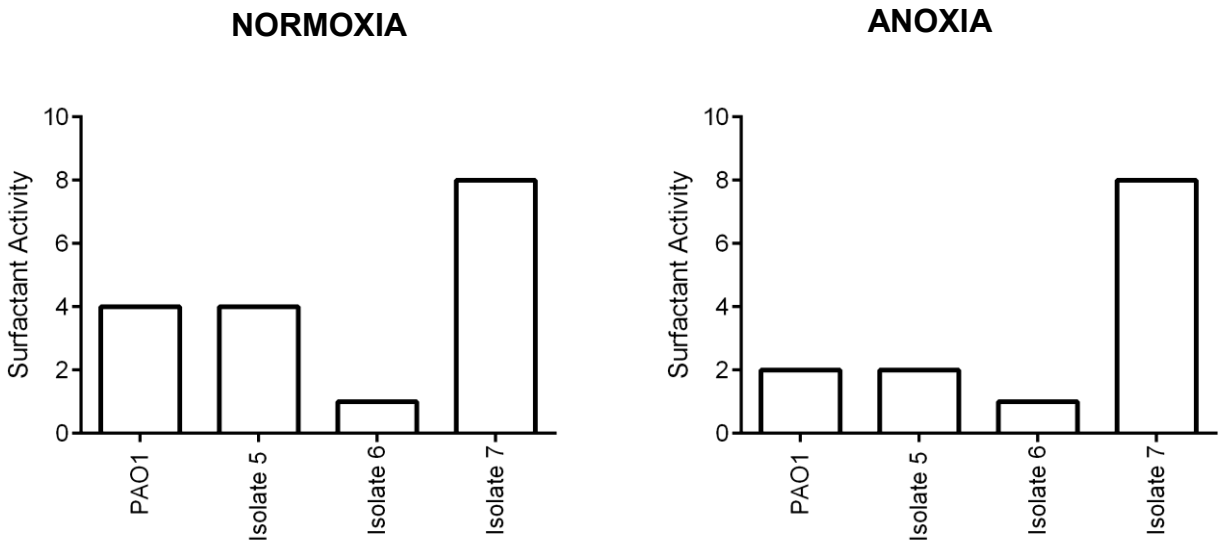


Figure 34. Drop collapse assay to measure surfactant activity of *P. aeruginosa*. The surfactant activity of *P. aeruginosa* cell-free supernatants from overnight cultures grown under normoxia or anoxia was determined using the drop collapse assay. Cell-free supernatants were serially diluted two-fold in dH_2O containing 0.0005% (w/v) crystal violet for visualisation. Small 20 µL drops were added to the underside of a petri plate lid and tilted at a 90° angle. Surfactant scores are expressed as the reciprocal of the greatest dilution to which surfactant activity was quantifiable. Columns represent the mean of three independent experiments (*N*=3), each performed in triplicate.

4.5.8 The impact of anoxia upon the secretome of *P. aeruginosa*

This study also investigated the impact of anoxia upon the cell-free secretome of PAO1 and CF isolates 5, 6 and 7. Whilst this study was only preliminary (*N*=1 from 5 pooled samples for each isolate and each environmental condition), it aimed to provide an insight into the effects of oxygen on the *P. aeruginosa* secretome.

Initial studies showed that a large number of proteins were identified for each isolate (<100), with the total number varying across isolates and following growth under normoxia and anoxia. The secretome data for laboratory strain PAO1 obtained in this study was compared to the online Swissprot database. One of the difficulties regarding the interpretation of data for these CF clinical isolates was selecting a *P. aeruginosa* reference strain in the database (e.g. PAO1 or PA14). To prevent bias and potential errors, the results were not filtered for a specific reference strain of *P. aeruginosa* within the database.

Due to the large number of proteins identified for each isolate tested, many of which were housekeeping proteins (essential for survival, including DNA replication, respiration, protein synthesis and cell division), a total of 20 were selected for analysis. These proteins focused particularly upon major *P. aeruginosa* virulence factors and those likely to play a role in withstanding host defences. As 0.22 µm sterile filters were used to remove cells and cell debris, it is likely that this may influence the total abundance and range of proteins detected within the bacterial culture supernatants.

Considering the enzymes involved in anaerobic respiration, as shown in Figure 35, PAO1 demonstrated a 17.5-fold increase in the presence of denitrification regulatory protein NirQ under anoxia, compared to normoxia. Conversely, there was a 39.5-fold decrease in periplasmic nitrate reductase under anoxia, along with a 2.8-fold decrease for nitrite reductase under anoxia. CF isolate 5 failed to produce any detectable denitrification regulatory protein NirQ or periplasmic nitrate reductase under both conditions tested, whilst there was a minimal fold change regarding the production of nitrite reductase under both environmental conditions. A 4.2-fold increase in NirQ was seen under anoxia for CF isolate 6, whilst periplasmic nitrite reductase was not detected. A minimal fold change for nitrite reductase was seen under anoxia for CF isolate 6. CF isolate 7 failed to produce any detectable denitrification regulatory protein NirQ, whilst there was a minimal fold change in periplasmic nitrate reductase under anoxia and a 1.7-fold increase in nitrite reductase under anoxia.

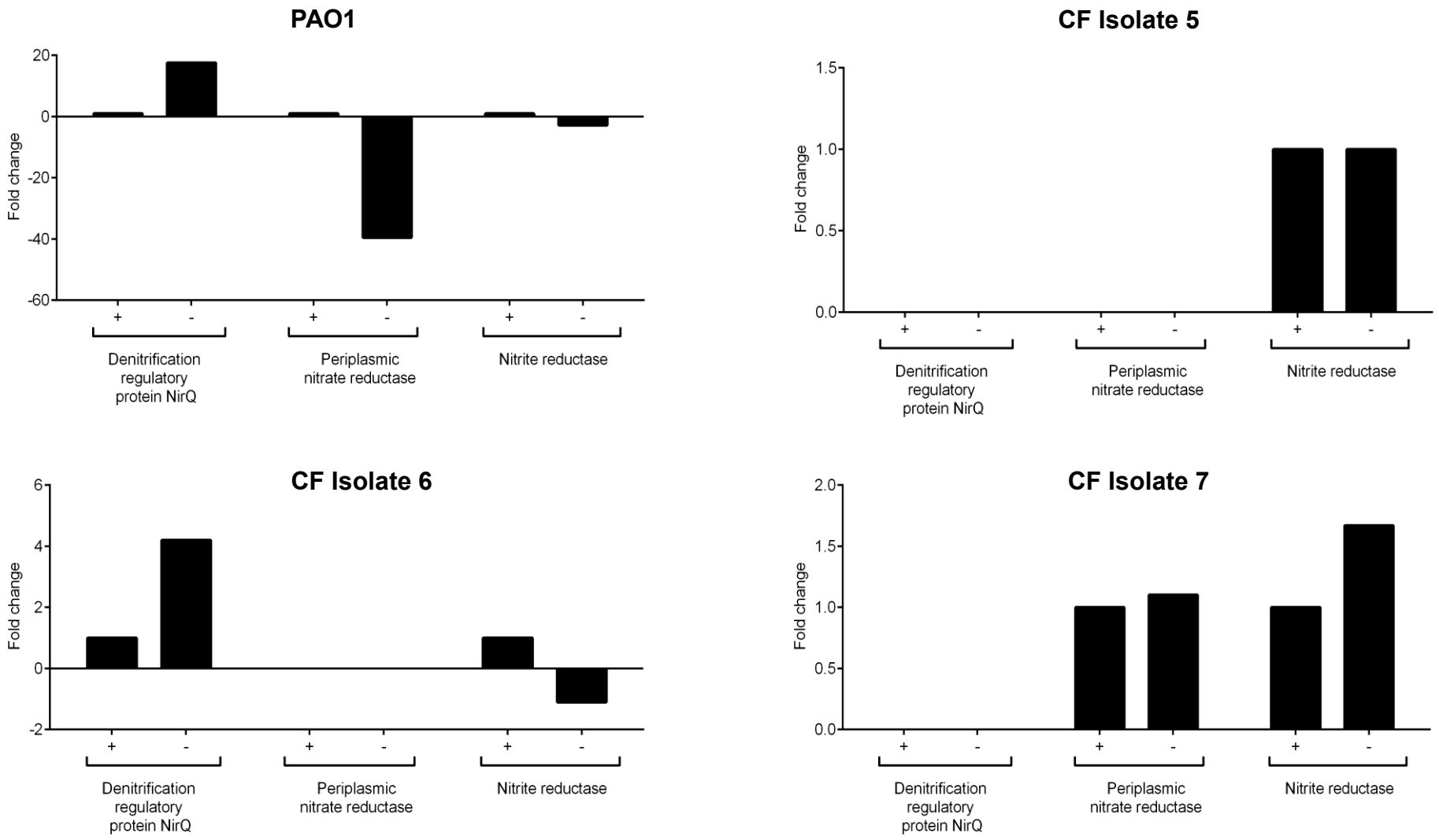


Figure 35. *P. aeruginosa* secretome analysis relating to anaerobic respiration. Cell-free supernatants obtained from 24 h cultures of PAO1 and CF isolates 5, 6 and 7 grown under normoxia and anoxia were subject to protein precipitation, SDS-PAGE electrophoresis and MS analysis. Data represents *N*=1 from 5 pooled samples. The abundance of each protein is expressed as fold-change compared to normoxia.

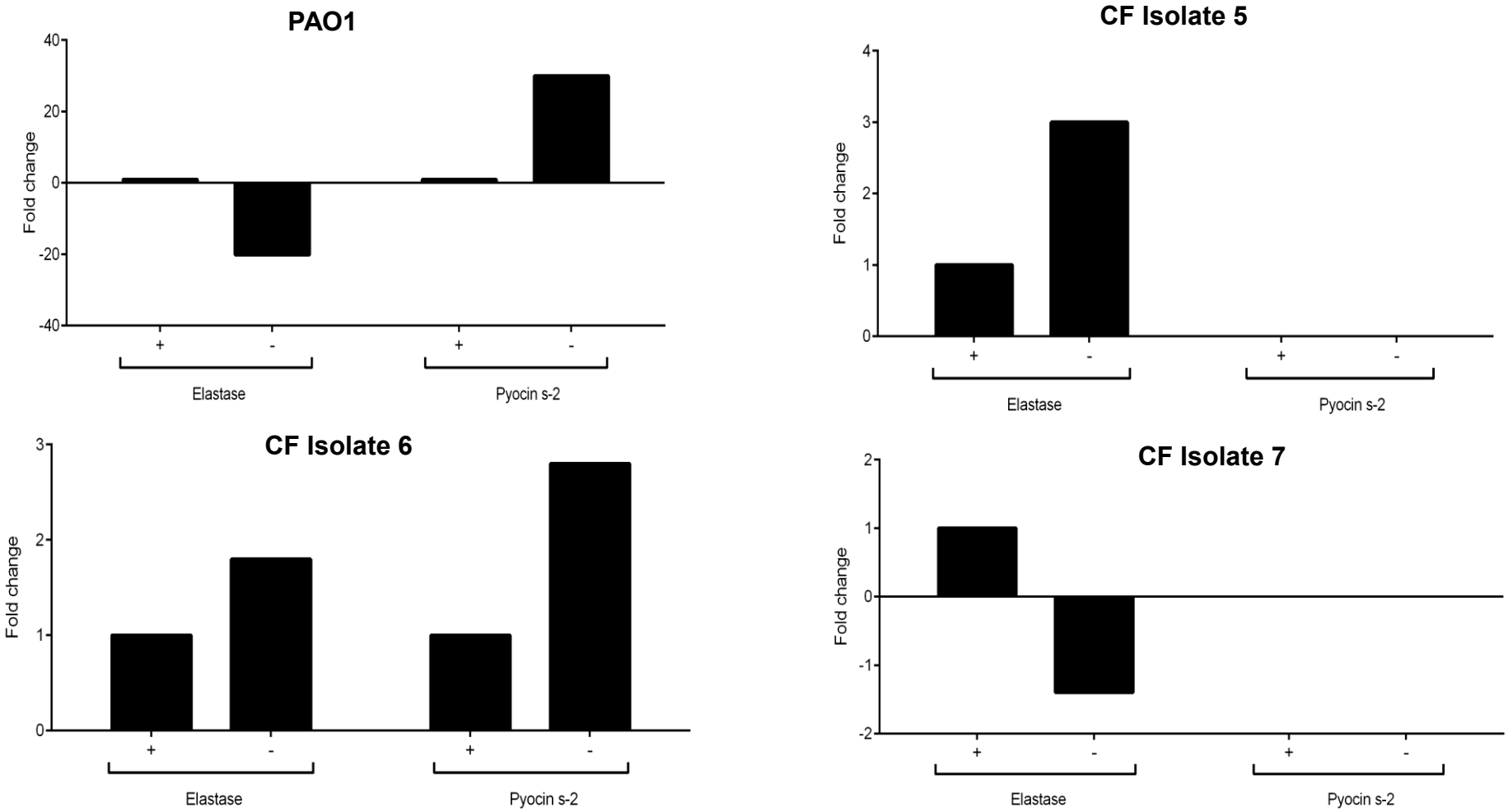


Figure 36. *P. aeruginosa* secretome analysis relating to elastase production and pyocin s-2. Cell-free supernatants obtained from 24 h cultures of PAO1 and CF isolates 5, 6 and 7 grown under normoxia and anoxia were subject to protein precipitation, SDS-PAGE electrophoresis and MS analysis. Data represents *N*=1 from 5 pooled samples. The abundance of each protein is expressed as fold-change compared to normoxia.

As shown in Figure 36, a 20.2-fold decrease in elastase was detected under anoxia for PAO1, compared to normoxia. CF clinical isolate 5 exhibited a 3.0-fold increase of elastase under anoxia, whilst CF isolate 6 exhibited a 1.8-fold increase in elastase under anoxia. CF isolate 7 exhibited a 1.4-fold decrease in the production of elastase under anoxia.

PAO1 was shown to produce the anti-pseudomonal compound pyocin-S2, with a 30-fold increase under anoxia. Whilst pyocin was not detected for CF isolate 5, there was a 2.8-fold increase under anoxia by CF isolate 6 compared to normoxia. Like CF isolate 5, CF isolate 7 failed to produce any detectable pyocin under normoxia and anoxia.

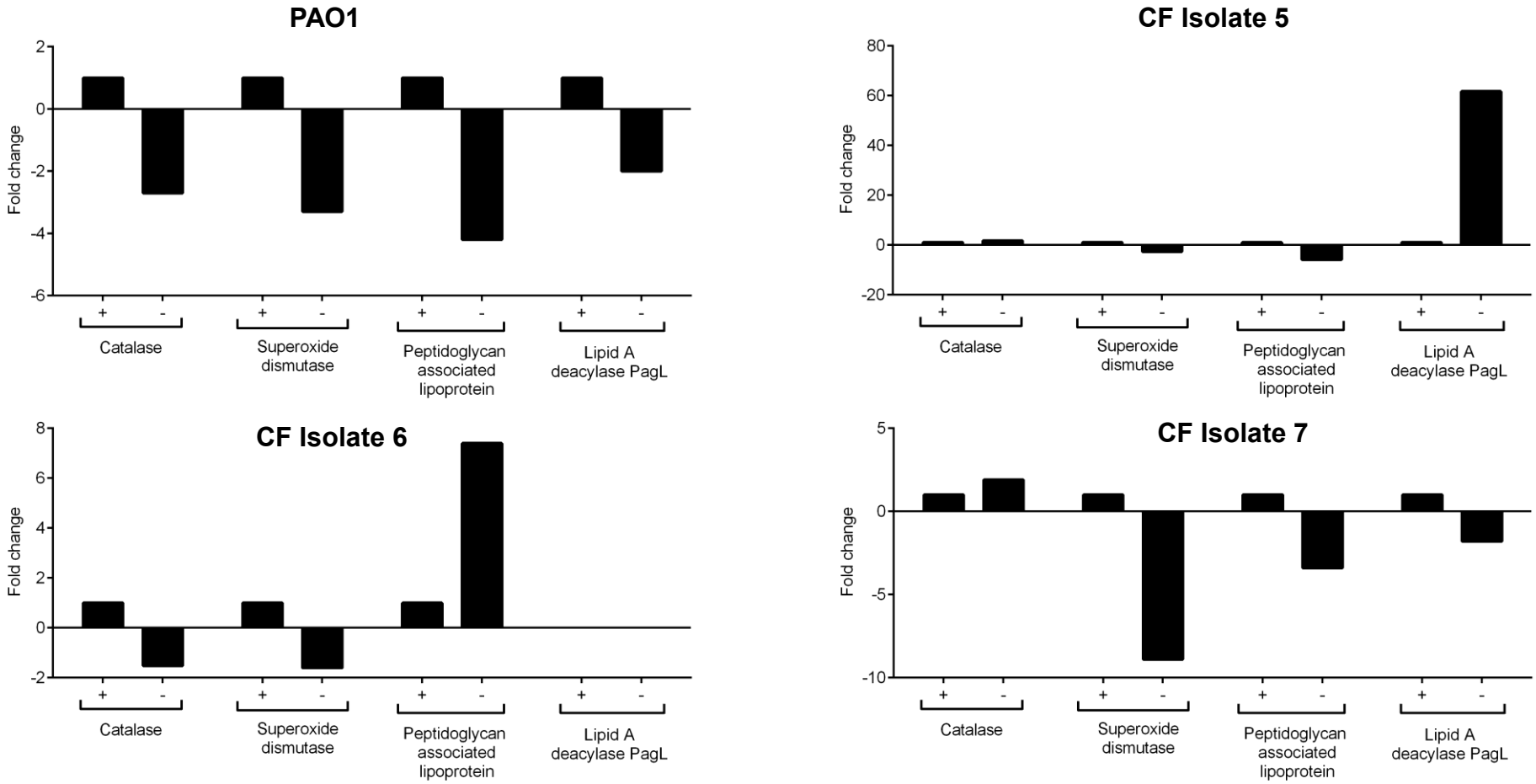


Figure 37. *P. aeruginosa* secretome analysis relating to immune detection. Cell-free supernatants obtained from 24 h cultures of PAO1 and CF isolates 5, 6 and 7 grown under normoxia and anoxia were subject to protein precipitation, SDS-PAGE electrophoresis and MS analysis. Data represents *N*=1 from 5 pooled samples. The abundance of each protein is expressed as fold-change compared to normoxia.

As shown in Figure 37, PAO1 exhibited a 2.7-fold decrease in catalase under anoxia, whilst a 3.3-fold decrease in superoxide dismutase was detected under anoxia. CF isolate 5 secreted detectable catalase, with a 1.8-fold increase under anoxia, whilst exhibiting a 2.7-fold decrease in superoxide dismutase under anoxia. CF isolate 6 also produced detectable catalase, with a 1.5-fold decrease under anoxia and a 1.6-fold decrease in superoxide dismutase under anoxia. CF isolate 7 exhibited a 1.9-fold increase in the production of catalase under anoxia and a 8.9-fold decrease in superoxide dismutase under anoxia.

Analysis of the secretome also focused upon the extracellular components known to be involved in airway inflammation. Whilst the database does not specify the exact lipoprotein, there was a 4.2-fold decrease in peptidoglycan associated lipoprotein under anoxia compared to normoxia for PAO1, whilst CF isolate 5 also exhibited a 5.9-decrease under anoxia. Interestingly, CF isolate 6 exhibited a 7.4-fold higher abundance of lipoprotein under anoxia than normoxia. CF isolate 7 demonstrated a 3.4-fold decrease in peptidoglycan associated lipoprotein under anoxia.

Finally, lipid A deacylase is an enzyme known to be involved in the modification of the major surface antigen LPS. PAO1 exhibited a 2.0-fold decrease of lipid A deacylase under anoxia compared to normoxia. However, CF isolate 5 exhibited a striking 61.7-fold increase in lipid A deacylase under anoxia compared to normoxia. This enzyme was not detected for CF isolate 6. There was a 1.8-fold decrease detected in lipid A deacylase under anoxia for CF isolate 7.

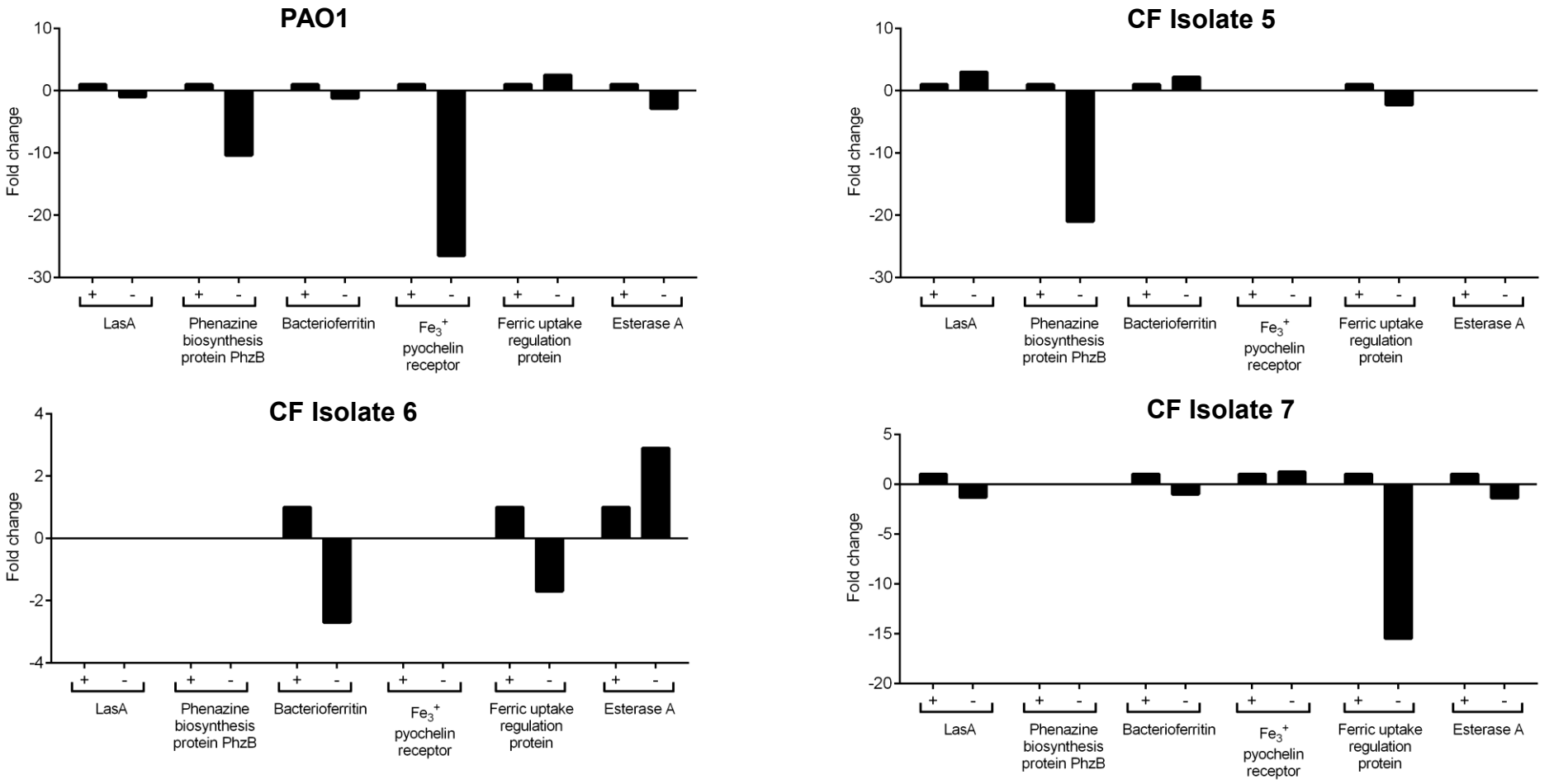


Figure 38. *P. aeruginosa* secretome analysis relating to the antagonism of *S. aureus*. Cell-free supernatants obtained from 24 h cultures of PAO1 and CF isolates 5, 6 and 7 grown under normoxia and anoxia were subject to protein precipitation, SDS-PAGE electrophoresis and MS analysis. Data represents *N*=1 from 5 pooled samples. The abundance of each protein is expressed as fold-change compared to normoxia.

As shown in Figure 38, regarding the production of the staphylolysin LasA, there was a minimal change in its production under anoxia for PAO1, whilst CF isolate 5 demonstrated a 3.0-fold increase in LasA under anoxia. LasA was not detected for CF isolate 6, whilst there was a minimal fold change in LasA for CF isolate 7.

As mentioned previously, pyocyanin is a green phenazine. As shown in Figure 38, the phenazine biosynthesis protein PhzB was detected for PAO1, with a 10.4-fold decrease under anoxia. CF isolate 5 demonstrated a 21.0-fold decrease in the phenazine biosynthesis protein, whilst CF isolates 6 and 7 failed to produce detectable PhzB.

As previously mentioned, iron is an essential micronutrient for *P. aeruginosa* growth. Analysis of the secretome of PAO1 showed that whilst there is a 1.2-fold decrease in the production of bacterioferritin under anoxia, there was a 26.5-fold decrease in the Fe^{3+} pyochelin receptor compared to anoxia. Conversely, a 2.5-fold increase in ferric uptake regulation protein was detected under anoxia compared to normoxia. CF isolate 5 demonstrated a 2.2-fold increase in the production of bacterioferritin under anoxia compared to normoxia. Whilst Fe^{3+} pyochelin receptor was not detected for CF isolate 5, a 2.3-fold decrease in the ferric uptake regulation protein was seen under anoxia. CF isolate 6 exhibited a 2.7-fold decrease in the production of bacterioferritin under anoxia compared to normoxia. Whilst Fe^{3+} pyochelin receptor was not detected for CF isolate 6 under normoxia or anoxia, there was a 1.7-fold decrease in ferric uptake regulation protein under anoxia. There was a minimal change in the production of bacterioferritin under anoxia for CF isolate 7 compared to normoxia, as well as a minimal fold change in Fe^{3+} pyochelin receptor under anoxia. CF isolate 7 exhibited a 15.5-fold decrease in the production of ferric uptake regulation protein under anoxia.

Secretome analysis of PAO1 demonstrated that there was a 2.9-fold decrease in the rhamnolipid esterase A under anoxia. Conversely, whilst CF isolate 5 produced no detectable esterase A under both conditions, CF isolate 6 exhibited a 2.9-fold increase in esterase A under anoxia. CF isolate 7 demonstrated a minimal fold change in esterase A under anoxia.

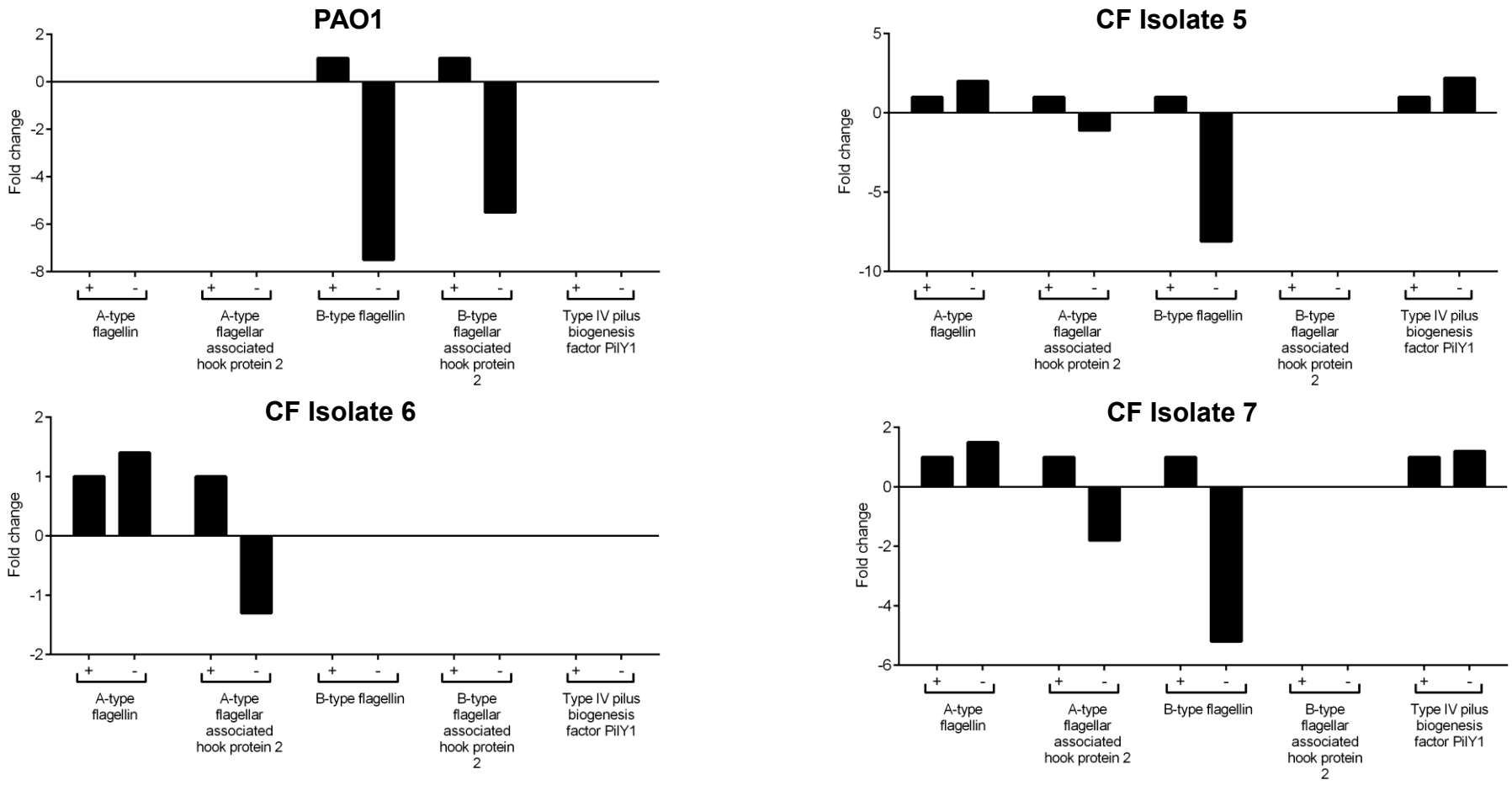


Figure 39. *P. aeruginosa* secretome analysis relating to *P. aeruginosa* motility. Cell-free supernatants obtained from 24 h cultures of PAO1 and CF isolates 5, 6 and 7 grown under normoxia and anoxia were subject to protein precipitation, SDS-PAGE electrophoresis and MS analysis. Data represents *N*=1 from 5 pooled samples. The abundance of each protein is expressed as fold-change compared to normoxia.

Regarding *P. aeruginosa* motility, as shown in Figure 39, A-type flagellin and A-type flagellar associated hook protein was not detected for PAO1. Anaerobiosis induced a 7.5-fold decrease in the production of B-type flagellin in PAO1 and a 5.5-fold decrease in B-type flagellar associated hook protein 2 under anoxia. Type IV pilus biogenesis factor was not detected for PAO1 under normoxia or anoxia.

A 2.0-fold increase was seen in A-type flagellin for CF isolate 5 under anoxia whilst changes in A-type flagellar associated hook protein 2 was minimal. An 8.1-fold decrease in B-type flagellin was seen under anoxia, whilst B-type flagellar associated hook protein 2 was not detected. A 2.2-fold increase was seen Type IV pilus biogenesis factor under anoxia for CF isolate 5.

CF isolate 6 exhibited a minimal fold change in A-type flagellin under anoxia, along with a minimal fold change in A-type flagellar associated hook protein 2. B-type flagellin, B-type flagellar associated hook protein 2 and Type IV pilus biogenesis factor were not detected.

CF isolate 7 exhibited a minimal fold change in A-type flagellin under anoxia and a 1.8-fold decrease in A-type flagellar associated hook protein 2 under anoxia. CF isolate 7 demonstrated a 5.2-fold decrease in B-type flagellin under anoxia, whilst B-type flagellar associated hook protein 2 was not detected. There was a minimal fold change in Type IV pilus biogenesis factor under anoxia.

4.5.9 Preliminary analysis of the impact of anoxia upon the production of AHLs

The production of many *P. aeruginosa* virulence factors is regulated by QS. The *E. coli* biosensors pSB536 and pSB1142 were used to detect and semi-quantify the production of C_4-HSL and 3-oxo-C_{12}-HSL respectively in cell-free culture supernatants of *P. aeruginosa,* following growth under normoxia and anoxia. In response to external AHLs, the biosensor strains emit light which can consequently be detected by measuring bioluminescence.

As shown in Figure 40, the production of C_4-HSL detected within *P. aeruginosa* cell-free culture supernatants varies across the CF isolates. CF isolate 5 and 6 under normoxia produced similar levels of C_4-HSL compared to PAO1 under normoxia, whilst CF isolate 7 appeared to produce lower levels under normoxia compared to PAO1. Whilst anoxia appeared to exert a minimal impact upon C_4-HSL production compared to normoxia for each isolate, further repeats are required to confirm this trend.

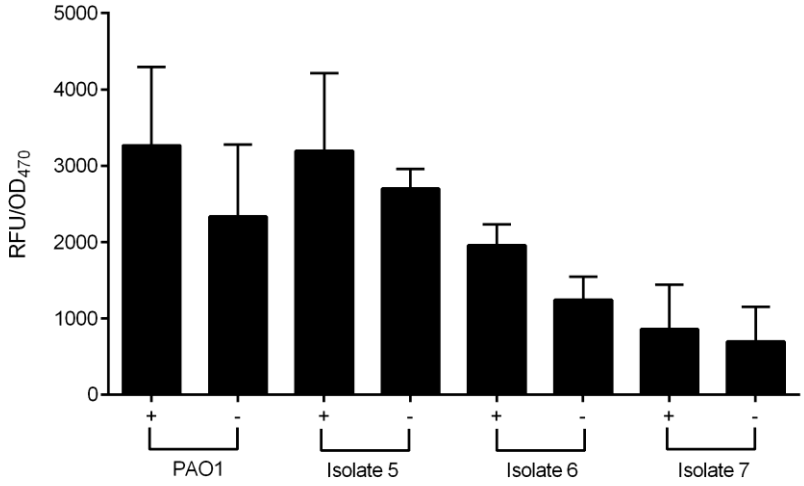


Figure 40. C_4-HSL production by PAO1 and *P. aeruginosa* CF clinical isolates following growth under normoxia and anoxia. Cell-free supernatants of *P. aeruginosa* were added to the *E. coli* biosensor pSB536 for 6 h, prior to the luminescence being read. Bioluminescence values were normalised to the OD_{470} of the biosensor strain to account for differences in their growth and final cell densities. Luminescence values were additionally subtracted from the control (pSB523 and LBN broth only). Bars represent the mean ± S.D of one individual experiment (*N*=1) performed in triplicate. **+** represents normoxia, whilst **–** represents anoxia.

Next, the production of the longer chain 3-oxo-C_{12}-HSL was assessed following the growth of *P. aeruginosa* under normoxia and anoxia. As shown in Figure 41**,** CF isolates 5, 6 and 7 appeared to produce similar levels of 3-oxo-C_{12}-HSL compared to PAO1. Anoxia only appeared to affect PAO1 QS, although further repeats are needed.

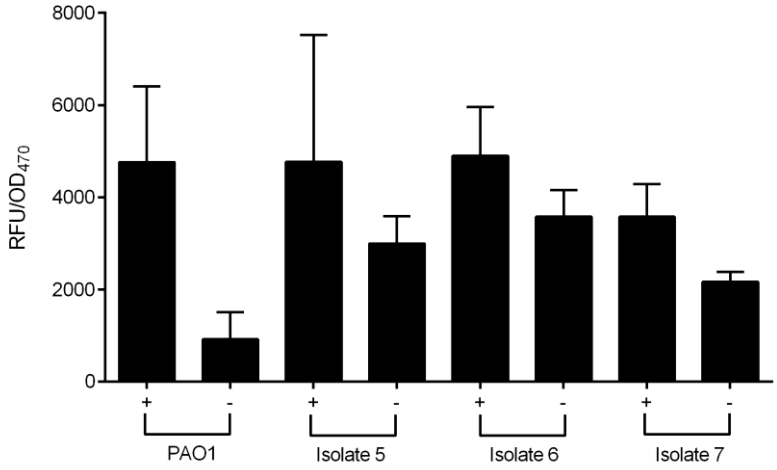


Figure 41. C_{12}-HSL production by PAO1 and *P. aeruginosa* CF clinical isolates following growth under normoxia and anoxia. Cell-free supernatants of *P. aeruginosa* were added to the *E. coli* biosensor pSB1142 for 6 h, prior to luminescence being read. Bioluminescence values were normalised to the OD_{470} of the biosensor strain to account for differences in growth and final cell densities and were additionally subtracted from the control (pSB1142 with LBN broth only). Bars represent the mean ± S.D of one individual experiment (*N*=1) performed in triplicate. **+** represents normoxia, whilst **–** represents anoxia.

4.5.10 Effect of *P. aeruginosa* extracellular products upon the planktonic growth of *S. aureus*

Whilst *P. aeruginosa* CF isolates produce a range of anti-staphylococcal compounds, a series of experiments were conducted in an attempt to determine the size of the anti-staphylococcal compound(s) likely to mediate *P. aeruginosa* dominance. Heat treatment was also performed to determine if the compound(s) were heat-labile. Cell-free supernatants from cultures of *P. aeruginosa* grown under normoxia or anoxia were subjected to size fractionation using 3 kDa molecular weight cut off filters, with select fractions then being subjected to heat-treatment (95 °C for 10 min). Culture supernatants were subsequently added to normalised *S. aureus* cultures, to assess their ability to inhibit *S. aureus* growth over 14 h. Whilst most proteins would be retained within the >3 kDa fraction, smaller molecules such as pyocyanin, rhamnolipids, pyoverdine and AHLs would be expected to be present within the <3 kDa fraction.

As shown in Figure 42, all *P. aeruginosa* PAO1 fractions following growth under normoxia, including those which had undergone heat treatment were able to antagonise *S. aureus* growth at 14 h. The degree of *S. aureus* antagonism was reduced for CF isolate 5 however, where exposure to the <3 kDa fraction and <3 kDa heat-treated fractions caused *S. aureus* to grow to densities similar to the untreated *S. aureus* only control (*S. aureus* with LBN broth only). These findings were also seen for CF isolates 6 and 7 under normoxia.

For all three of the *P. aeruginosa* CF isolates, the heat treated whole supernatant fraction and >3 kDa fraction appeared to be better at reducing *S. aureus* growth, compared to the same fractions which had not been heat treated.

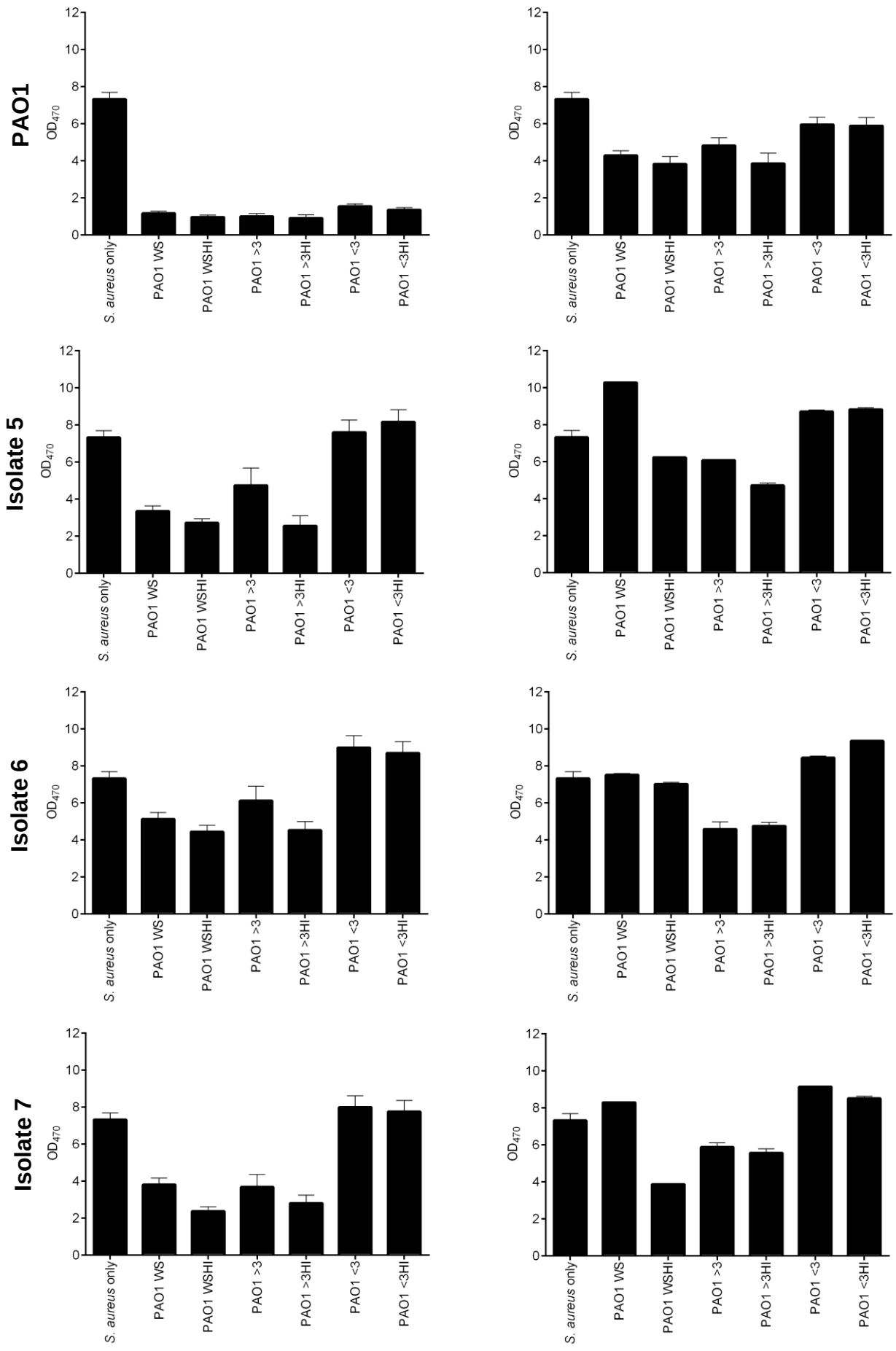

NORMOXIA
ANOXIA
PAO1
Isolate 5
Isolate 6
Isolate 7
OD470
S. aureus only
PAO1 WS
PAO1 WSHI
PAO1 >3
PAO1 >3HI
PAO1 <3
PAO1 <3HI

Figure 42. Determining the effect of heat-treatment and size fractionation of *P. aeruginosa* cell-free supernatants upon the growth of *S. aureus*. *P. aeruginosa* cell-free supernatants were added to standardised *S. aureus* culture either directly as whole supernatant (WS), or following size fractionation (>3 and <3 kDa) and/or heat treatment (HI). Plates were incubated at 37 °C for 14 h and the OD_{470} read at hourly intervals. Plots represent the mean OD_{470} at the 14 h time point ± S.D of two individual experiments (*N*=2), each performed in duplicate.

In contrast, only the whole supernatant and >3 kDa fractions of PAO1 following growth under anoxia, were able to better antagonise the growth of *S. aureus*. Once more, heat treatment failed to abolish this effect. For CF isolate 5, the >3 kDa fractions were also the most inhibitory and this effect was not abolished following heat-treatment. Of particular interest is how heat-treatment of the whole supernatant caused *S. aureus* to grow to a lower final density at 14 h, compared to the whole-supernatant which had not been subject to boiling. Both >3 kDa fractions produced by *P. aeruginosa* CF isolate 6 were the only fractions which caused *S. aureus* to grow to a lower bacterial density than *S. aureus* alone. Lastly, the >3 kDa fractions of CF isolate 7 also reduced *S. aureus* growth at 14 h. As seen with CF isolate 5 under anoxia, heat treatment of the whole supernatant produced by CF isolate 7, caused *S. aureus* to grow to a lower final density at 14 h, compared to whole-supernatant. Together, this data suggests that the anti-staphylococcal compound(s) produced by *P. aeruginosa* is largely contained within the >3 kDa fraction and its activity is not abrogated by heat-treatment.

Such findings are in contradiction to the data shown in Figure 27 and Figure 28, where PAO1 and CF isolates 5 and 6 all failed to exert an antagonistic effect upon the growth of *S. aureus* under anoxia, in planktonic co-culture and mixed species biofilms. Such a discrepancy is likely to be due to differences in assay set up. In this assay (Figure 42), PAO1 cell-free supernatants obtained following overnight growth under anoxia were added to *S. aureus* at time point zero. Thus, *S. aureus* would be exposed to pre-made *P. aeruginosa* virulence properties from time point zero. In the mixed planktonic culture and biofilm co-culture experiments however, both bacterial species were pelleted and resuspended in fresh LBN broth at time point zero, removing any pre-formed anti-staphylococcal products.

Furthermore, due to the plate reader set up, *S. aureus* grew under normoxia regardless of whether it was exposed to *P. aeruginosa* cell-free supernatants obtained following growth normoxia or anoxia. In the planktonic co-culture and mixed species biofilms, both *S. aureus* and *P. aeruginosa* cultures grew either under normoxia or anoxia.

4.5.11 *S. aureus* biofilm disruption by *P. aeruginosa* exoproducts

The ability of *P. aeruginosa* extracellular products to disrupt an established *S. aureus* biofilm was determined. As shown in Figure 43**,** under normoxia PAO1 and most of the CF isolates caused a significant reduction in *S. aureus* biofilm biomass (*P*<0.001) compared to *S. aureus* only control. Conversely, CF isolates 6 and 8 both significantly increased *S. aureus* biofilm biomass compared to *S. aureus* alone (*P*<0.001). The use of cell-free supernatants obtained from anoxic cultures of *P. aeruginosa* demonstrated that four of the isolates retained their ability to disrupt *S. aureus* biofilm, whilst PAO1 and CF isolates 1 and 3 lost this ability.

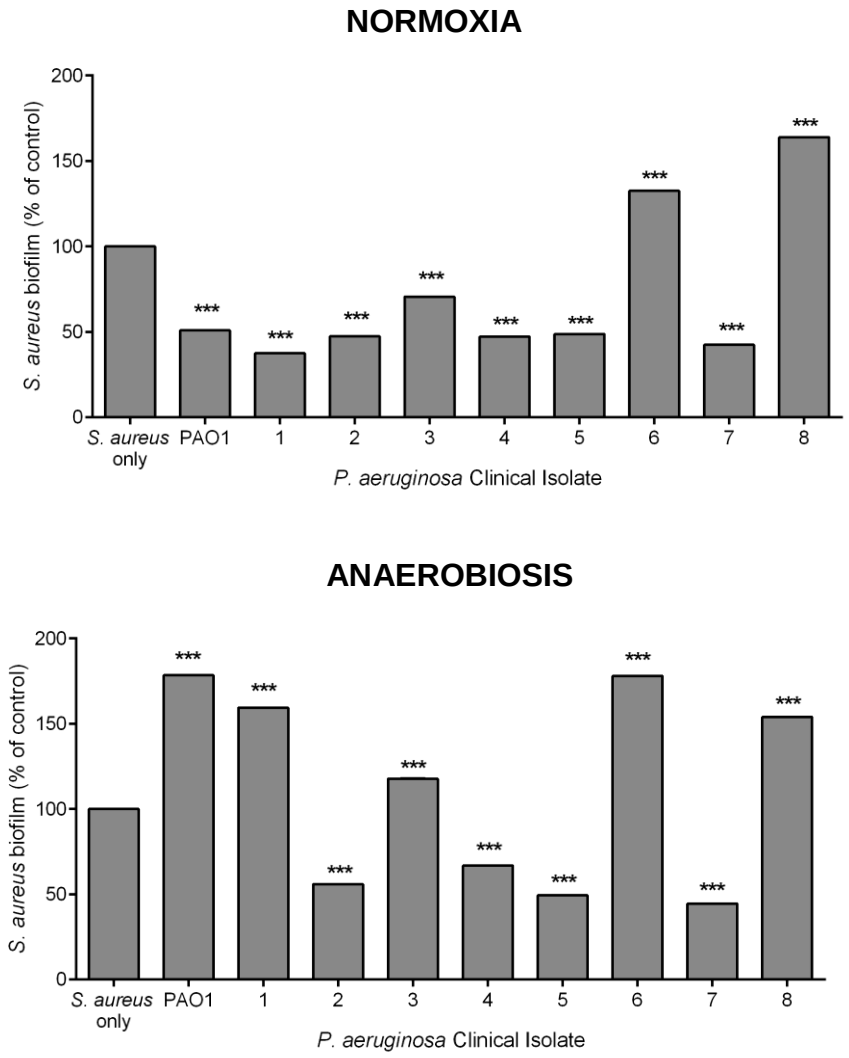


Figure 43. Ability of *P. aeruginosa* CF isolate exoproducts to disrupt *S. aureus* biofilm under normoxia and anoxia. *S. aureus* biofilm was grown under normoxia for 24 h, prior to the addition of 100 µL of each *P. aeruginosa* cell-free supernatant. Plates were incubated under normoxia for a further 5 h at 37 °C. Biofilms were washed with PBS and stained with 1% (w/v) crystal violet and read at OD_{570}. Data has been normalised to a percentage of *S. aureus* only biofilm (the control). Columns represent the mean ± S.E.M. for three independent experiments (*N*=3), each performed in triplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc*, comparing values to *S. aureus* only biofilm. ****P*<0.001.

4.5.12 *S. aureus* biofilm inhibition by *P. aeruginosa* exoproducts

The ability of *P. aeruginosa* cell-free supernatants to inhibit *S. aureus* biofilm formation was also investigated. Shown in Figure 44, cell-free supernatants obtained from PAO1 and most of the CF isolates, significantly inhibited *S. aureus* biofilm production (*P*<0.001). Conversely, exposure to exoproducts from CF isolate 6 significantly increased *S. aureus* biofilm biomass (*P*<0.001). Under anoxia, PAO1 and CF isolates 2, 4, 5 and 7 were able to inhibit *S. aureus* biofilm. Conversely CF isolates 1, 3, 6 and 8 significantly increased *S. aureus* biofilm production compared to the *S. aureus* only control (*P*<0.001).

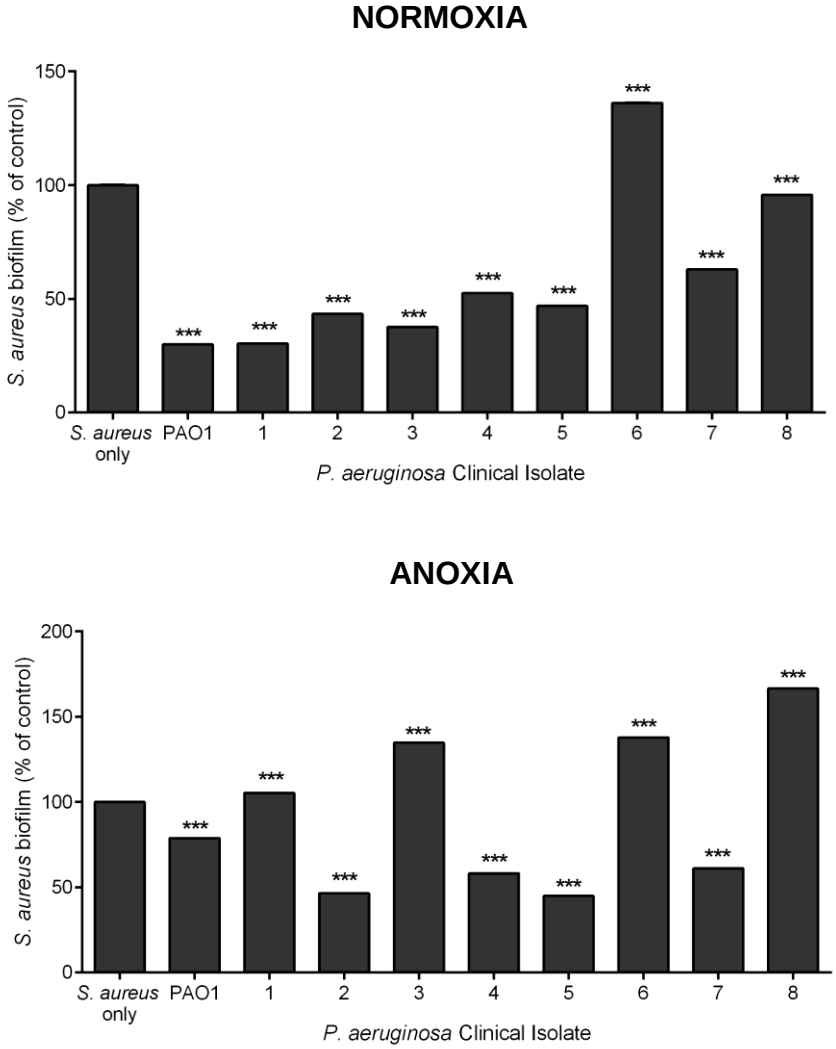


Figure 44. Ability of *P. aeruginosa* exoproducts to inhibit *S. aureus* biofilm formation under normoxia and anoxia. *S. aureus* was grown in the presence of each *P. aeruginosa* supernatant for 24 h. Biofilms were stained with 1% (w/v) crystal violet and read at OD_{570}. Data has been normalised to a percentage of *S. aureus* biofilm only (the control). Columns represent the mean ± S.E.M. for three independent experiments (*N*=3), each performed in triplicate. Statistical differences were determined using one-way ANOVA with Dunnett's *post-hoc*, comparing values to the *S. aureus* only biofilm. ****P*<0.001.

4.5.13 Influence of *S. aureus* extracellular products upon *P. aeruginosa* planktonic growth

The work shown above focused upon the ability of *P. aeruginosa* cell-free supernatants to antagonise the growth of *S. aureus.* The data presented in Figure 45 however, sought to determine the effect of *S. aureus* cell-free supernatants upon the planktonic growth of *P. aeruginosa*. The experiments aimed to mimic early *P. aeruginosa* infection, where *P. aeruginosa* is likely to colonise CF airways in an environment abundant in *S. aureus* and its exoproducts.

As shown in Figure 45**,** the addition *S. aureus* culture supernatants obtained under normoxia inhibited the planktonic growth of *P. aeruginosa* PAO1 and all the CF isolates (*P*<0.001), compared to growth in the absence of *S. aureus* exoproducts. This trend was also seen under anoxia, where *S. aureus* cell-free supernatant inhibited the planktonic growth of PAO1 and CF isolates 5 and isolate 7 (*P*<0.001), but not CF isolate 6.

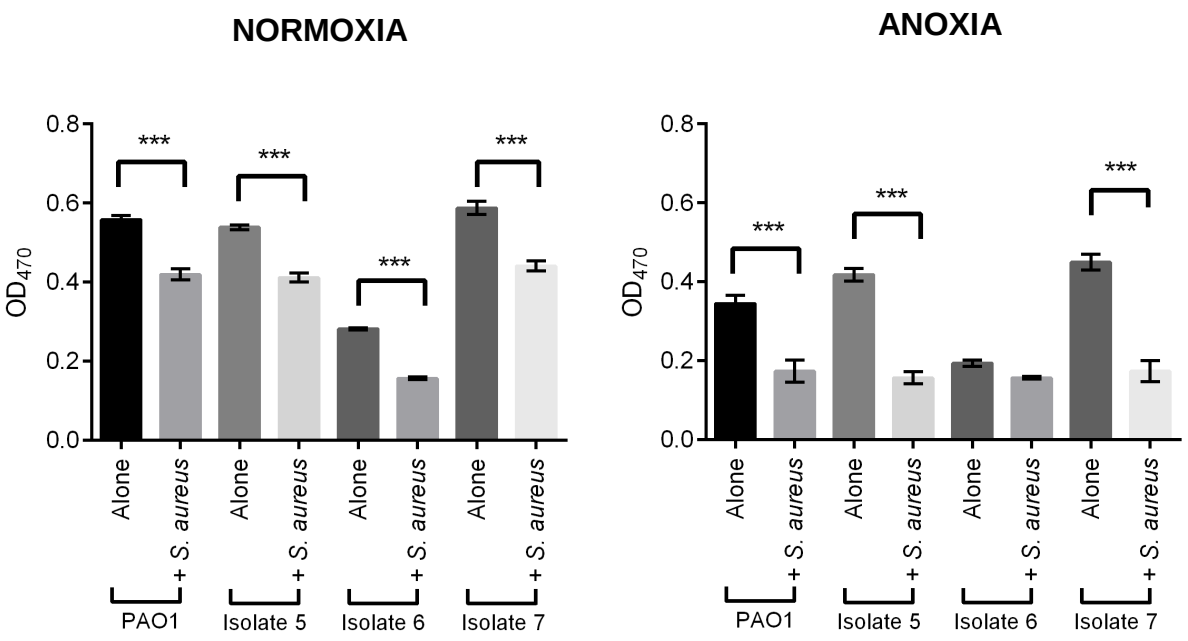


Figure 45. Influence of *S. aureus* cell-free supernatants upon *P. aeruginosa* planktonic growth. 100 µL of sterile filtered *S. aureus* culture supernatant was added to 100 µL planktonic *P. aeruginosa*. Alone represents the *P. aeruginosa* isolate cultured in the presence of 100 µL of LBN broth (acting as a control). Bacteria were grown statically at 37 °C for 8 h under normoxia or anoxia. Plates were read at OD_{470}. The data represents the mean ± S.E.M of three independent experiments (*N*=3), each performed in triplicate. Statistical differences were determined using one-way ANOVA with Tukey's *post-hoc* test. ***P<0.001.

4.5.14 Influence of oxygen and *S. aureus* exoproducts upon *P. aeruginosa* motility

The impact of anoxia upon *P. aeruginosa* swimming and swarming motilities was determined, along with the impact of *S. aureus* exoproducts upon *P. aeruginosa* motility. As shown in Figure 46**,** under normoxia all isolates exhibited a degree of swimming motility and this was unaffected by the inclusion of *S. aureus* exoproducts. CF isolate 5 alone exhibited significantly greater swimming motility under normoxia*,* compared to PAO1 and CF isolates 6 and 7 alone (P<0.001).

Under anoxia, PAO1 alone lost its swimming motility, whilst the addition of *S. aureus* cell-free supernatant significantly restored this (P<0.001). *S. aureus* cell-free supernatant also enhanced the swimming motility of CF isolate 7 (P<0.01), compared to CF isolate 7 alone. CF isolate 5 alone also exhibited the greatest swimming motility under anoxia compared to PAO1 alone (P<0.01), along with CF isolates 6 and 7 alone (P<0.05). Compared to normoxia, anoxia reduced the swimming motility of PAO1 alone (P<0.01), CF isolate 5 alone (P<0.001) and CF isolate 5 in the presence of *S. aureus* (P<0.001).

Under normoxia, PAO1 and CF isolates 6 and 7 displayed minimal swarming motility, whilst the addition of *S. aureus* exoproducts enhanced the swarming motility of PAO1 (P<0.001). CF isolate 5 alone exhibited the greatest swarming motility compared to PAO1 alone and CF isolates 6 and 7 alone (P<0.001).

Under anoxia, CF isolate 5 exhibited the greatest swarming motility compared to PAO1, as well as CF isolates 6 and 7 alone (P<0.001). *S. aureus* exoproducts did not exert any effects upon *P. aeruginosa* swarming motility under anoxia. Only CF isolate 5 alone exhibited a significant reduction in swarming motility under anoxia, compared to normoxia (P<0.01).

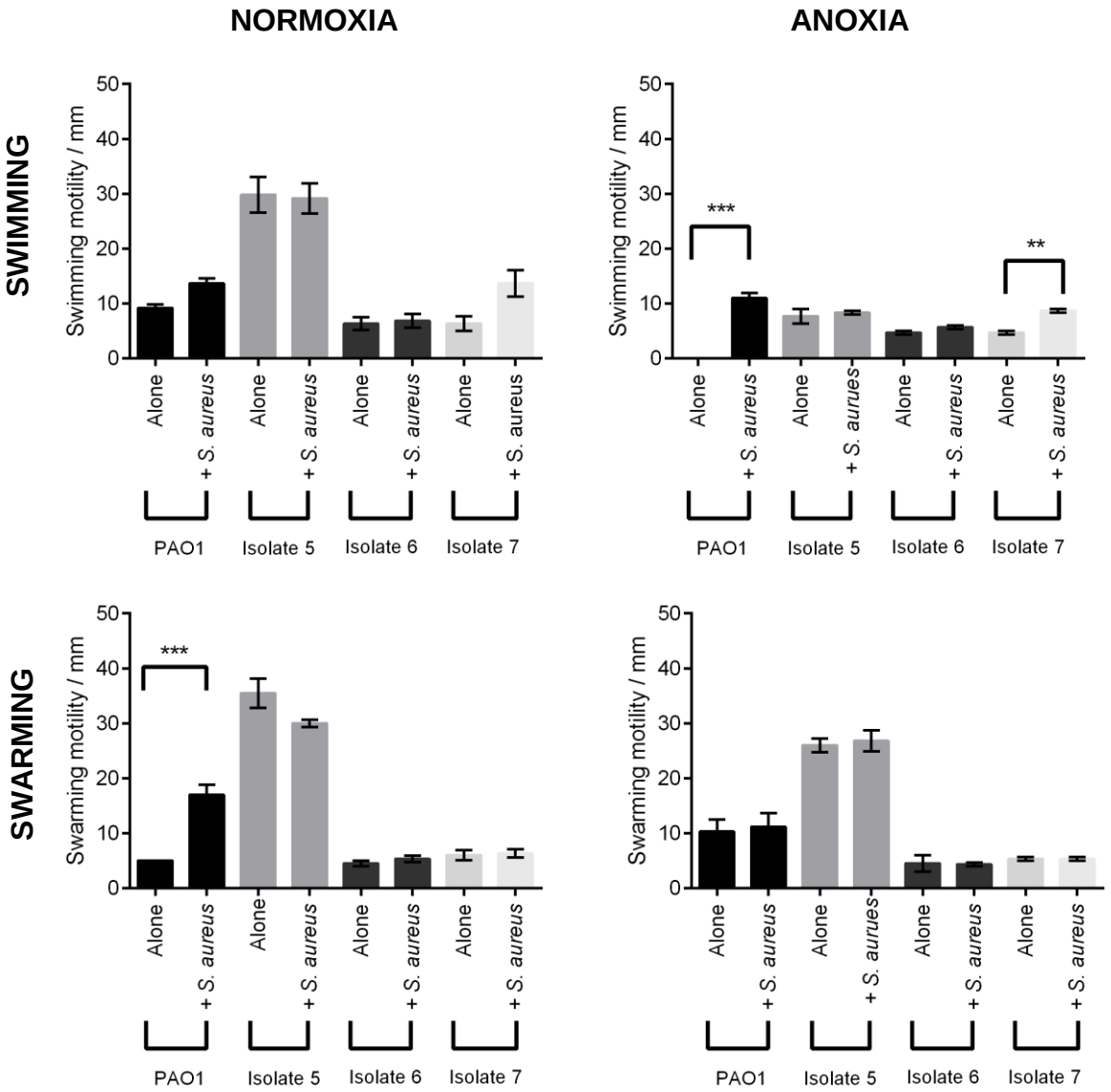


Figure 46. Effect of oxygen and *S. aureus* culture supernatant upon *P. aeruginosa* swimming and swarming motility. Swimming and swarming motilities of *P. aeruginosa* isolates were assessed following their overnight incubation upon swimming and swarming plates. To test the effects of *S. aureus* culture supernatant upon these two forms of motility, a 1:100 dilution of the culture supernatant was added to the plates before the agar set. Data are presented as mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. Statistical differences were determined using one-way ANOVA with Tukey's *post-hoc*. ***P*<0.01 ****P*<0.001.

The *S. aureus* cell-free supernatants were subsequently subjected to size fractionation and heat-treatment in order to gain a greater insight into the compound(s) that are either restoring, or modulating PAO1 and CF isolate 7 swimming motility under anoxia and the compound that is modulating the swarming activity of PAO1 under normoxia.

As shown in Figure 47, all fractions of *S. aureus* cell-free culture supernatant, regardless of size and heat-treatment were able to modulate *P. aeruginosa* swimming and swarming motility to the same extent as the whole culture supernatants (Figure 46).

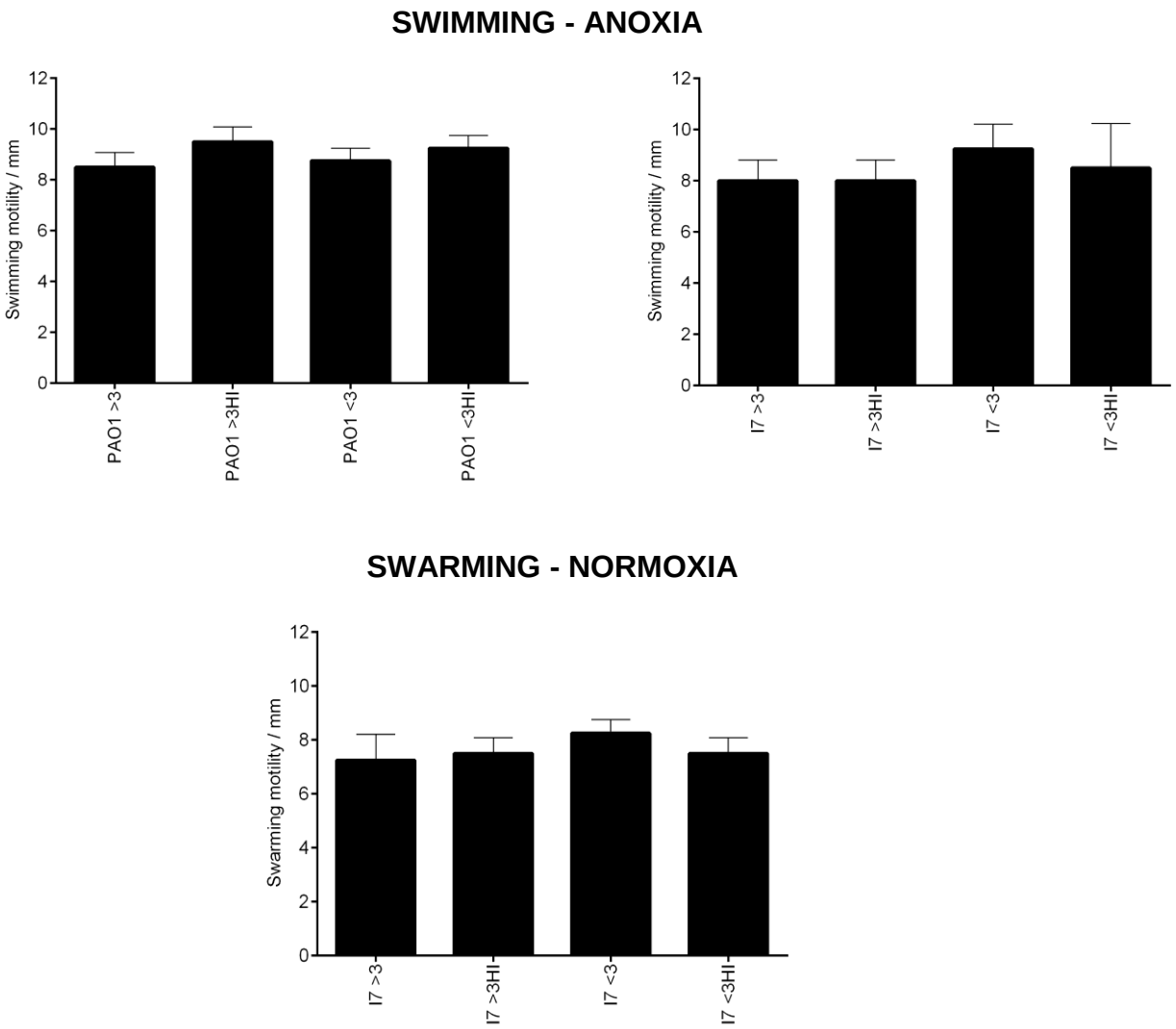


Figure 47. Heat-treatment and size fractionation of *S. aureus* cell-free supernatants upon *P. aeruginosa* swimming and swarming motility. Swimming and swarming motilities of *P. aeruginosa* PAO1 and CF isolate 7 were assessed following an overnight incubation on swimming and swarming agar plates. Plates were supplemented with a 1:100 dilution of *S. aureus* culture supernatant fractions (>3 or <3 kDa), with select fractions also being subject to heat-treatment. Data are presented as mean ± S.D from two independent experiments (*N=2*) each performed in duplicate.

4.5.15 Effect of oxygen upon the sensitivity of *P. aeruginosa* CF isolates to tobramycin

Lastly, the impact of oxygen availability upon the susceptibility of *P. aeruginosa* to the major anti-pseudomonal antibiotic tobramycin was determined. Overnight cultures of *P. aeruginosa* grown in LBN broth under normoxia or anoxia were normalised to an OD_{470} of 1.0, diluted to 10^6 CFU/mL and 100 µL was added to a sterile 96-well plate. A microbroth dilution method was used to determine the MIC of tobramycin. Plates were incubated for 24 h at 37 °C under normoxia or anoxia, prior to the MIC being determined by visual inspection.

As shown in Figure 48, anoxia resulted in a 2-fold increase in the MIC of each CF isolate compared to normoxia. As the isolates were cultured in LBN broth, the breakpoints for susceptibility or resistance to tobramycin were not used as NCCLS guidelines recommend the use of Mueller Hinton broth for antibiotic susceptibility testing (European Committee on Antimicrobial Susceptibility Testing, 2018).

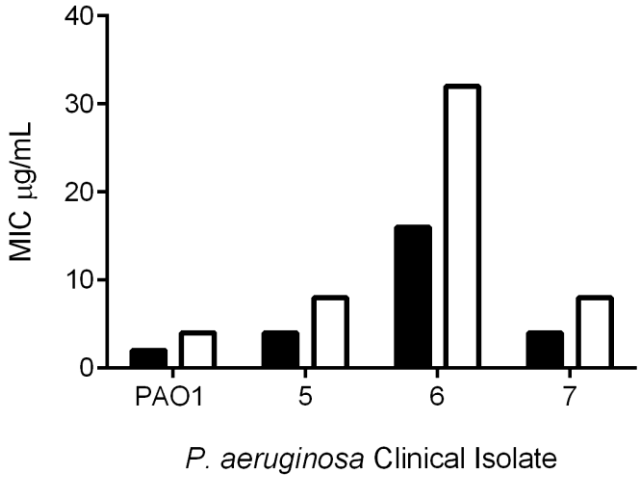


Figure 48. Impact of oxygen availability upon the susceptibility of *P. aeruginosa* to tobramycin. Overnight cultures of *P. aeruginosa* grown under normoxia or anoxia were normalised and 100 µL of each culture was added to wells of a 96-well plate containing serially diluted concentrations of tobramycin (64-0.125 µg/mL). Plates were incubated statically for 24 h at 37 °C under normoxia or anoxia, prior to the MIC being determined by visual inspection. Black bars represent the MIC under normoxia and the white bars represent the MIC under anoxia. Data are presented as mean ± S.E.M from three independent experiments (*N*=3), each performed in duplicate.

4.6 Discussion

4.6.1 Influence of anoxia upon *S. aureus*-*P. aeruginosa* bacterial competition

Employing environmental conditions designed to mimic more closely those found within regions of the CF lung, this chapter investigated the effects of static growth and oxygen availability upon the phenotype of *P. aeruginosa* CF isolates, as well as upon their interspecies interactions with *S. aureus*.

The polymicrobial nature of infection has been shown increasingly to be a key driver of disease severity in CF airways (Peters *et al.*, 2012, Rogers *et al.*, 2009, Rosenbluth *et al.*, 2004, Sibley *et al.*, 2006). Whilst the localised co-existence of *P. aeruginosa* and *S. aureus* within the CF lung is associated with a worsening of pulmonary function and increased exacerbations (Hubert *et al.*, 2013, Hogan *et al.*, 2016, Fugere *et al.*, 2014a, Limoli *et al.*, 2016), interspecies interactions between these two major CF pathogens is poorly understood, particularly under anoxia.

The growth competition data in Figure 27 illustrates that oxygen availability plays a major role in influencing interspecies interactions and community composition. In agreement with previous studies (Baldan *et al.*, 2014a, Kluge *et al.*, 2012, Filkins *et al.*, 2015, Korgaonkar *et al.*, 2013) *P. aeruginosa* PAO1 and CF clinical isolates 5, 6 and 7 were able to outcompete *S. aureus* in static planktonic co-culture at 24 h, without their own growth being adversely affected. However, in mixed species biofilms (Figure 28), CF isolate 6 was the only isolate unable to reduce *S. aureus* viability under normoxia. The introduction of anoxia caused PAO1 and CF isolates 5 and 6 to lose their ability to dominate at 24 h in both planktonic co-culture and mixed species biofilms, with *S. aureus* being detected in co-culture densities similar to those obtained in pure culture (Figure 28). Meanwhile, CF isolate 7 retained its ability to reduce *S. aureus* viability under anoxia at 24 h (Figure 28). Thus, the absence of oxygen appears to provide *S. aureus* with a survival advantage in the presence of *P. aeruginosa* and may explain in part why *S. aureus* can co-exist with *P. aeruginosa* (Limoli *et al.*, 2016).

In addition to oxygen availability, the mode of bacterial growth appears to influence microbial competition. Whilst *P. aeruginosa* CF isolate 6 predominated over *S. aureus* in mixed planktonic culture under normoxia (Figure 27), it was unable to outcompete on both solid agar (Figure 26) and in mixed species biofilms (Figure 28). Previous reports however have shown bacteria grown as a biofilm exhibit differences in virulence, compared to those grown in planktonic culture (Yadav *et al.*, 2004, Secor *et al.*, 2011, Waite *et al.*, 2005). It is also known that bacteria within biofilms are a heterogenous population varying in their growth and physiological state, ranging from rapidly growing cells, to those which are in stationary phase

(Borriello *et al.*, 2004). It is likely that variations nutrient availability, pH and rates of oxygen consumption will influence this, although further work is required to support this. It is also important to reiterate that the microbiological data for these CF clinical isolates of *P. aeruginosa* is lacking and no information is available as to whether these patients were also culture positive for *S. aureus.* The presence or absence of *S. aureus* in the airways of the individuals with CF is likely to influence *P. aeruginosa* virulence, where *P. aeruginosa* isolates obtained from CF patients co-infected with *S. aureus* have been shown to be less antagonistic towards *S. aureus* (Orazi and O'Toole, 2017).

4.6.2 The effect of anoxia upon the production of *P. aeruginosa* anti-staphylococcal virulence properties

This chapter also aimed to evaluate the impact of anoxia upon a number of *P. aeruginosa* virulence properties, to greater understand the mechanisms which govern *P. aeruginosa* dominance. The production of several known anti-staphylococcal exoproducts in the supernatants of *P. aeruginosa* were studied and further subjected to heat-treatment and size fractionation in an attempt to determine the size of the compound(s) and determine whether it is heat-labile. The secretome of *P. aeruginosa* was also analysed following mass spectrometric analysis.

Laboratory strain PAO1 has previously been shown to produce LasA, responsible for cleaving the peptidoglycan cell wall of *S. aureus* (Kessler *et al.*, 1993b, Barequet *et al.*, 2004). In turn, this provides *P. aeruginosa* with the essential micronutrient iron, which it can use for its own growth (Mashburn *et al.*, 2005b). This study demonstrated that PAO1 and CF isolates 5 and 7 lysed heat-killed *S. aureus* under normoxia, whilst CF isolate 6 did not (Figure 31). PAO1 subsequently lost its staphylolytic ability under anoxia, a finding supported by reports of a decrease in the transcription of LasA for PAO1 (Filiatrault *et al.*, 2005) and a reduction in PAO1 elastase (Lee *et al.*, 2011) under anoxia.

Mass spectrometric analysis of the *P. aeruginosa* secretome (Figure 38) further support these findings. Mass spectrometric score is a measure of ”goodness” of the all peptide identifications for a given protein, and it rises with the number of positively identified proteins. Protein score approximately reflects the relative protein abundance in a given dataset. Whilst PAO1 demonstrated a minimal fold change in its LasA production under anoxia, using the score as an approximation of protein abundance, LasA was shown to be five times less abundant (score: 69.96) than LasA secreted by CF isolate 5 and 7 (score: 397.57 and 354.17 respectively). Unlike PAO1, CF isolates 5 and 7 both retained their staphylolytic activity under anoxia (Figure 38). Thus, it is reasonable to suggest that the LasA produced by PAO1 under anoxia is likely to be below a threshold to exert a considerable effect upon *S. aureus* viability. *S. aureus* has also previously been shown to exhibit an increase in cell wall thickness under

anoxia (Conti *et al.*, 1968) and whilst this was not investigated in this study, this mechanism may further reduce *S. aureus* susceptibility to *P. aeruginosa* LasA in the absence of oxygen.

Whilst LasA may be advantageous to *P. aeruginosa* under growth in polymicrobial communities with *S. aureus,* collectively, LasA mediated lysis of *S. aureus* alone doesn't appear to be essential for reducing *S. aureus* growth under normoxia. All CF isolates were able to outcompete *S. aureus* in planktonic co-culture (Figure 27), yet CF clinical isolate 6 failed to exhibit detectable staphylolytic activity (Figure 31) or secrete detectable LasA as determined by mass spectrometry (Figure 38). The ability of select CF isolates to lyse *S. aureus* under anoxia however, may well provide competitive advantages to *P. aeruginosa* in the presence *S. aureus,* which unlike *P. aeruginosa,* is better adapted to thriving in environments low or devoid of oxygen (Yoon *et al.*, 2002).

LasA also doesn't appear to be essential for modulating *P. aeruginosa* dominance under anoxia, as CF isolates 5 and 7 both retained their staphylolytic activity under in the absence of oxygen (Figure 31). Only CF isolate 7 was able to outcompete in planktonic co-culture (Figure 27) and mixed species biofilm (Figure 28). Furthermore, whilst whole culture supernatants and >3 kDa fractions from PAO1 and the CF isolates were able to antagonise *S. aureus* growth, heat-treatment failed to abolish their inhibitory activity (Figure 47). As staphylolysis is known to be mediated by LasA, boiling such fractions is expected to denature this 20 kDa protease. Despite this, the heat-treated whole supernatant and >3 kDa fraction retained their anti-staphylococcal activity. It is likely that the anti-staphylococcal factor influencing interspecies interactions is another compound retained within this fraction.

The protease data shown in Figure 30 is in agreement with a previous study which demonstrated that protease production by PAO1 was below the limit of detection following growth under anoxia (Lee *et al.*, 2011). Conversely, CF isolates 5 and 7 retained their protease activity under anoxia. Interestingly, only those isolates which produced detectable levels of protease and exhibited staphylolytic activity under normoxia and anoxia were able to exert a detrimental effect upon *S. aureus* biofilm (Figure 43 and Figure 44). Meanwhile, those isolates which failed to demonstrate any protease activity, also failed to disrupt or inhibit *S. aureus* biofilm production. Although the mechanisms which facilitate the disruption and inhibition of *S. aureus* biofilm requires further study, current literature suggests that it is a protease. Qin *et al.* demonstrated that extracellular products from wildtype PAO1 were able to disrupt *S. epidermidis* biofilm formation (Qin *et al.*, 2009), whilst Park *et al.* demonstrated that the presence of external protease proteinase K induced *S. aureus* autolysis, leading to degradation of its own biofilm (Park *et al.*, 2012). With *S. aureus* often being the first opportunistic pathogen to colonise CF airways (Lyczak *et al.*, 2002), the ability for incoming *P.*

aeruginosa to disrupt and inhibit *S. aureus* biofilm may make *S. aureus* vulnerable to the arsenal of *P. aeruginosa* virulence factors.

The ability of select CF isolates of *P. aeruginosa* to secrete proteases under anoxia may extend beyond interspecies competition and facilitate host colonisation regardless of the dynamic changes in oxygen availability within the CF lung overtime. This includes facilitating the degradation of tight junctions between airway epithelia (de Bentzmann *et al.*, 2000), the degradation of proteins of the ECM (Yang *et al.*, 2015) and the breakdown of airway mucins (Henke *et al.*, 2011), even when regions of the lung become anoxic. Protease secretion is also likely to provide protection against the host's immune response, facilitating the degradation of sIgA (Diebel *et al.*, 2009) and pulmonary surfactant A (Beatty *et al.*, 2005) The ability to also degrade pro-inflammatory cytokines and chemokines including IL-8 (Okuda *et al.*, 2011) will further impair neutrophil chemotaxis. Protease-mediated degradation of host lysozyme may also provide additional protection against *P. aeruginosa* killing (Jacquot *et al.*, 1985). The ability to damage, colonise and survive in regions of CF airways devoid of oxygen may in turn protect *P. aeruginosa* from the anti-pseudomonal antibiotic tobramycin, shown to be ineffective under anoxia (Borriello *et al.*, 2004), with the data presented in Figure 48 showing a two-fold increase in the MIC of this antibiotic under anoxia.

An intriguing question regarding bacterial virulence is how *P. aeruginosa* is protected from the damaging effects of its own virulence factors? *P. aeruginosa* secretes a series of proteases, including alkaline protease, protease IV, along with LasA, LasB and LasD (Engel *et al.*, 1998, Blackwood *et al.*, 1983, Suter, 1994). LasA, LasB and protease IV are known to exist as intracellular pre-pro-enzymes, preventing harmful protease activity in the cytoplasm and periplasm (Kessler and Safrin, 1994). Upon secretion to the environment, it is believed that LasB activates LasA and protease IV (Oh *et al.*, 2017, Kessler *et al.*, 1998), as well as activating itself, through degradation of its own pro-peptide (Kessler *et al.*, 1998).

Pyocyanin is detected in high concentrations in CF sputum and has been shown to inhibit the respiratory chain of *S. aureus,* leading to the emergence of a sub-population of slow growing small colony variants (SCVs) (Hoffman *et al.*, 2006, Biswas *et al.*, 2009a). Pyocyanin is also known to target the host, inducing neutrophil apoptosis, including inhibiting airway epithelia respiration, calcium signalling and the beating of airway cilia (Wilson *et al.*, 1988, Denning *et al.*, 1998a, Usher *et al.*, 2002, Kerr, 1994).

PAO1 and CF isolates 5 and 6 produced detectable levels of pyocyanin under normoxia following phenol-chloroform extraction, whilst this was below the limit of detection for CF isolate 7 (

Figure **33**). Despite this, CF isolate 7 was still able to dominate over *S. aureus* under normoxia (Figure 27). Furthermore, pyocyanin was below the limit of detection for all culture supernatants cultured under anoxia following phenol-chloroform extraction (

Figure **33**).

Secretome analysis demonstrated that there was a 10-fold decrease in the phenazine biosynthesis protein under anoxia (Figure 38) and a 21-fold decrease for CF isolate 5. This protein was not detected for CF isolates 6 and 7. However, mass spectrometry was only performed on samples collected from one independent repeat and pyocyanin production is mediated by the formation of numerous intermediates (Ho Sui *et al.*, 2012). Furthermore, size exclusion and heat treatment also demonstrated that *S. aureus* antagonism was restricted to the >3 kDa fraction and was not abolished following heat-treatment (Figure 42). As pyocyanin is a protein, it is expected to be present in the <3 kDa fraction (due to it being 0.21 kDa size) its activity abolished by heat-treatment.

Whilst a lack of pyocyanin under anoxia may be due to the redox nature of the phenazine (Rada and Leto, 2013), a previous study demonstrated that hypoxia significantly reduced pyocyanin production by *P. aeruginosa* (Schwank *et al.*, 2013). Additionally, a lack of detectable pyocyanin under anoxia may have a minimal impact upon *P. aeruginosa* survival. The production of this respiratory inhibitor under anoxic conditions would fail to inhibit the respiratory chain of *S. aureus* due to undergoing anaerobic respiration. Furthermore, as molecular oxygen is also required for the neutrophil respiratory burst, the absence of oxygen will severely impair this antimicrobial defence (Mandell, 1974). The data in

Figure **33** also shows that the presence of *S. aureus* failed to enhance the production of pyocyanin by the *P. aeruginosa* isolates tested. This is in contrast to a previous study which illustrated that the presence of *S. aureus* induced the production of pyocyanin by PAO1 under normoxia (Kluge *et al.*, 2012). Further work is required to assess the impact of *S. aureus* extracellular products upon pyocyanin mRNA synthesis and whether *S. aureus* influences intracellular signalling and pyocyanin gene transcription.

In addition to targeting *S. aureus,* pyocyanin may also act as an alternative electron acceptor for *P. aeruginosa* under normoxia*,* where steep electron acceptor gradients are known to exist within the airway lumen (Worlitzsch *et al.*, 2002). This phenazine in turn could be re-oxidised following diffusion to an oxygen rich surface and thus be involved in redox homeostasis (Arai, 2011). Pyocyanin has also been shown to be involved in QS, as well as in the uptake of iron during growth within biofilms (Dietrich *et al.*, 2006, Koley *et al.*, 2011). *P. aeruginosa* is able to protect itself from the damaging effects of pyocyanin as a highly active pyocyanin intermediate is transported through the MexgHIOpmD efflux pump, preventing oxidation of its

own proteins and metabolites, whilst the intermediate, including pyocyanin itself upregulates expression of this efflux pump (Sakhtah *et al.*, 2016, Dietrich *et al.*, 2006).

The ability of *P. aeruginosa* to cause chronic CF lung infections is also owed to its ability to chelate iron (Fe^{3+}) through the secretion of pyoverdine. Siderophore production and consequent iron depletion by *P. aeruginosa* has been shown to reduce *S. aureus* growth, as well as enhance *P. aeruginosa* virulence (Nguyen *et al.*, 2015, Mashburn *et al.*, 2005b, Filkins *et al.*, 2015). Shown in Figure 32, pyoverdine secretion varies across CF isolates of *P. aeruginosa,* a finding highlighted previously (Nguyen *et al.*, 2015). Both PAO1 and CF isolate 5 produced elevated levels of pyoverdine (Figure 32) and outcompeted *S. aureus* (Figure 27), whilst CF isolates 6 and 7 produced minimal amounts of pyoverdine and were still able to outcompete *S. aureus* in planktonic co-culture under normoxia (Figure 27). Under anoxia, pyoverdine production by PAO1 and CF isolate 5 was significantly reduced compared to normoxia, a finding also reported following the growth of *P. aeruginosa* under hypoxia (Schwank *et al.*, 2013). Moreover, CF isolate 7 retained its ability to dominate over *S. aureus* in mixed planktonic culture (Figure 27), despite producing low levels of this siderophore.

Analysis of the PAO1 secretome regarding iron uptake (Figure 38), demonstrated that whilst bacterioferritin was not detected, there was a 26-fold increase in the pyochelin receptor under normoxia compared to anoxia, whilst there was a 2-fold increase in the ferric uptake regulator protein under anoxia. CF isolate 5 demonstrated a 2-fold increase in bacterioferritin under anoxia, including a 2-fold decrease in the ferric uptake regulator protein. For CF isolate 6, only bacterioferritin was detected, with there being a 2-fold increase under anoxia. CF isolate 7 exhibited a minimal fold change in the production of bacterioferritin and Fe^{3+} pyochelin receptor under anoxia, whilst exhibiting a 15.5-fold decrease in the production of ferric uptake regulation protein under anoxia.

As the antimicrobial activity of *P. aeruginosa* cell-free supernatants was restricted to the >3 kDa fractions (Figure 42), together with the secretome data, it is unlikely that siderophore production in isolation drives *S. aureus* antagonism. Furthermore, with iron being required by *S. aureus* for respiratory chain cytochromes (Voggu *et al.*, 2006, Kogut and Lightbown, 1962), the ability of pyoverdine to chelate iron under anoxia may also elicit a minimal effect on *S. aureus* when it is not undergoing aerobic respiration. However, it is also likely that as iron-depleted growth media was not used in this study, the importance of pyoverdine in governing interspecies interactions may be underestimated. Furthermore, as the assay used is solely based upon the inherent fluorescent properties of pyoverdine, a more specific approach such as high performance liquid chromatography (HPLC) would be required to accurately measure the pyoverdine abundance.

Surfactants (rhamnolipids) have previously been shown to target *S. aureus* (Haba *et al.*, 2003, Bharali *et al.*, 2013) and induce rapid necrosis of host neutrophils (Jensen *et al.*, 2007) *P. aeruginosa* is known to secrete a number of rhamnolipids (Rendell *et al.*, 1990), that have been shown to be important in facilitating bacterial detachment from biofilms (Boles *et al.*, 2005) facilitating bacterial swarming (Deziel *et al.*, 2003). Rhamnolipids have also been shown to target the host, including inhibiting macrophage-mediated phagocytosis (McClure and Schiller, 1996), reducing airway epithelial cell cilia beat frequency (Kanthakumar *et al.*, 1996) along with reducing cell polarity and barrier function (Zulianello *et al.*, 2006).

Assessing surfactant activity using the established drop collapse assay (Limoli *et al.*, 2017, Price *et al.*, 2015) the data shown in Figure 34 demonstrated that PAO1 and the three CF *P. aeruginosa* isolates tested harboured and retained their surfactant activity, although this was reduced under anoxia for PAO1 and CF isolate 5. Surfactant activity was the greatest for CF isolate 7 (Figure 34) under both normoxia and anoxia.

Secretome analysis of PAO1 demonstrated that there was a 2.9-fold decrease in esterase A under anoxia (Figure 38)**,** supported by reductions in the drop collapse assay (Figure 34), where PAO1 surfactant activity was reduced in the absence of oxygen. Conversely, CF isolate 5 exhibited a 2.9-fold increase under anoxia, although this was not reflected in the drop collapse assay (Figure 34). CF isolate 6 failed to produce any detectable esterase A (Figure 38), despite exhibiting surfactant activity (Figure 34). Additional repeats are required, to allow more meaningful comparisons and conclusions to be made from this data. Whilst CF isolate 7 exhibited the greatest surfactant score (Figure 34), surfactants are expected to be in the <3 kDa. However, only the >3 kDa fractions of the cell-free supernatant obtained from CF isolate 7 antagonised *S. aureus* growth.

P. aeruginosa employs several other virulence properties which were not characterised in this study and may contribute to the differences seen in bacterial competition. Whilst hydrogen cyanide has been shown to target the respiratory chain of *S. aureus* under normoxia, previous research has demonstrated that hydrogen cyanide is rapidly inactivated under anoxia (Blumer and Haas, 2000) and is therefore unlikely to govern interspecies interactions in the absence of oxygen. Whilst *P. aeruginosa* is also known to produce the small respiratory inhibitor HQNO (Machan *et al.*, 1992), its impact under anoxia is likely to be minimal when *S. aureus* is undergoing anaerobic respiration. Furthermore, PQS is known to regulate the production of itself, as well as regulate the secretion of virulence factors, including elastase (Diggle *et al.*, 2003, Wade *et al.*, 2005). However, its inhibition under anoxia means its impact in anoxic regions of the CF lung is likely to be minimal (Schertzer *et al.*, 2010, Toyofuku *et al.*, 2008). The antibiotic mupirocin has been shown to target *S. aureus* (Matthijs *et al.*, 2014), however its production by CF isolates of *P. aeruginosa* and its production under anoxia has yet to be

determined. Whilst this study and others have shown that *P. aeruginosa* exoproducts are effective at antagonising the growth of *S. aureus,* the ability of type III and IV secretion systems to deliver virulence properties intracellularly to host cells and competing bacteria are also likely to play a role in bacterial competition (Engel and Balachandran, 2009, Hood *et al.*, 2010).

A study which removed *P. aeruginosa* exoproducts using a biofilm flow model demonstrated that mucoid isolates were able to outcompete *S. aureus,* whilst non-mucoid isolates facilitated *S. aureus* biofilm formation (Yang *et al.*, 2011). This finding contrasts with other studies which demonstrate that mucoid isolates exhibit reduced antagonism towards *S. aureus* (Limoli *et al.*, 2017). Thus, not only are virulence factors important for bacterial competition, but growth conditions are also likely to influence this further.

Results from this study suggest that the major anti-staphylococcal compound(s) governing interspecies interactions under normoxia and anoxia is restricted to the >3 kDa fraction, is heat stable and is likely to be an anti-staphylococcal factors not studied. An interesting observation in Figure 42 is that the heat-treated whole supernatant and >3 kDa fractions for most CF isolates under normoxia and anoxia appeared to more greatly reduce *S. aureus* growth, compared to those which had not been heat-treated. Perhaps heating inactivates a factor within the complex culture supernatant which may attenuate the potency of the anti-staphylococcal factor(s) or attenuate a compound which promotes growth. It is also entirely possible that the compound(s) modulating *P. aeruginosa* dominance under normoxia varies to the factor(s) governing bacterial community dynamics under anoxia. A limitation of this study is that the absolute concentration of the *P. aeruginosa* exoproducts in cell-free culture supernatants was not determined and it may be that some of the virulence factors were at concentrations too low to exert an antagonistic effect upon *S. aureus.*

The inability to identify a single virulence factor which facilitates *P. aeruginosa* dominance is a well-recognised phenomenon within CF (Bragonzi *et al.*, 2012, Baldan *et al.*, 2014a). It is likely that the ability of *P. aeruginosa* to dominate under both environmental conditions is not due to the secretion of a single virulence property, but is due to the production of several (Filkins *et al.*, 2015, Michelsen *et al.*, 2014, Limoli *et al.*, 2016, Bhagirath *et al.*, 2016, Bragonzi *et al.*, 2012). Previous authors have reported that deletion of a single virulence property has been shown to reduce *P. aeruginosa* antagonism towards *S. aureus* (Limoli *et al.*, 2017). Despite this, it is interesting to note that CF isolate 7 which produced the smallest array of anti-staphylococcal factors studied and a minimal amount of protein for mass spectrometry analysis, was the only isolate able to outcompete *S. aureus* under anoxia.

Additional work is required to gain a better understanding of the mechanisms behind these findings. One possible approach would be to generate mutants of the *P. aeruginosa* CF isolates, systematically targeting individual virulence factors, including LasA. As co-isolated isolates of *S. aureus* and *P. aeruginosa* have previously been shown to be less antagonistic compared to those from mono-infected patients (Fugere *et al.*, 2014a, Limoli *et al.*, 2017), the effects of anoxia upon co-infected isolates also warrants further investigation.

4.6.3 Impact of anoxia and *S. aureus* exoproducts upon *P. aeruginosa* motility

The research presented in Figure 46 shows the effects of *S. aureus* supernatant, as well as anoxia upon *P. aeruginosa* CF isolate motility. Decreases in the production of proteins relating to swimming motility in PAO1 was identified following mass spectrometry analysis (Figure 39), which supports the swimming motility data shown in Figure 46 – where PAO1 exhibited a loss of detectable PAO1 swimming motility under anoxia. Conversely, anoxia exerted a minimal impact upon the production of proteins relating to swimming motility for CF isolates 5 and 6 (Figure 39), a finding supported by swim motility diameters shown in Figure 46.

Whilst *S. aureus* exoproducts failed to elicit any effect upon *P. aeruginosa* swimming motility under normoxia, the ability of these exoproducts to both restore and enhance swimming motility under anoxia may facilitate *P. aeruginosa* dissemination, colonisation and consequent survival into other areas of the CF lung, including those which are poorly oxygenated. PAO1 and each of the CF isolates tested demonstrated varying degrees of swarming motility under both normoxia and anoxia (Figure 46). The addition of *S. aureus* cell-free supernatant significantly increased the swarming motility of PAO1 under normoxia only, whilst it did not modulate swarming of the CF isolates under both oxygen conditions tested. This modulating activity of *S. aureus* culture supernatant could not be restricted to a single size fraction (Figure 47) and thus further work is required to characterise the *S. aureus* compound(s) modulating *P. aeruginosa* motility.

4.6.4 Impact of anoxia upon *P. aeruginosa* susceptibility to tobramycin

Antimicrobial treatment strategies against *P. aeruginosa* employ the use of inhaled antibiotics, to delay or prevent chronic CF airway infection. Despite this approach, current treatment strategies are relatively ineffective, with diagnostic laboratory antibiotic susceptibilities using planktonic cultures under normoxia, poorly translating in patients (Hassett *et al.*, 2002). Whilst many CF isolates of *P. aeruginosa* exhibit resistance to commonly used antibiotics due to long term exposure at sublethal MICs (Kohanski *et al.*, 2010), the fact that CF airways contain regions of anoxia is likely to influence treatment efficacy (Worlitzsch *et al.*, 2002, Hassett *et al.*, 2009). The frontline antibiotic tobramycin, as well as levofloxacin have both previously been shown to have reduced efficacy under anoxia (King *et al.*, 2010, Hill *et al.*, 2005).

Data in Figure 48 demonstrated that anoxia reduced the efficacy of tobramycin for PAO1 and all CF isolates tested. This is possibly due to poor antibiotic uptake, which requires energy from quinone-associated electron transport, which would not be function in the absence of oxygen (Bryan and Kwan, 1983). The presence of molecular oxygen has been shown to restore the bactericidal activity of aminoglycoside antibiotics which is lost under anoxia (Mader

et al., 1989). Whilst previous research has shown that the presence of nitrate itself reduces antibiotic susceptibility (Borriello *et al.*, 2004), LBN broth was used to conduct MIC testing under both normoxia and anoxia. Although this study tested tobramycin against planktonic cultures of *P. aeruginosa,* for antimicrobial treatments to be more effective, future studies would seek to use biofilm cultures of *P. aeruginosa* grown under anoxia, as well its impact upon mixed species biofilms. More closely mimicking the conditions of the CF lung may help to make current treatment approaches more efficacious in reducing the *P. aeruginosa* burden in CF airways.

4.6.5 The impact of oxygen upon *P. aeruginosa* survival: secretome analysis by mass spectrometry

Mass spectrometry analysis also demonstrated changes within the secretome of *P. aeruginosa.* PAO1 demonstrated a 17.4-fold increase in the presence of denitrification regulatory protein NirQ under anoxia, compared to normoxia (Figure 35**)**. As *P. aeruginosa* undergoes denitrification under anoxia, using nitrogen as an end terminal acceptor in the absence of molecular oxygen (Schreiber *et al.*, 2007), the induction of this protein under anoxia is likely to be involved in ATP synthesis. Conversely, there was a 39.4-fold increase in periplasmic nitrate reductase under normoxia (Figure 35), supporting previously findings that anoxia represses this enzyme (t Riet *et al.*, 1968). An additional study has also shown that under anoxia, membrane bound nitrate reductase is depressed, in favour of upregulating periplasmic nitrate reductase (Van Alst *et al.*, 2009).

Nitrite reductase is involved in the conversion of nitrite to nitric oxide during denitrification (Henry and Bessieres, 1984) and is produced only when the bacteria is growing under anaerobiosis in the presence of nitrate (Yamanaka *et al.*, 1961). Whilst the data in Figure 35 shows this enzyme is detected and is more abundant under normoxia than anoxia (a 2.8-fold increase), this is likely due to nitrate being present in LBN broth used to grow CF isolates under both environmental conditions tested.

In addition to producing extracellular proteases to target competing bacteria, such as LasA against *S. aureus* (Kessler *et al.*, 1993b), *P. aeruginosa* produces soluble pyocin-S2 which targets other competing *P. aeruginosa* strains and degrades their DNA. Protection of a given *P. aeruginosa* strain to its own pyocin arises due to the synthesis of a pyocin immunity protein (Sano, 1993, Parret and De Mot, 2000, Michel-Briand and Baysse, 2002). The ability to also secrete pyocyins under anoxia may be important for bacterial survival, particularly when resources such as nitrate availability are limited in a competitive environment. Whilst pyocyin was detected for PAO1 and CF isolate 6, it was not detected for CF isolates 5 and 7 (Figure 36). Pyocins are routinely detected in CF clinical isolates of *P. aeruginosa* and are used to type

strains, as well as act as a marker to investigate the spread of resistant strains between CF patients (Richardson *et al.*, 1991, Jones *et al.*, 2001).

Catalase and superoxide dismutase were both shown to be downregulated in PAO1 and CF isolates 5 and 6 under anoxia (Figure 37). As colonisation of CF airways by *P. aeruginosa* leads to a neutrophil dominated immune response, the production of catalase and superoxide dismutase is likely to provide protection against oxidative stress, due to the breakdown of the reactive oxygen intermediate hydrogen peroxide, which is formed as part of the neutrophil respiratory burst (Winterbourn *et al.*, 2006). As the absence of oxygen will significantly impair this immune response, the failure of PAO1 and the *P. aeruginosa* CF isolates to produce these two enzymes under anoxia (Figure 37) may prevent valuable nutrients and resources from being wasted.

Finally, lipid A deacylase is an enzyme known to be involved in the modification of the major surface antigen LPS, to escape TLR4 detection upon airway epithelia (Chow *et al.*, 1999). PAO1 exhibited a 2-fold decrease of lipid A deacylase under anoxia compared to normoxia (Figure 37), whilst CF isolate 5 exhibited a striking 61.65-fold increase in lipid A deacylase under anoxia. This enzyme was not detected for CF isolate 6, a finding which is known to occur in some CF isolates (Ernst *et al.*, 2006). Whilst there is no doubt that changes to the structure of lipid A provide survival advantages to *P. aeruginosa* in the CF lung (Ernst *et al.*, 1999, Ernst *et al.*, 2003, Cigana *et al.*, 2009), changes in the abundance of this enzyme under anoxia are complex and require further study.

4.6.6 The impact of anoxia upon quorum sensing (QS)

P. aeruginosa virulence is governed by two major AHL signals, 3-oxo-C_{12}-HSL by the las system and C_4-HSL by the rhl system. A third QS system PQS utilises 2-heptyl-3-hydroxy-4-quinolone (Pearson *et al.*, 1995, Gambello and Iglewski, 1991, McGrath *et al.*, 2004). Accumulation of these QS signal molecules regulate the production of over 300 virulence genes in *P. aeruginosa*, including bacterial swarming (Kohler *et al.*, 2000), biofilm formation (Yang *et al.*, 2009) the secretion of proteases (Schuster *et al.*, 2003) and anti-staphylococcal compounds such as lasA and pyocyanin (Pearson *et al.*, 1997, Kessler *et al.*, 1993b).

Preliminary experiments sought to determine the impact of normoxia and anoxia upon the production of 3-oxo-C_{12}-HSL and C_4-HSL by *P. aeruginosa*. The production of a third QS signal molecule PQS was not assessed, as it is reported to not be produced under anoxia (Schertzer *et al.*, 2010, Toyofuku *et al.*, 2008). As was shown in Figure 40, the production of C_4-HSL varied across the *P. aeruginosa* CF isolates, with its production appearing to be lowest for CF

isolate 7 under both environmental conditions. Whilst the QS data is preliminary and additional repeats are required, such a finding is of interest.

C_4-HSL governs the production of many *P. aeruginosa* virulence factors, including proteases, elastases and pyocyanin (Winson *et al.*, 1995, Pearson *et al.*, 1995, Bosgelmez-Tinaz and Ulusoy, 2008). Whilst PAO1 produced higher levels of C_4-HSL under both normoxia and anoxia, it lost its ability to lyse *S. aureus* under anoxia (Figure 31), as well as degrade skimmed milk agar (Figure 30). Conversely, CF isolate 7 exhibited staphylolytic ability (Figure 31) and protease activity under normoxia (Figure 30) and retained this under anoxia, despite appearing to produce low levels of C_4-HSL under both conditions.

Losses in *P. aeruginosa* virulence under anoxia have previously been reported to be due to attenuations in QS, where the addition of exogenous AHLs has been shown to restore virulence gene expression (Lee *et al.*, 2011). However, PAO1 produced comparable levels of C_4-HSL under normoxia and anoxia and thus questions remain as to why elastase and protease activity was below the limit of detection under anoxia. Unlike C_4-HSL, early findings suggest that the absence of oxygen attenuates C_{12}-HSL production for PAO1 and not the CF isolates (Figure 41). Whilst elastase production is governed by the las system, it has also been shown to be partially regulated by the rhl system (Pearson *et al.*, 1997).

The poor correlations between virulence and QS are most likely to be due to the preliminary nature of these findings, with results being obtained from a single independent experiment. It may also be possible that different AHL threshold concentrations exist across the CF isolates in order to activate virulence gene expression, a hypothesis that has been previously suggested (Fang *et al.*, 2013). Additionally, complex regulatory pathways are known to govern *P. aeruginosa* gene expression. For example, some strains of *P. aeruginosa* have been shown to produce the autoinducer C_{12}-HSL despite mutations in lasR, a phenomenon which results from the ability of the rhI system to override this mutation (Dekimpe and Deziel, 2009).

4.7 Limitations

The work presented in this chapter has several limitations. The first is that the experiments were performed using LBN broth and questions remain as to how closely this mimics the nutrient availability found within CF airways. Stationary phase growth of the bacterial cultures, however, led to the development of macroscopic clumps of *P. aeruginosa* characteristically seen in CF sputum (Sriramulu *et al.*, 2005) and also allowed comparisons to be made to published literature which has widely used LB broth to study *P. aeruginosa* physiology and interspecies interactions. Despite this, the growth medium chosen is likely to influence the bacterial physiology and virulence of *P. aeruginosa* and *S. aureus*. Previous studies have shown how the type of growth media and its nutrient availability influences both *P. aeruginosa*

motility (Wolfgang *et al.*, 2004) and the secretion of extracellular virulence factors (Palmer *et al.*, 2007a), including rhamnolipids (Mata-Sandoval *et al.*, 2001). Whilst some studies have employed expectorated sputum samples from CF and non-CF patients (Palmer *et al.*, 2005, Palmer *et al.*, 2007a), there have been recent developments in the development of synthetic CF sputum, supplemented with salmon sperm DNA and mucin (Palmer *et al.*, 2007a, Turner *et al.*, 2015, Fung *et al.*, 2010).

The levels of nitrate within CF sputum has been reported to be approximately 400 µM (Linnane *et al.*, 1998). However, this study used 100 mM potassium nitrate (equivalent to 1% (w/v) potassium nitrate), a concentration that has been widely used to study *P. aeruginosa* under anoxia (Filiatrault *et al.*, 2006, Alvarez-Ortega and Harwood, 2007, Van Alst *et al.*, 2009, Williams *et al.*, 1978, Frederick *et al.*, 2001).

4.8 Future work

Future work would seek to use artificial CF sputum as the growth medium for the study of *S. aureus-P. aeruginosa* interspecies interactions, in attempt to more closely mimic the physiology of the CF lung. It is likely that the growth medium will require optimisation in order to facilitate *P. aeruginosa* growth under anoxia. The use of a benchtop oxygen meter or luminescent dissolved oxygen sensor would also allow measurements to be made regarding the amounts of dissolved oxygen within artificial sputum over the course of co-infection (Alvarez-Ortega and Harwood, 2007, Chen *et al.*, 2003a), something which was not addressed in this work.

The use of *P. aeruginosa* mutants (i.e. with abolished QS or virulence factors), would also provide a more targeted approach to studying individual *P. aeruginosa* virulence properties and their impact upon governing *S. aureus-P. aeruginosa* interspecies interactions. A similar approach of could be used to generate *S. aureus* mutants in order to determine the compound(s) which are likely to modulate *P. aeruginosa* swimming and swarming motility in an isolate dependent manner under normoxia and anoxia.

As mentioned previously, co-isolated strains of *S. aureus* and *P. aeruginosa* have been shown to be less antagonistic than those from mono-infected patients. A longitudinal study of select *S. aureus* and *P. aeruginosa* co-isolates would allow the pathoadaptive mechanisms that occur overtime in both *S. aureus* and *P. aeruginosa* to be studied in greater detail. This could be complimented by further mass spectrometry work addressing the secretome of *P. aeruginosa* CF isolates, to provide a greater insight into the impact of oxygen availability upon bacterial physiology and virulence.

4.9 Conclusion

The originality of this research is that it demonstrates the impact of oxygen availability upon *S. aureus-P. aeruginosa* interspecies interactions in both planktonic co-culture and mixed species biofilms. Whilst *P. aeruginosa* CF isolates were shown to dominate under normoxia in both planktonic and biofilm culture, anoxia reduced *P. aeruginosa* antagonism in an isolate dependent manner and thus may be one mechanism which facilitates *S. aureus-P. aeruginosa* co-existence within CF airways, a finding reported clinically and one that is associated with poorer clinical outcomes. The proposed mechanism of how anoxia may influence *S. aureus-P. aeruginosa* interspecies interactions is summarised in Figure 49. The results presented also suggest that the compound(s) which mediates *P. aeruginosa* dominance under normoxia and anoxia is an extracellular factor which is >3 kDa in size and is heat stable. Perhaps eventual identification of this compound may open up new therapeutic avenues to interfere with these interspecies interactions, to provide more favourable outcomes. The results also extend to their likely impact upon the host. The ability of select CF *P. aeruginosa* isolates to retain their virulence properties, including protease production and motility under anoxia, may provide *P. aeruginosa* with a survival advantage, permitting airway persistence in an environment where oxygen availability is likely to decrease overtime.

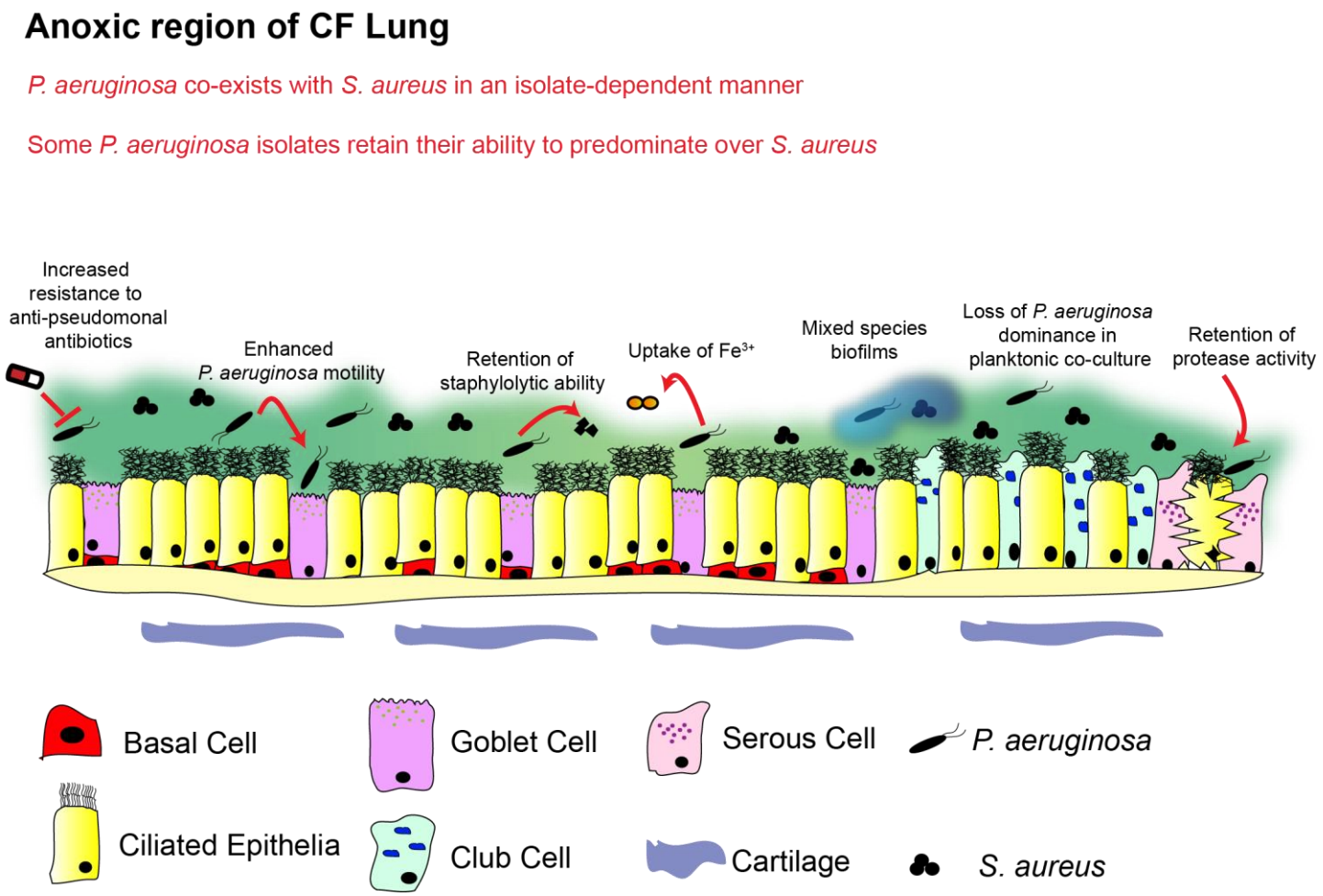


Figure 49. Effects of anoxia upon *S. aureus-P. aeruginosa* interactions in the CF lung. Schematic diagram from the data obtained from this chapter, as to the numerous effects that anoxia may have upon *S. aureus-P. aeruginosa* interspecies interactions in CF airways.

5 Determining the impact of *S. aureus-P. aeruginosa* co-infection upon the airway inflammatory response

5.1 Chapter Transition

Although several previous studies have demonstrated the ability of *P. aeruginosa* to reduce the viability of *S. aureus in vitro*, chapter 4 revealed how changes in oxygen availability influence the secretion of a number of *P. aeruginosa* virulence properties and shapes *S. aureus-P. aeruginosa* interspecies interactions in planktonic co-culture and mixed species biofilms. This polymicrobial nature of CF airways infection is likely to not only impact upon the survival and persistence of each bacterial species within the dynamic microbial community, but also influence the host's airway inflammatory response.

5.2 Introduction

In addition to acting as a physical barrier, airway epithelia play an essential role in surveying and orchestrating an innate immune response to inhaled pathogens within the conducting airways. Their ability to detect a broad range of microbial components and diffusible extracellular products arises due to the expression of a diverse repertoire of PRR's upon their cell surface (Gomez and Prince, 2008). Whilst airway epithelia express a number of receptor families including cytosolic NOD-like receptors (Uehara *et al.*, 2007) and C-type lectins (Rust *et al.*, 1991), the most abundant family are the transmembrane TLRs. TLRs are glycoproteins which consist of an extracellular leucine rich repeat domain and a conserved cytoplasmic toll-IL-1 receptor (TIR) domain (O'Neill and Bowie, 2007). Airway epithelia are able to detect a diverse array of PAMPs, not only due to airway epithelia expressing all ten known TLRs (Sasai and Yamamoto, 2013), but also due to their ability to form both homo- and heterodimers (Hajjar *et al.*, 2001). The predominant TLRs involved in bacterial detection are the cell surface associated TLR2, -4 and -5 (Muir *et al.*, 2004).

TLR2 recognises the widest variety of bacterial products of the TLRs due to its ability to form heterodimers with other TLRs (Muir *et al.*, 2004, Greene and McElvaney, 2005, Wetzler, 2003). This includes the detection of *P. aeruginosa*-derived ExoS toxin via its C-terminus (Epelman *et al.*, 2004) along with specific structural arrangements of LPS and lipoproteins from Gram-negative bacteria (Erridge *et al.*, 2004) following co-localisation with the high affinity LPS binding protein CD14 (Yang *et al.*, 1999, Kirschning *et al.*, 1998).

TLR2 is also involved in the detection of mannuronic acid – a component of *P. aeruginosa* alginate (Flo *et al.*, 2002). In addition to being involved in the detection of the flagellin protein required for *P. aeruginosa* swimming motility (Adamo *et al.*, 2004), TLR2 detects peptidoglycan and lipoteichoic acid (LTA) from the cell wall of Gram-positive bacteria (Yoshimura *et al.*, 1999, Schwandner *et al.*, 1999), including the major CF pathogen *S. aureus* (Iwaki *et al.*, 2002). Furthermore, TLR2 also detects Panton-Valentine Leucocidin (PVL) secreted by *S. aureus* (Zivkovic *et al.*, 2011), responsible for inducing neutrophil apoptosis (Genestier *et al.*, 2005). Following infection, TLR2 is promptly transported to the apical surface of airway epithelia in lipid rafts, associating with the glycolipid co-receptor asialoGM_1 (Soong *et al.*, 2004). The absence of TLR2 is associated with an increased susceptibility to *S. aureus* (Takeuchi *et al.*, 2000). TLR2 expression has been shown to be elevated in the immortalised CF cell line CFBE41o–, compared to the non-CF cell line, 16HBE14o (Shuto *et al.*, 2006). However, TLR2 expression in bronchial biopsies from individuals with CF were shown to comparable to healthy controls (Hauber *et al.*, 2005)

TLR4 plays role in the recognition of the lipid A moiety of LPS (Chow *et al.*, 1999, Poltorak *et al.*, 1998). However, receptor activation requires the formation of a complex including MD2, CD14 and LPS-binding proteins, with MD2 being involved in the binding to LPS (Pugin *et al.*, 1993, Park *et al.*, 2009, Nagai *et al.*, 2002). Interestingly, the CFTR corrected cell line corrCFBE41o- has been shown to express higher levels of TLR4 compared to the isogenic CF cell line CFBE41o-, which is supported by histological analysis of airway epithelia from CF lungs, which exhibited reduced TLR4 expression, compared to healthy controls (John *et al.*, 2010). Conversely, another study demonstrated that TLR4 expression in bronchial biopsies from individuals with CF were significantly higher than those in healthy controls (Hauber *et al.*, 2005).

TLR5 detects a highly conserved site of *P. aeruginosa* bacterial flagellin (Adamo *et al.*, 2004, Hayashi *et al.*, 2001), the protein monomer responsible for flagella-mediated motility. Flagellin deficient mutants of *P. aeruginosa* subsequently do not initiate an airway inflammatory response by TLR5 (Zhang *et al.*, 2005, Blohmke *et al.*, 2008, Hybiske *et al.*, 2004). IB3-1 CF epithelia have previously been demonstrate to have increased mRNA expression of TLR5 (Blohmke *et al.*, 2008), whilst the addition of an anti-TLR5 antibody was shown to reduce IL-6 production by CF IB3-1 epithelia (Blohmke *et al.*, 2008). There is also evidence that TLR2 is involved in the detection of bacterial flagellin, possibly due to the ability of TLRs to heterodimerise (Adamo *et al.*, 2004). Residing intracellularly and mobilised to the apical surface during infection (Gewirtz *et al.*, 2001), TLR5 is able to recognise flagellar from both the major Gram-negative CF pathogen *P. aeruginosa* (Zhang *et al.*, 2005), along with flagellar from Gram-positive bacteria (Hayashi *et al.*, 2001). Both TLRs are likely to play an essential role in pulmonary airway defence, as a lack of TLR4 and TLR5 expression has been associated with

a hypersusceptibility to *P. aeruginosa* infection (Ramphal *et al.*, 2008, Raoust *et al.*, 2009). This protective role of TLRs within the airways is further supported by a study which silenced MYD88, an adaptor protein associated with downstream signalling following activation of most TLRs. Silencing its expression resulted in a blunting of an early airway inflammatory response, along with uncontrolled *P. aeruginosa* proliferation and necrosis of the respiratory epithelium (Skerrett *et al.*, 2004).

Whilst TLR activation by lipoproteins, peptidoglycan and LPS lead to the upregulation of antimicrobial peptides including HBD-2 (Hertz *et al.*, 2003, Homma *et al.*, 2004, Jia *et al.*, 2004), the CF airway response to infection is dominated primarily by the release of IL-8, with CF bronchial airway epithelia (Tabary *et al.*, 1998, Kammouni *et al.*, 1997) and alveolar macrophages (Bonfield *et al.*, 1995b, Khan *et al.*, 1995) being principle sources of this pro-inflammatory chemokine. IL-8 is a potent activator and recruiter of neutrophils to the basolateral lumen of airway epithelia (Huber *et al.*, 1991, Hammond *et al.*, 1995). In non-CF airways, neutrophils form 1% of all inflammatory cells, whilst making up 70% in CF airways (Kelly *et al.*, 2008). This immune cell recruitment is exacerbated further as neutrophils exposed to a pro-inflammatory cytokine environment release of monocyte chemoattractant protein-1 (MCP-1), leading to late-stage monocyte recruitment (Yamashiro *et al.*, 1999) and further amplifying the inflammatory cycle.

The exceedingly high levels of IL-8 in CF sputum, BALF and serum (Dean *et al.*, 1993, Bonfield *et al.*, 1995b) are known to exacerbate damage to the CF lung, through the release of NE. Detected in micromolar concentrations (Konstan *et al.*, 1994), NE tips the protease-antiprotease balance, stimulates mucus hypersecretion (Kohri *et al.*, 2002, Fischer and Voynow, 2002) and induces IL-8 gene expression in airway epithelia (Nakamura *et al.*, 1992). In turn, this leads to a chronic cycle of pulmonary inflammation, irreversible tissue damage and decreases within lung function. The development of the oral NE protease inhibitor AZD9668 has been shown to be associated with reductions in the sputum biomarkers including TNF-α, IL-6 and IL-8 and elastin degradation markers, despite eliciting no effect upon lung function, neutrophil counts, or NE activity (Elborn *et al.*, 2011).

The secretion of another pro-inflammatory cytokine IL-6 plays an important role in orchestrating the airway inflammatory response (Hurst *et al.*, 2001). Acting in an endocrine fashion, it is a potent activator of acute phase protein production by the liver, as well as in the maturation of B lymphocytes and activation of T lymphocytes as part of the adaptive immune system (Bettelli *et al.*, 2006, Courtney *et al.*, 2004, Fattori *et al.*, 1994). IL-6 has also been shown to play an important role in recruiting leukocytes, through stimulating IL-8 and MCP-1 release from endothelial cells, as well as enhancing the expression of molecules important for leukocyte adhesion (Romano *et al.*, 1997). The levels of IL-6 in the serum of individuals with

CF have been shown to increase during airway inflammation (Horsley *et al.*, 2013) and the concentrations detected are significantly correlated with *S. aureus* bacterial density in CF sputa (Junge *et al.*, 2016), making it a potential future biomarker.

The release of IL-10 from airway epithelia is involved in the resolution of inflammation. Increasing the synthesis of I-κB, this serves to subsequently reduce NF-κB activity and in turn, downregulate the production of pro-inflammatory cytokines, including IL-8 (Barnes and Karin, 1997, Tabary *et al.*, 2003). An absence of IL-10 has been associated with a prolonged pro-inflammatory response and neutrophil persistence within CF airways (Chmiel *et al.*, 2002).

Numerous studies to date have examined the impact of whole live *S. aureus* or *P. aeruginosa* upon the airway inflammatory response, as well as the impact of their secreted products (Moreilhon *et al.*, 2005, Hawdon *et al.*, 2010, Beaudoin *et al.*, 2013, Massion *et al.*, 1994, Pena *et al.*, 2009, Delgado *et al.*, 2006, Zhang *et al.*, 2005, LaFayette *et al.*, 2015, DiMango *et al.*, 1998). Indirect interactions between the products of CF pathogens and the host are important. *P. aeruginosa*-derived pyocyanin has been detected at concentrations of up to 27 µg/mL in CF sputum (Wilson *et al.*, 1988), whilst host antibodies to extracellular proteases and exotoxin A secreted by *P. aeruginosa* have also been detected (Doring *et al.*, 1985). Furthermore, CF patients have been shown to mount IgG antibodies to *S. aureus* exoproducts, including leucotoxins LukED (Junge *et al.*, 2016), LukAB (Thomsen *et al.*, 2014) and PVL (Chadha *et al.*, 2016) the pore forming alpha toxin (Ericsson *et al.*, 1986) and cell-wall derived teichoic acid (Hollsing *et al.*, 1987a). Although it is appreciated that CF airway infections are polymicrobial in nature, how airway epithelia respond to multiple bacterial stimuli shed and secreted by different CF pathogens is poorly understood.

5.3 Aims and objectives

Chapter 4 aimed to examine the impact of *S. aureus* and *P. aeruginosa* extracellular products upon the release of the major pro-inflammatory mediators IL-8 and IL-6, as well as the anti-inflammatory cytokine IL-10 by CF and non-CF bronchial epithelia. Monoculture stimulation with *S. aureus* and/or *P. aeruginosa* cell-free supernatants were performed, prior to co-stimulation with *S. aureus* and *P. aeruginosa* exoproducts simultaneously. The impact of these challenges upon bronchial epithelia metabolism (as a measure of viability) and cellular morphology were also determined.

5.4 Methods

Bacterial strains and growth conditions. *S. aureus* ATCC 6538, *P. aeruginosa* PAO1 and *P. aeruginosa* CF clinical isolates 5, 6 and 7 were used for all experiments. Single colonies of each isolate grown routinely on LB agar were inoculated into 10 mL of LB2 broth and were grown overnight (approximately 16 h), under normoxia at 37 °C. The following day, the overnight cultures were diluted 1:100 into 100 mL of LB broth in 250 mL conical flasks and were grown under normoxia at 37 °C for 24 h. At 24 h, the density of the cultures were determined at OD_{470} and recorded. All cultures were used after 24 h despite differences in the final bacterial cell density.

Preparation of cell-free supernatants. Twenty four h cultures of *S. aureus* and *P. aeruginosa* grown under normoxia were centrifuged at 4,000 x *g* for 30 min at 4 °C to pellet the bacterial cells. Each supernatant was sterile filtered with a low-binding 0.22 µm polyethersulfone membrane filter and stored at −20 °C until use. To confirm sterility after each preparation, a small volume of the supernatant was streaked out on LB agar plate and incubated for approximately 20 h prior to reading. All cell-free supernatants were heat-inactivated for 10 min at 95 °C to minimise airway epithelial cell toxicity, unless otherwise specified.

Airway epithelial cell culture. For airway inflammation studies, IB3-1 and C38 cells were seeded into 24-well polystyrene tissue culture treated plates at $1x10^5$ cells/mL, with 1 mL of the cell suspension being added to each well. Plates were incubated overnight at 37 °C with 5% CO_2. The following day at confluence, cell culture media was aspirated and replaced with 1 mL/well of starvation medium (DMEM/F12 supplemented with 1% (v/v) FBS) for a minimum of 16 h prior to stimulation. For cell morphology assays, cells were seeded into 96-well plates at $2.5x10^4$ cells/well in a total of volume of 200 µL and incubated overnight at 37 °C with 5% CO_2.

Airway epithelial cell bacterial product challenge. Heat-inactivated cell-free supernatants of *S. aureus* and/or *P. aeruginosa* were added to the epithelial cells at a 1:10 dilution. LB broth was used as the negative control, whilst stimulation with LPS from *E. coli* 0111:B4 was used at a final concentration of 10 µg/mL. IB3-1 and C38 were then incubated at 37 °C with 5% CO_2 for 24 h. After stimulation, the cell culture supernatants were centrifuged at 14,000 x *g* for 10 min to pellet any cell debris, transferred to a new sterile microtube and stored at −20 °C until further analysis. For the cell morphology study, cell-free supernatants of *S. aureus* and/or *P. aeruginosa* were added to the cells at a 1:10 dilution, with LB broth being added as a negative control. Plates were incubated 37 °C with 5% CO2 for 24 h.

Airway epithelial cell metabolism. CellTiter Blue™ (CTB™) is an endpoint assay based on fluorometry, which measures cell metabolism as a function of cell viability. Metabolically active viable cells are able to convert resazurin (blue) reagent to its highly fluorescent product resorufin (pink). CTB™ was added to the cell culture medium using 20 µL for each 100 µL of cell culture medium. The reagent was incubated for 2 h at 37 °C (as per manufacturer's instructions). The fluorescence was subsequently read on a multiwell fluorescent plate reader using $560/590_{nm}$ excitation/emission wavelength.

Detection of IL-8, IL-6 and IL-10 by ELISA. For the quantification of human IL-8, IL-6 and IL-10, clarified cell culture supernatants from CF and non-CF airway epithelia were assayed using IL-8, IL-6 and IL-10 ELISA Ready-Set-Go® kits. All reagents were part of each kit unless otherwise stated and were prepared according to the manufacturer's product information.

Morphology images. Cell-free supernatants from *S. aureus* and/or *P. aeruginosa* were added to IB3-1 and C38 cells and incubated for 24 h at 37 °C. After this, epithelial cell morphology was assessed using an inverted light microscope, under a x20 objective.

Statistical analysis. All results unless otherwise specified are expressed as mean ±S.E.M. Data for each experiment were collected from three independent experiments (N=3), each performed in triplicate. All statistical analyses were performed using GraphPad Prism 6 software with significance being set to P<0.05. The specific tests and *post-hoc* used for each experiment are described in the figure legends.

5.5 Results

5.5.1 *S. aureus-P. aeruginosa* co-infection, CF airway epithelia and IL-8 release

Bronchial airway epithelia play an essential role in the detection of inhaled pathogens within the lungs. The secretion and accumulation of a diverse array of diffusible extracellular products by CF pathogens activate CF airway epithelia and the release of pro-inflammatory mediators. IB3-1 CF airway epithelia were stimulated with *S. aureus* cell-free supernatants (10% v/v) and/or *P. aeruginosa* exoproducts (10% v/v) for 24 h. The IL-8 protein concentration following mono- and co-stimulation was subsequently determined from the airway epithelial cell culture supernatants.

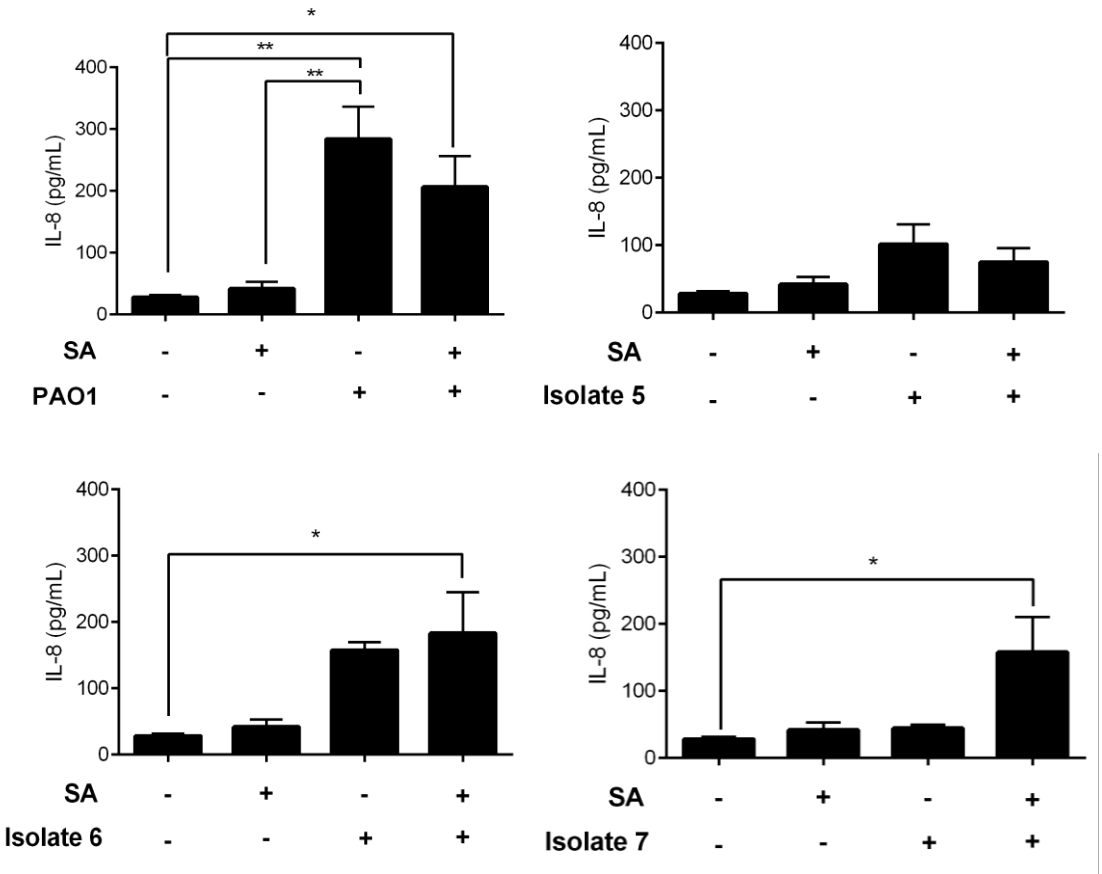


Figure 50. IL-8 responses of CF epithelia to cell-free supernatants of *S. aureus* and CF isolates of *P. aeruginosa*. IL-8 production by IB3-1 epithelia is shown following stimulation with cell-free supernatants of *S. aureus* (SA) at 10% (v/v) and/or *P. aeruginosa* (PAO1 and CF isolates 5, 6 and 7) at 10% (v/v). Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-8 in airway epithelial cell culture supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. **P*<0.05, ***P*<0.01 (One-way ANOVA with Tukey's *post-hoc* test).

As shown in Figure 50, *S. aureus* exoproducts induced a very low IL-8 response in CF airway epithelia (41.4 pg/mL ± 19.3), which was not significantly different in comparison to the LB broth only control (27.9 pg/mL ± 5.9). However, exposure to *P. aeruginosa* exoproducts led to the heightened release of IL-8 in an isolate dependent manner. PAO1 exoproducts significantly increased IL-8 release in CF airway epithelia compared to the LB broth only control ($P<0.01$) and *S. aureus* alone ($P<0.01$), whilst exoproducts from CF isolates 5, 6 and 7 did not.

Co-stimulation with exoproducts from *S. aureus* and *P. aeruginosa* PAO1 together induced significantly higher concentrations of IL-8 compared to the LB broth only control ($P<0.05$). However, co-stimulation had no significant effect upon the release of IL-8 compared to *S. aureus* only and PAO1 only. Whilst there appeared to be a decrease in the amount of IL-8 produced following co-stimulation compared to PAO1 alone, this was not significant ($P>0.05$).

Co-stimulation with exoproducts products from *S. aureus* and *P. aeruginosa* CF isolates 6 and 7 was significantly higher than the LB broth only control ($P<0.05$), a finding not seen for CF isolate 5. However, co-stimulation did not induce a significantly higher IL-8 response compared to *S. aureus* alone or *P. aeruginosa* alone for all of the CF isolates. In summary, *P. aeruginosa* cell-free supernatants can induce IL-8 responses from CF airway epithelia in an isolate dependent manner, but challenge in association with *S. aureus* did not further increase that response.

5.5.2 *S. aureus-P. aeruginosa* co-infection, non-CF airway epithelia and IL-8 release

Given the responses seen in CF airway epithelia, as a comparison the IL-8 responses from non-CF airway epithelia were also analysed. C38 monolayers were challenged with *S. aureus* cell-free supernatants (10% v/v) and/or *P. aeruginosa* cell-free supernatants (10% v/v) for 24 h as indicated.

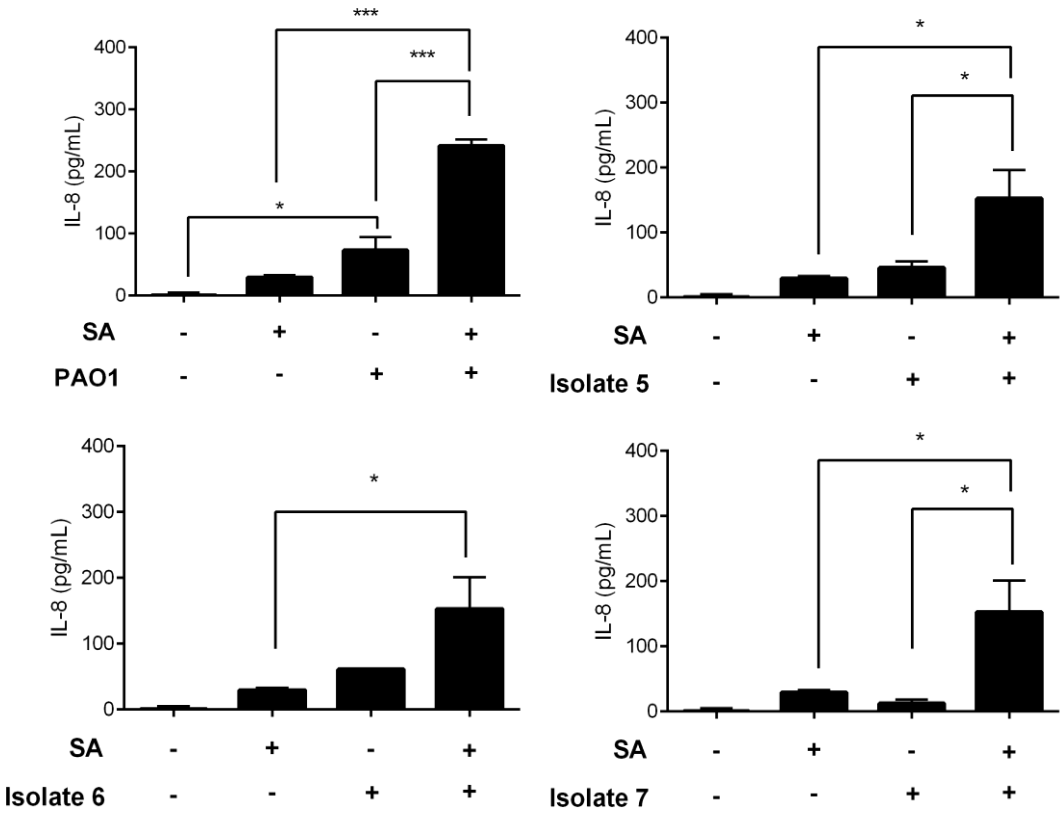


Figure 51. IL-8 responses of non-CF airway epithelia to cell-free supernatants of *S. aureus* and CF isolates of *P. aeruginosa*. IL-8 production by C38 cells is shown following stimulation with cell-free supernatants (10% v/v) of *S. aureus* (SA) and/or (10% v/v) *P. aeruginosa* (PAO1 and CF isolates 5, 6 and 7) as indicated. Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-8 in airway epithelial cell supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. **P*<0.05, ****P*<0.001 (One-way ANOVA with Tukey's *post-hoc* test).

As shown in Figure 51, the LB broth only control elicited a minimal IL-8 response in non-CF epithelia (0.52 pg/mL ± 7.012) which was significantly lower than the concentration measured in CF epithelia (*P*<0.01). Furthermore, *S. aureus* exoproducts did not elicit a significant increase in the IL-8 response (29.32 pg/mL ± 6.30) compared to the LB broth only control. The levels of IL-8 induced by *S. aureus* exoproducts in non-CF epithelia were also not significantly different to those measured in CF epithelia. Mono-stimulation with *P. aeruginosa* PAO1 exoproducts led to the heightened production of IL-8, which was significantly higher than LB

broth alone (P<0.05), but not compared to *S. aureus* exoproducts alone. The amount of IL-8 produced in response to PAO1 mono-stimulation was significantly lower in non-CF epithelia (P<0.05), compared to CF epithelia.

P. aeruginosa CF isolate 5 exoproducts failed to induce a significant increase in IL-8 compared to the LB broth only control and *S. aureus* alone, with the IL-8 concentration being found at a concentration which was not significantly different to that induced CF airways. CF isolate 6 exoproducts failed to elicit a significant change in the production of IL-8 in non-CF airways compared to the LB broth only control and *S. aureus* alone. The production of IL-8 following a 24 h challenge with CF isolate 6 exoproducts was also significantly lower in non-CF airways, compared to CF airways (P<0.01). Challenge with CF isolate 7 exoproducts failed to induce a robust IL-8 response compared to the LB broth only control and *S. aureus* alone in non-CF airways. The concentration of IL-8 induced by CF isolate 7 cell-free supernatants in non-CF epithelia was also significantly lower than those induced in CF airway epithelia (P<0.05).

Co-stimulation of non-CF epithelia with *S. aureus* and PAO1 exoproducts significantly increased the release of IL-8 compared to both *S. aureus* alone (P<0.001) and PAO1 alone (P<0.001). The concentration of IL-8 detected in non-CF epithelia following co-stimulation however, was not significantly different to that induced in CF airway epithelia. Co-stimulation of non-CF epithelia with *S. aureus* and CF isolate 5 exoproducts also induced a significant increase in IL-8 release compared to both *S. aureus* mono-stimulation (P<0.05) and *P. aeruginosa* mono-stimulation (P<0.05). The concentration of IL-8 induced in non-CF epithelia by co-stimulation with *S. aureus* and CF isolate 5 exoproducts was not significantly different to that induced in CF airway epithelia.

Co-stimulation with *S. aureus* and CF isolate 6 induced a significant increase in the release of IL-8 in non-CF epithelia compared to *S. aureus* alone (P<0.05), but not *P. aeruginosa* CF isolate 6 alone. Once more, co-stimulation of non-CF airway epithelia to *S. aureus* and *P. aeruginosa* CF isolate 6 failed to induce a significant change in IL-8 compared to the response seen in CF airway epithelia. Lastly, co-stimulation of non-CF epithelia with *S. aureus* and CF isolate 7 exoproducts elicited a significant increase in the production of IL-8 compared to both *S. aureus* alone (P<0.05) and CF isolate 7 alone (P<0.05). The concentration of IL-8 released in non-CF epithelia following co-stimulation with *S. aureus* and CF isolate 7 exoproducts was not significantly different to that induced in CF airway epithelia.

In summary, unlike CF airway epithelia, the co-stimulation of non-CF epithelia with *S. aureus* and *P. aeruginosa* exoproducts, lead to a significant increase in the production of IL-8 compared to both *S. aureus* alone and *P. aeruginosa* alone. However, the levels of IL-8 produced following co-stimulation with *S. aureus* and *P. aeruginosa* exoproducts are comparable in CF and non-CF epithelia.

5.5.3 Effect of LPS upon the IL-8 inflammatory response in CF and non-CF airway epithelia

Whilst bronchial epithelia detect and respond to accumulated diffusible extracellular products, they also respond to shed cell wall components, including the TLR4 agonist LPS. This assay determined the impact of purified LPS from the Gram-negative bacterium *E. coli* 0111:B4 strain upon the release of IL-8 from CF epithelia and whether *S. aureus* extracellular products influenced airway inflammation.

As shown in Figure 52, LPS induced a potent IL-8 response in CF airway epithelia, which was significantly higher compared to the LB broth only control (*P*<0.001). Co-stimulation of LPS with *S. aureus* cell-free supernatants at a concentration of 10% (v/v) also led to a robust IL-8 response compared to the LB broth only control (*P*<0.001), although this was not significantly different compared to LPS alone. LPS also induced a significant increase in the release of IL-8 from non-CF airway epithelia compared to the LB broth only control (*P*<0.001), with *S. aureus* exoproducts failing to elicit any change in the production of IL-8. LPS alone and co-stimulation with *S. aureus* extracellular products induced a more potent response in CF airway epithelia, compared to the non-CF counterpart (*P*<0.001).

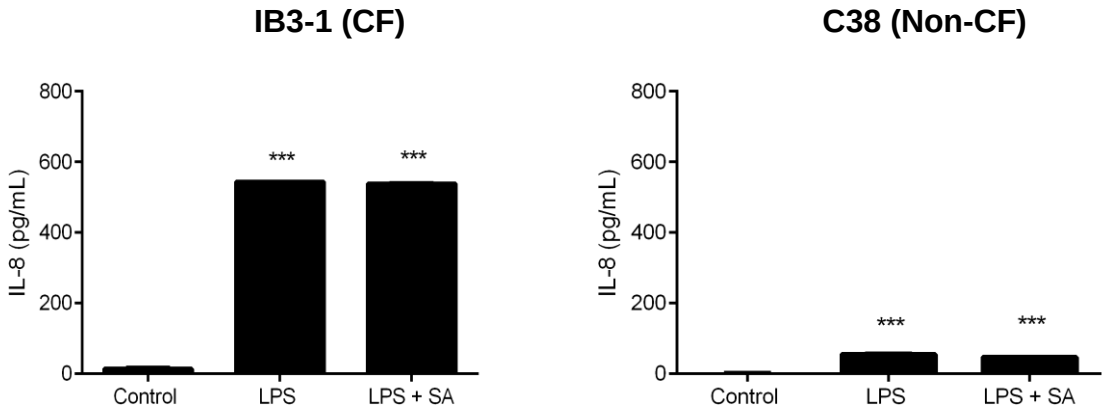


Figure 52. Induction of IL-8 by LPS alone versus LPS with *S. aureus* extracellular products. IL-8 production by IB3-1 (left) and C38 (right) airway epithelia following stimulation with *E. coli* 0111:B4 derived LPS and *S. aureus* cell-free supernatants (10% v/v). Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-8 in airway epithelial cell supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. ****P*<0.001, (One-way ANOVA with Tukey's *post-hoc* test).

5.5.4 *S. aureus-P. aeruginosa* co-infection, CF airway epithelia and IL-6 release

The impact of bacterial extracellular products upon the release of the pro-inflammatory cytokine IL-6 from CF bronchial epithelia was also determined (Figure 53). Submerged monolayers of IB3-1 were challenged with *S. aureus* cell-free supernatants (10% v/v) and/or *P. aeruginosa* cell-free supernatants (10% v/v) and the plates incubated at 37 °C for 24 h. The concentrations of IL-6 released by the CF airway epithelia were measured by ELISA.

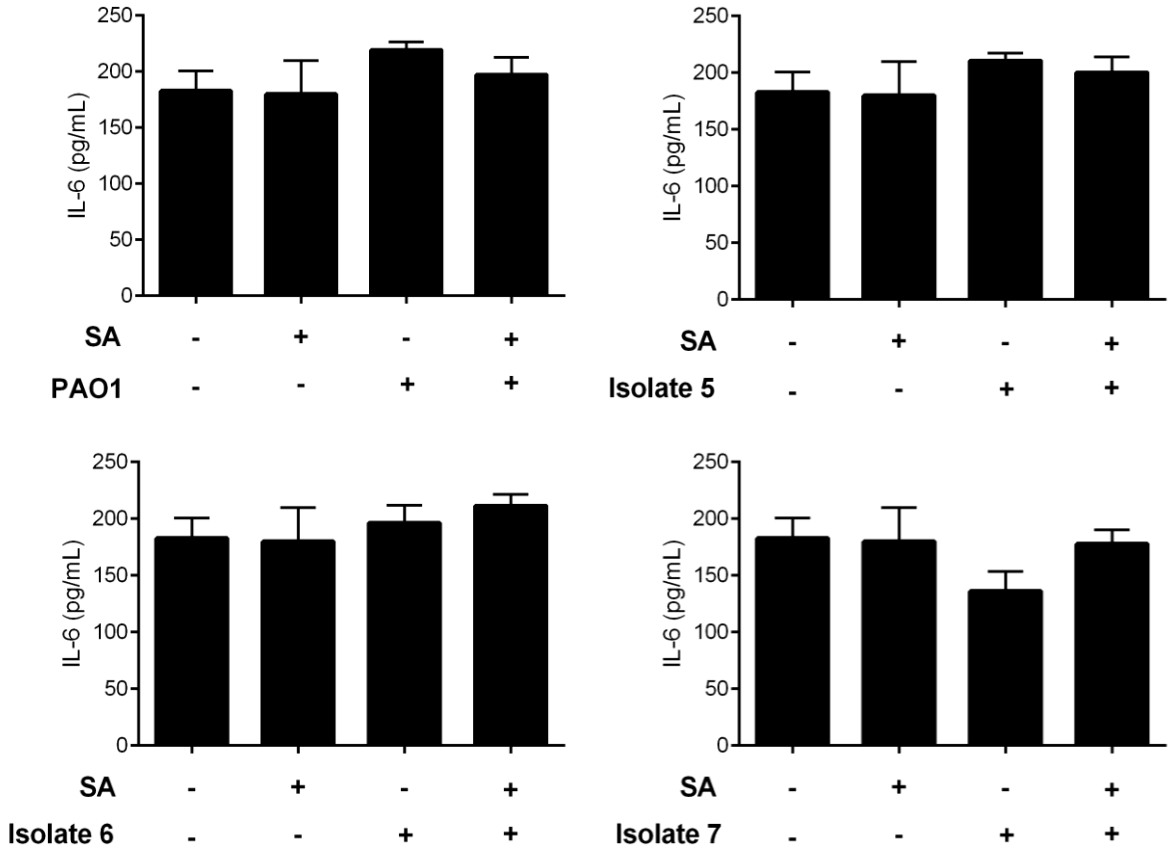


Figure 53. IL-6 responses of CF airway epithelia to cell-free supernatants of *S. aureus* and CF isolates of *P. aeruginosa*. IL-6 production by IB3-1 airway epithelia following was determined following stimulation with cell-free filtrates of *S. aureus* (SA) (10% v/v) and/or *P. aeruginosa* (PAO1 and CF isolates 5, 6 and 7) (10% v/v) as indicated. Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-6 in airway epithelial cell supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. (One-way ANOVA with Tukey's *post-hoc* test).

As seen with IL-8, CF epithelia demonstrated a hyper-inflammatory phenotype, with the release of high concentrations of IL-6 in the presence of the LB broth only control (182.66 pg/mL ± 30.962) (Figure 53). *S. aureus* exoproducts failed to induce any effect upon the release of IL-6 compared to LB broth only control. Single challenges with PAO1 and CF isolates 5, 6 and 7 exoproducts products did not significantly affect the production of IL-6 compared to single challenges with the LBN broth only control or *S. aureus*. Co-stimulation did

not significantly influence the IL-6 inflammatory response in relation to *S. aureus* alone or *P. aeruginosa* alone for PAO1 and the CF clinical isolates.

5.5.5 *S. aureus-P. aeruginosa* co-infection, non-CF airway epithelia and IL-6 release

Given the responses seen in CF airway epithelia, the IL-6 responses from non-CF airway epithelia were also determined. Submerged monolayers of C38 were challenged with *S. aureus* cell-free supernatants (10% v/v) and/or *P. aeruginosa* cell-free supernatants (10% v/v) and the plates incubated at 37 °C for 24 h. The concentrations of IL-6 released by the non-CF airway epithelia were measured by ELISA.

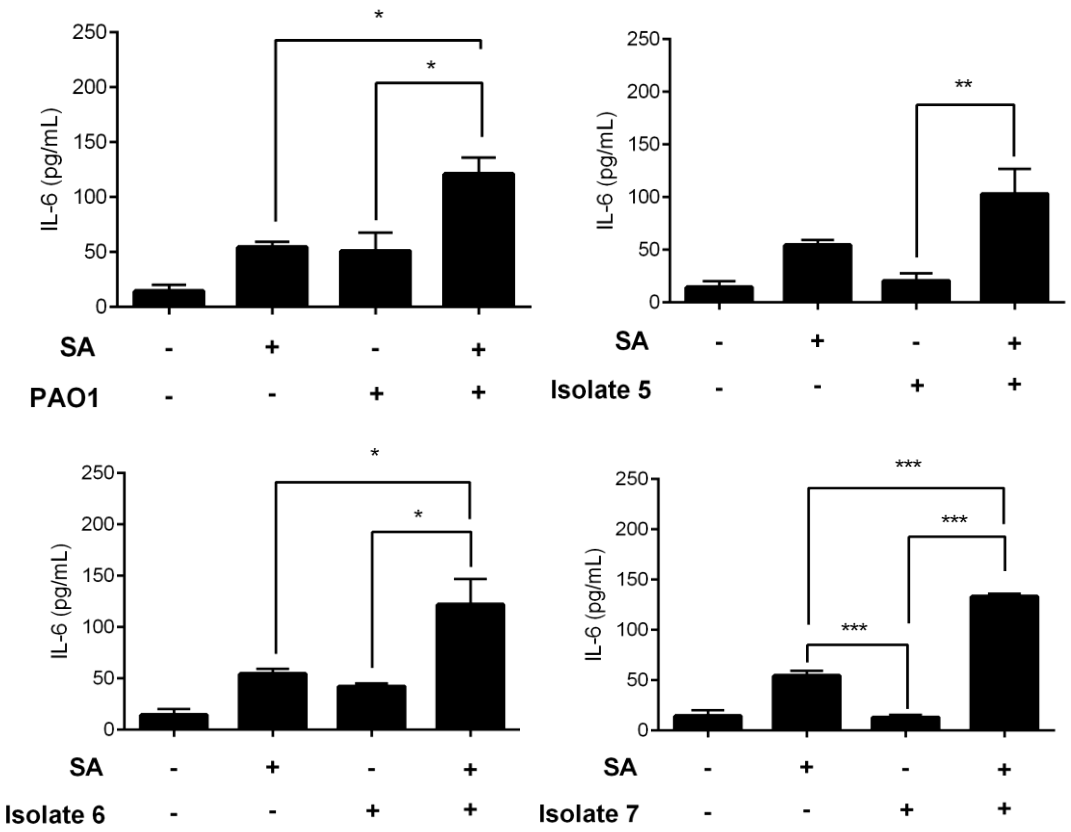


Figure 54. IL-6 responses of non-CF airway epithelia to sterile filtrates of *S. aureus* and CF isolates of *P. aeruginosa*. IL-6 production by C38 airway epithelia following stimulation with cell-free supernatants of *S. aureus* (10% v/v) and/ or *P. aeruginosa* (PAO1 and CF isolates 5, 6 and 7) (10% v/v) as indicated. Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-6 in airway epithelial cell supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. **P*<0.05 and ***P*<0.01 (One-way ANOVA with Tukey's *post-hoc* test).

As shown in Figure 54, LB broth alone induced a small amount of IL-6 (14.3 pg/mL ± 9.79) in non-CF airway epithelia, which was significantly lower than that induced in CF airway epithelia (*P*<0.001). Single challenge with *S. aureus* exoproducts did not induce a significant increase in IL-6 release compared to the LB broth only control. Single challenge with PAO1 exoproducts also did not induce a significant change in the production of IL-6 compared to LB broth alone and *S. aureus* alone. Both *S. aureus* and PAO1 induced significantly lower amounts of IL-6 in non-CF epithelia compared to CF airway epithelia (*P*<0.05 and *P*<0.001 respectively). Exoproducts from CF isolates 5 and 6 did not elicit an effect upon the release of IL-6 compared to LB broth only control and *S. aureus* exoproducts alone. Interestingly, CF isolate 7 exoproducts did not significantly affect the production of IL-6 compared to the LB broth only control, whilst it did induce significantly lower levels of IL-6 compared to *S. aureus* alone (*P*<0.001). Additionally, the concentrations of IL-6 induced by CF isolates 5, 6 and 7 exoproducts were all significantly lower than those induced in CF epithelia (*P*<0.001 for CF isolate 5 and 6 and *P*<0.01 for CF isolate 7).

Co-stimulation with *S. aureus* and PAO1 extracellular products significantly increased IL-6 response compared to *S. aureus* (*P*<0.05) and PAO1 mono-challenges (*P*<0.05), whilst the total amount of IL-6 released following co-stimulation was lower than that induced in CF airway epithelia (*P*<0.05). Co-stimulation with *S. aureus* and CF isolate 5 exoproducts significantly increased the release of IL-6, compared to stimulation with CF isolate 5 alone (*P*<0.01), but not *S. aureus* alone. Co-stimulation of non-CF epithelia with *S. aureus* and CF isolate 6 exoproducts significantly increased the concentration of IL-6 released compared to *S. aureus* alone (*P*<0.05) and CF isolate 6 alone (*P*<0.05). Co-stimulation with *S. aureus* and extracellular products from either CF isolate 5 and 6 induced lower concentrations of IL-6 than those measured in CF airways (*P*<0.05). Lastly, co-stimulation with *S. aureus* and CF isolate 7 extracellular products led to a significant increase in the release of IL-6 compared to *S. aureus* alone (*P*<0.001) and CF isolate 7 alone (*P*<0.001). The concentration of IL-6 induced in non-CF epithelia following co-stimulation with *S. aureus* and CF isolate 7 exoproducts was significantly lower than the concentration measured in CF epithelia (*P*<0.05).

In summary, the concentration of IL-6 produced in CF airway epithelia was significantly elevated at baseline compared non-CF airway, whilst stimulation with *S. aureus* and/or *P. aeruginosa* exoproducts did not increase this further. Conversely, non-CF airway epithelia produced a low level of IL-6 at baseline, where co-stimulation of non-CF epithelia with *S. aureus* and *P. aeruginosa* exoproducts significantly increased the production of IL-6, compared to mono-stimulation with *S. aureus* exoproducts and *P. aeruginosa* exoproducts.

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5.5.6 Effect of LPS upon the release of IL-6 from bronchial airway epithelia

The effect of purified LPS and *S. aureus* extracellular products was also determined upon the release of IL-6 (Figure 55). The TLR4 agonist LPS induced a potent IL-6 response in CF airway epithelia, which was significantly higher compared to the LB broth only control (*P*<0.001). As seen with IL-8, co-stimulation of LPS with *S. aureus* cell-free supernatant led to a robust IL-6 response in CF epithelia, although this was not significantly different compared to LPS alone. LPS also induced a significant increase in the release of IL-6 in non-CF airway epithelia compared to the LB broth only control (*P*<0.001), where co-stimulation with *S. aureus* did not elicit any significant change in the inflammatory response compared to LPS alone. IL-6 release from LPS alone and co-stimulation with *S. aureus* was significantly greater in CF bronchial epithelia, than that produced by non-CF airway epithelia (*P*<0.01).

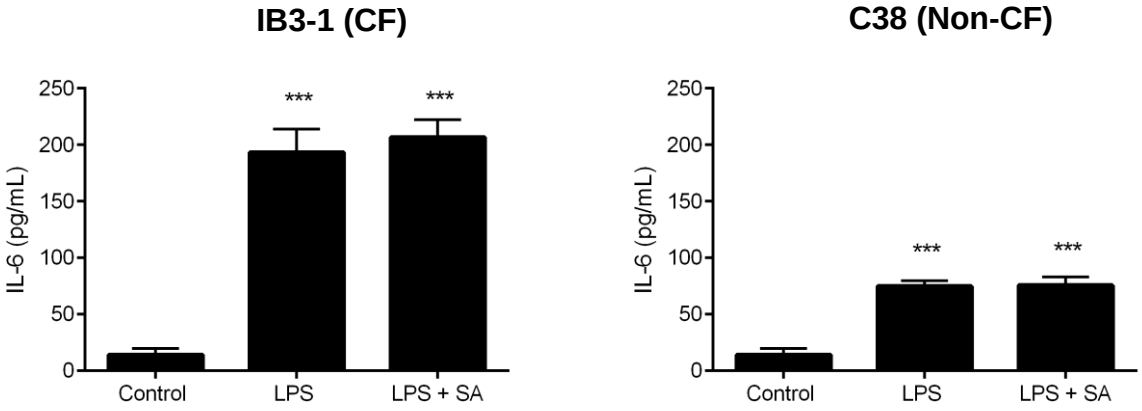


Figure 55. Influence of LPS alone versus LPS with *S. aureus* exoproducts upon the release of IL-6 from bronchial airway epithelia. IL-6 production by IB3-1 (left) and C38 (right) airway epithelia following stimulation with *E. coli* 0111:B4 derived LPS and *S. aureus* cell-free supernatant (10% v/v). Plates were incubated at 37 °C and 5 % CO_2 for 24 h, prior to quantifying extracellular IL-6 in airway epithelial cell supernatants by ELISA. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. ****P*<0.001 (One-way ANOVA with Tukey's *post-hoc* test).

5.5.7 Release of IL-10 from CF and non-CF airway epithelia following challenge with *S. aureus* and *P. aeruginosa* exoproducts and LPS

After determining the impact of single and dual challenges of *S. aureus* and *P. aeruginosa* exoproducts upon the production of the pro-inflammatory mediators IL-8 and IL-6 in CF and non-CF epithelia, their impact upon the production of IL-10, involved in airway inflammation resolution was also determined.

Mono- and co-stimulation with *S. aureus* and *P. aeruginosa* cell-free supernatants in CF and non-CF airway epithelia failed to induce any detectable IL-10 after 24 h (data not shown). Stimulation of airway epithelia with LPS alone and in combination with *S. aureus* exoproducts also failed to elicit a detectable IL-10 response in both cell lines (data not shown).

5.5.8 Cytotoxicity of *S. aureus* and *P. aeruginosa* extracellular products upon CF and non-CF airways

Although *S. aureus* and *P. aeruginosa* cell-free supernatants were heat-inactivated to minimise airway epithelial cell toxicity, the impact of these challenges upon cell viability was determined using CTB™. As shown in Figure 56 below, co-stimulation with *S. aureus* and CF isolate 6 exoproducts induced a significant decrease in airway cell metabolism in CF epithelia compared to the LB broth only control ($P<0.05$). The remaining isolates exhibited no effect upon cell viability in either CF or non-CF airway epithelia.

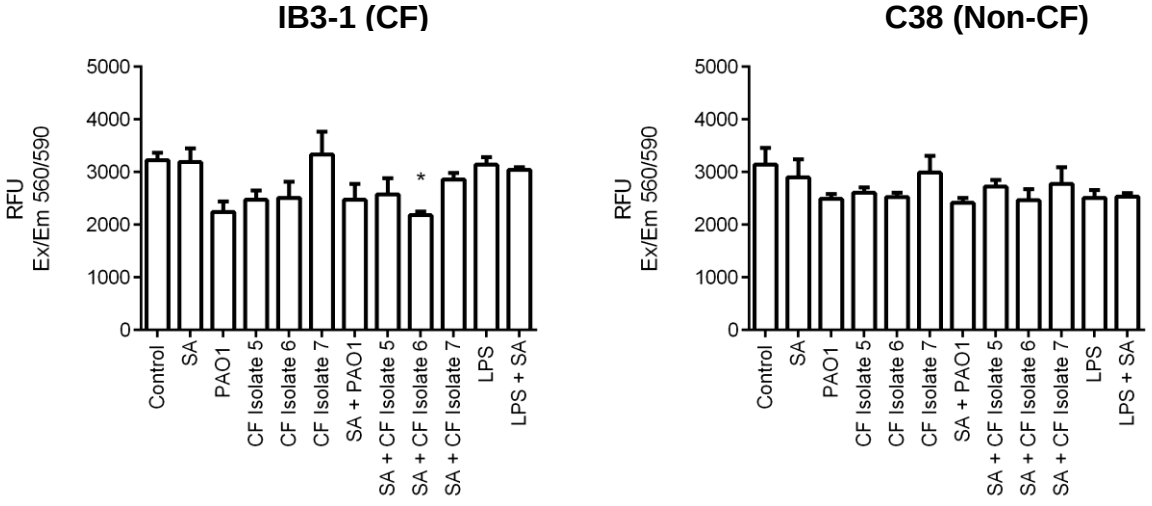


Figure 56. Airway epithelial cell metabolic activity following challenge with *S. aureus* and *P. aeruginosa* exoproducts. CF (left) and non-CF (right) bronchial epithelia exposed to *S. aureus* and *P. aeruginosa* exoproducts (each at 10% v/v) for 24 h were treated with CTB™ for 2 h to assess cell metabolic activity. Sterile LB broth was added to CF and non-CF epithelia as a control. The airway epithelial supernatants were collected and the RFU measured. Results are expressed as the mean ± S.E.M of three individual experiments ($N=3$), each performed in triplicate. (One-way ANOVA with Dunnett's *post-hoc*, versus control). *$P<0.05$.

5.5.9 Effect of *S. aureus* and *P. aeruginosa* supernatants upon airway cell morphology

Finally, *S. aureus* and *P. aeruginosa* cell-free supernatants which were not heat inactivated were exposed to both CF and non-CF airway epithelia to determine their effects airway cell morphology after 24 h. As shown in Figure 57, untreated bacterial filtrates from *S. aureus* induced cell elongation and a loss of adhesion compared to the LB broth only control, whilst PAO1 exoproducts induced cell rounding in IB3-1 monolayers. CF isolates 5, 6 and 7 induced no apparent changes to CF airway epithelial cell morphology.

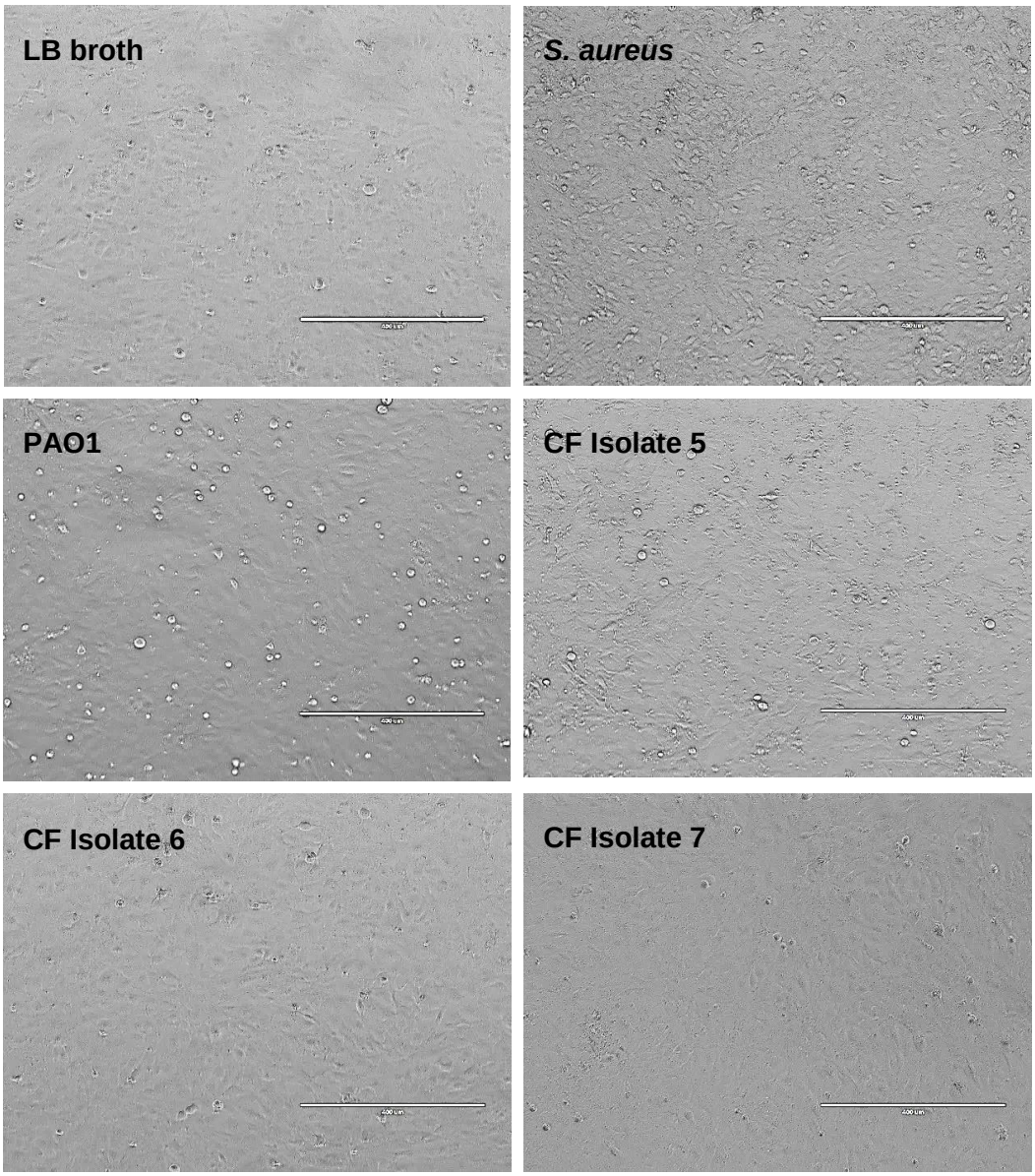


Figure 57. Impact of bacterial exoproducts upon CF airway epithelial morphology. Submerged monolayers of IB3-1 were exposed to either LB broth alone, or cell-free supernatants from *S. aureus* or *P. aeruginosa* CF isolates at 10% (v/v) for 24 h as indicated. Any changes to cellular morphology were detected using light microscopy. Images are representative of three independent experiments (*N*=3), each performed in duplicate. Scale bar represents 400 µm.

The same changes to cell morphology were seen in non-CF airway epithelia (Figure 58). *S. aureus* exoproducts induced cell elongation and a loss of adhesion, whilst PAO1 exoproducts caused cell rounding, as shown below.

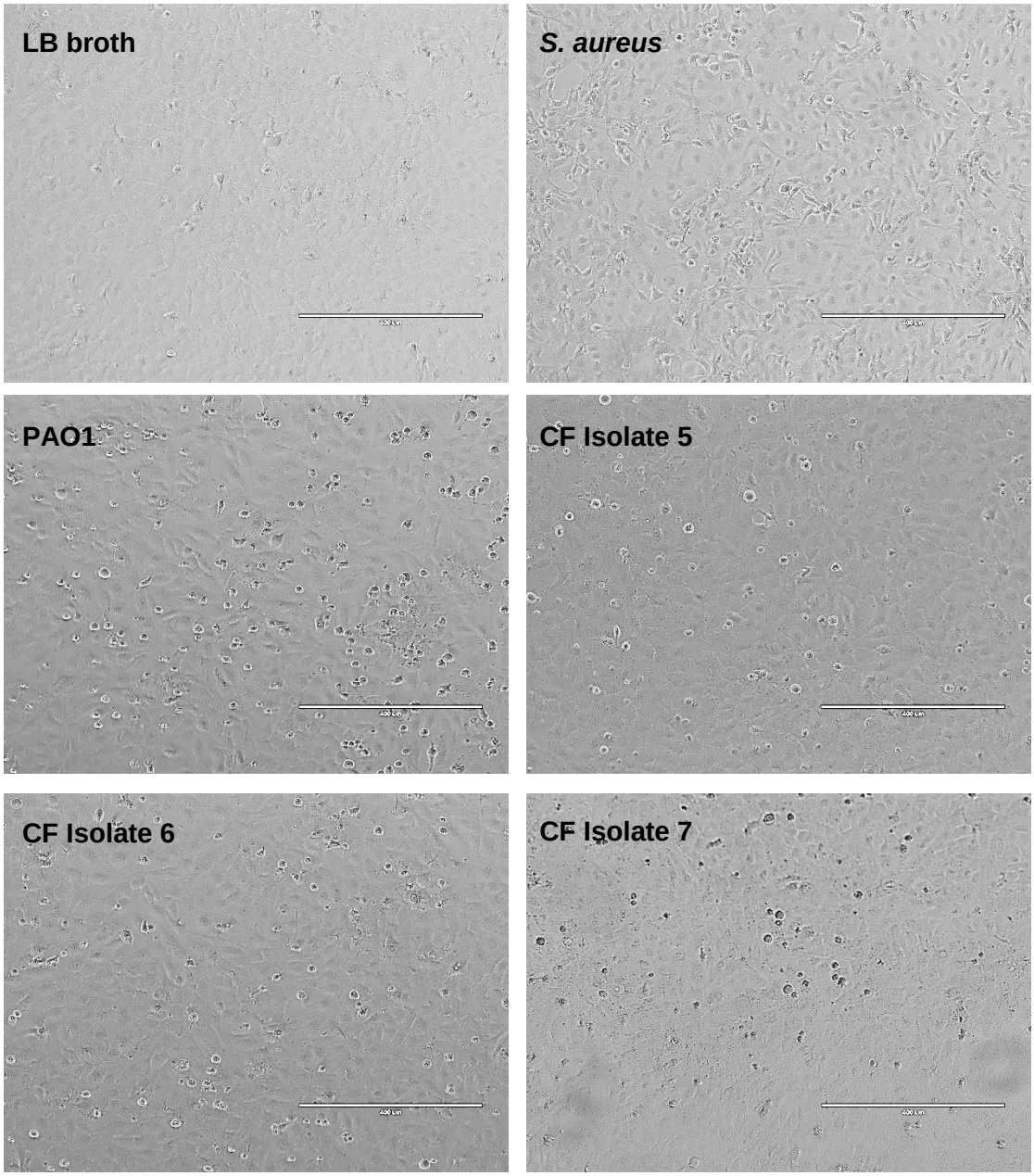


Figure 58. Impact of bacterial exoproducts upon non-CF airway epithelial morphology. Submerged monolayers of C38 were exposed to either LB broth alone (control) or cell-free supernatants from *S. aureus* or *P. aeruginosa* CF isolates (10% v/v) for 24 h as indicated. Any changes to cellular morphology were detected using light microscopy. Images are representative of three independent experiments *(N*=3*)*, each performed in duplicate. Scale bar represents 400 µm.

5.6 Discussion

S. aureus and *P. aeruginosa* have previously been shown to co-exist within the endobronchial lumen of CF airways (Rudkjobing *et al.*, 2012, Wakeman *et al.*, 2016, Biswas *et al.*, 2009a, Hogan *et al.*, 2016). The accumulation of diffusible extracellular products within the airway lumen secreted and shed by both *S. aureus* and *P. aeruginosa* interact with the surface of airway epithelia and drive inflammation (DiMango *et al.*, 1995). Whilst numerous studies have used heat-inactivated cell-free supernatants to study indirect interactions between airway epithelia and CF pathogens (Fink *et al.*, 2003, Berube *et al.*, 2010, Pena *et al.*, 2009, Wu *et al.*, 2005, LaFayette *et al.*, 2015), most focus upon challenges with a single CF pathogen. How airway epithelia respond to stimuli received as part of polymicrobial infections such as those seen in CF airways is poorly understood.

At present, it appears that only one previous study to date has addressed the effects of *S. aureus-P. aeruginosa* co-stimulation upon the release of the major chemokine IL-8 *in vitro* (Chekabab *et al.*, 2015). The study exposed non-CF Beas-2B airway epithelia to CF clinical isolates of *S. aureus* and laboratory strains of *P. aeruginosa* as single and dual challenges, with a final experiment used to validate their findings using the CF derived cell line, CFBE41o- (Chekabab *et al.*, 2015). This chapter subsequently employed the use of CF clinical isolates of *P. aeruginosa* along with *P. aeruginosa* reference strain PAO1 and *S. aureus* reference strain ATCC 6538 to determine the impact of bacterial co-stimulation upon the release of the primary neutrophil chemokine IL-8, the pro-inflammatory cytokine IL-6 and the anti-inflammatory, pro-resolving cytokine IL-10. Furthermore, paired isogenic epithelial cell lines were used, using the IB3-1 (CF) cell line and the CFTR corrected non-CF cell line, C38.

The bacterial cultures used in this assay were all grown for 24 h, with final densities reaching approximately 10^8 CFU/mL for *P. aeruginosa* and approximately 10^7 CFU/mL for *S. aureus,* as shown in the previous chapter (Figure 17). Such densities have been detected in CF sputum, with *P. aeruginosa* being detected at densities up to approximately 10^7-10^9 CFU/mL (Knibbs *et al.*, 2014, Hoiby, 1998, Palmer *et al.*, 2005, Bauernfeind *et al.*, 1987), whilst *S. aureus* has been detected in abundancies of up to approximately 10^7-10^8 CFU/mL (Johnson *et al.*, 2016, Hammerschlag *et al.*, 1980, Bauernfeind *et al.*, 1987, Osika *et al.*, 1999). This high abundancy in the CF lung is believed to be due to the nutritional composition of CF airway sputum which supports bacterial growth, rich in protein and amino acids (Ohman and Chakrabarty, 1982, Barth and Pitt, 1996).

Immortalised CF and non-CF airway bronchial epithelial cell lines were plated and grown to confluence, prior to being stimulated with LB broth only (control), *S. aureus* and/or *P. aeruginosa* exoproducts. During the course of chronic infection, airway epithelia are likely to respond to the diffusible exoproducts secreted by chronically infecting CF pathogens (Baltimore *et al.*, 1989) where extracellular elastase, pyocyanin and flagellin have all been shown to damage the airways, as well as induce airway inflammation (Nomura *et al.*, 2014, Denning *et al.*, 1998b, DiMango *et al.*, 1995). Individuals with CF have also been shown to develop antibodies to a range of bacterial exoproducts, including proteases, elastase, flagellin, LPS and LTA (Doring and Hoiby, 1983, Doring *et al.*, 1984, Anderson *et al.*, 1989, Fomsgaard *et al.*, 1988, Hollsing *et al.*, 1987a). In CF airways, cell debris, mucus, bacterial biofilms, excessive neutrophil influx and the absence of ASL are all likely to limit the concentration and diffusion of bacterial products reaching the epithelial cell surface.

As shown in Figure 50, the CF bronchial epithelia demonstrated a significantly increased IL-8 response in the absence of bacterial product stimulation, compared to non-CF airways (Figure 51) ($P<0.01$). This heightened baseline secretion of IL-8 by CF airway epithelia has been reported in primary bronchial and tracheal gland cells, primary bronchial epithelia and clinically, with CF neonates and infants producing elevated levels of IL-8, neutrophils and NE within BALF in the absence of any detectable infection (Khan *et al.*, 1995, Balough *et al.*, 1995, Noah *et al.*, 1997, Armstrong *et al.*, 1997, Carrabino *et al.*, 2006, Kube *et al.*, 2001, Kammouni *et al.*, 1997, Bonfield *et al.*, 1999, Muhlebach *et al.*, 2006, Tirouvanziam *et al.*, 2000, Black *et al.*, 1998). The basal and mRNA expression of IL-8 from the submucosal glands in CF patients has also been shown to be constitutively upregulated compared to non-CF bronchial gland cells (Tabary *et al.*, 1998).

This inflammatory response has been associated with constitutively activated NF-κB and the absence of IκB kinase α (IκBα) (Tabary *et al.*, 1998, Tabary *et al.*, 1999). Moreover, a higher co-localisation of NF-κB p65 has been reported in CF nasal polyps, compared to nasal polyps obtained from healthy controls (Raia *et al.*, 2005). The CF epithelial cell line used in this study (IB3-1) has previously been shown to exhibit changes to IκBβ regulation, facilitating the transcription of pro-inflammatory genes in the absence of infection (Venkatakrishnan *et al.*, 2000). The continuous inhibition of CFTR with CFTR(inh)-172 in primary airway epithelia grown at ALI has been shown to lead to a significant increase in IL-8 secretion at baseline, as well as upon exposure to *P. aeruginosa*. Losses in chloride conductivity gave rise to this finding (Perez *et al.*, 2007).

IB3-1 epithelia also exhibited a hyper-inflammatory phenotype at baseline regarding the production of IL-6 (Figure 53), which was significantly higher compared to the baseline production by non-CF epithelia (Figure 54) (P<0.001). This is supported by a study which reported that fresh CF bronchial epithelia (homozygous for Phe508del) exhibited an 8-fold increase in the production of IL-6 at baseline, compared to those isolated from heathy controls (Escotte *et al.*, 2003). CF-derived human tracheal gland cells have also been shown to secrete significantly higher levels of IL-6 at baseline, compared to those obtained from healthy individuals (Kammouni *et al.*, 1997).

A number of groups have sought to determine the underlying mechanisms regarding this phenotype. Vij *et al.* reported that functional wtCFTR localisation within cell surface lipid rafts negatively regulates NF-κB activity and subsequent IL-8 production (Vij *et al.*, 2009). An additional study by Hunter *et al.* demonstrated that the transfection of wtCFTR into pulmonary H441 and the non-pulmonary H57 cell lines (which lack CFTR expression), significantly reduced NF-κB activity and inflammation (Hunter *et al.*, 2010). TRADD is an adaptor protein which mediates phosphorylation of the inhibitory protein IκBα. This subsequently facilitates the translocation of NF-κB to the cell nucleus and the transcription of pro-inflammatory cytokines (Pobezinskaya and Liu, 2012). TRADD is known to bind to wtCFTR, where it subsequently sent for lysosomal degradation (Wang *et al.*, 2016). The lack of mature or functional CFTR in CF airway epithelia subsequently alleviates TRADD degradation, thus facilitating increased NF-κB activity and the production of pro-inflammatory mediators. Thus, detection of this hyper-inflammatory phenotype in this study is not unique to the specific cell line used, or that the cells were grown under submerged culture.

This hyper-inflammatory phenotype of CF epithelia in the absence of infection is highly debated within the CF field however, due to conflicting reports. Blau *et al.* demonstrated that the baseline secretion of IL-8 by IB3-1 airway epithelia was not significant compared to healthy C38 epithelia (Blau *et al.*, 2007), whilst Black *et al.* also reported that the baseline secretion of IL-8 was not significant between freshly isolated nasal epithelia from young children with CF and those obtained from healthy controls (Black *et al.*, 1998). Interestingly, another study reported that immortalised CFTR-corrected airway epithelia have secrete higher concentrations of IL-8 at baseline compared to CF epithelia which are homozygous for the Phe508del mutation (Massengale *et al.*, 1999).

S. aureus exoproducts alone failed to elicit a robust IL-8 response in both CF and non-CF bronchial epithelia (Figure 51 and Figure 52). Previous research has reported that IL-8 release from IB3-1 and C38 cells requires asialoGM1 and TLR2 co-mobilisation in a lipid raft (Soong *et al.*, 2004, DiMango *et al.*, 1995). The increased expression of asialoGM1 and TLR2 expression upon the surface of CF airway epithelia (DiMango *et al.*, 1995, Muir *et al.*, 2004)

gives rise to the expectation that CF epithelia would demonstrate a significant increase in IL-8 release in response to *S. aureus* products, a finding shown in previous studies (Ratner *et al.*, 2001, Below *et al.*, 2009, Moreilhon *et al.*, 2005). In spite of this, airway epithelia have also been shown to exhibit a dampened response to Gram-positive bacteria (Ivarsson *et al.*, 2013) compared to the response induced by Gram-negative bacteria (Mayer *et al.*, 2007). Furthermore, whilst TLR2 has been shown to initiate an inflammatory response to PVL (Zivkovic *et al.*, 2011), whether *S. aureus* ATCC 6538 secretes this toxin in detectable levels requires investigation. Bielemeier (2012) also demonstrated how C38 bronchial epithelia grown as co-cultures with fibroblasts at ALI secreted low levels of IL-8 following live bacterial apical challenges with *S. aureus* ATCC 6538, which was not significant compared to baseline (no bacteria) (Bielemeier, 2012b).

These data are also in contrast to previous reports however, which reported that *S. aureus* extracellular products induced a strong IL-8 response in human airway cells *in vitro* (Ratner *et al.*, 2001, Below *et al.*, 2009, Moreilhon *et al.*, 2005). It is possible that the bacterial growth media used in these studies (LB broth versus Tryptic Soy broth) is likely to influence the airway inflammatory response, a finding reported previously (Chekabab *et al.*, 2015). Moreover, Bielemeier (2012) reported how apical infection with *S. aureus* caused a significant increase in the release of apical and basal IL-8 compared to baseline (Bielemeier, 2012a) , whilst *S. aureus* strain Newman was shown to induce a significant increase in the IL-8 homologs MIP1α and MIP1β in a murine model of infection (Cigana *et al.*, 2017). However, in both of these studies, authors exposed airway epithelia to whole-live bacteria and not their cell-free exoproducts.

Cell-free supernatants from *P. aeruginosa* PAO1 induced the secretion of high levels of IL-8 in CF and non-CF airway epithelia after 24 h compared to baseline (Figure 50 and Figure 51). The ability of *P. aeruginosa* extracellular products to stimulate a potent pro-inflammatory response in airway epithelia has been reported previously (Kube *et al.*, 2001, Zhang *et al.*, 2005, Firoved *et al.*, 2004, Massion *et al.*, 1994, Oishi *et al.*, 1997), with *P. aeruginosa* colonisation also being associated with increases in the detection of pro-inflammatory markers in CF BALF (Noah *et al.*, 1997, Muhlebach *et al.*, 1999, Pukhalsky *et al.*, 1999).

Furthermore, the IL-8 response induced in CF epithelia following incubation with PAO1 exoproducts was significantly higher than that induced in non-CF epithelia ($P<0.01$). Previous reports have demonstrated how CF airway epithelia secrete high concentrations of IL-8 when challenged with *P. aeruginosa* (Scheid *et al.*, 2001, Joseph *et al.*, 2005, Balloy *et al.*, 2015, Virella-Lowell *et al.*, 2004, Kelly *et al.*, 2013, DiMango *et al.*, 1995). However, the evidence is also conflicting, where CF cells have been shown to express IL-8 at levels close to, or lower than non-CF airway epithelia (Reiniger *et al.*, 2005). Though additional work is required to

address this disparity, differences in cell lines, CF genotype and genetic components outside of this are all likely to have an influence upon IL-8 release. Surprisingly, cell-free supernatants from CF isolates 5, 6 and 7 all failed to induce significant increases in the release of IL-8 from CF and non-CF airway epithelia compared to the LB broth control and *S. aureus* alone.

It is possible that the findings obtained are due to the concentrations of the challenges used (10% v/v), which may have been too low cause a significant increase in the release of IL-8. Furthermore, PAO1 and the CF isolates tested exhibited wide variations in their virulence profile (as shown in Chapter 3). These wide variety of PAMPs will also lead to variations regarding the activation of airway epithelia TLRs and the downstream activation of NF-κB and p38 MAPK, as reported previously (Zhang *et al.*, 2005, Denning *et al.*, 1998b, Smith *et al.*, 2001, Mayer *et al.*, 2011, Rada *et al.*, 2011, Massion *et al.*, 1994). The impact of these exoproducts upon inducing airway inflammation will also be influenced by heat treatment, where *P. aeruginosa* flagellin for example is heat-labile (Ansorg, 1978).

In CF airways, bacterial colonisation induces an excessively high, yet highly variable IL-8 response (Colombo *et al.*, 2005, Mayer-Hamblett *et al.*, 2007, Osika *et al.*, 1999, Bodini *et al.*, 2005). Whilst one study demonstrated that the mean IL-8 concentration in CF sputum was approximately 145.4 ng/mL (± 75.3-337.3 ng/mL) in patients infected with *P. aeruginosa* (Watt *et al.*, 2005) another detected approximately 1087.5 ng/mL of IL-8 (Husson *et al.*, 2005). One study which had recruited twenty six CF patients aged 8-47 years old, illustrated that prior to antibiotic treatment, IL-8 concentrations in CF sputa ranged between 5,000-33,000 pg/mL, which was significantly reduced following the administration of intravenous antibiotics (Chiron *et al.*, 2008). BALF analysis from eighteen CF patients (mean age of 19.66 ± 5.2) had IL-8 concentrations of 325 ± 81.96 pg/mL, compared to 125.92 ± 43.95 pg/mL in healthy control subjects (Reeves *et al.*, 2011). An additional study assessing effect of antibiotics upon cytokine levels within CF sputum demonstrated that the average concentration of IL-8 in CF patients with a mean age of 8.6 ±5.4 was 7,165 pg/mL (3,400-13,770 pg/mL) prior to the administration of antibiotics (Colombo *et al.*, 2005). Another study assessing lung disease in 57 infants newly diagnosed with CF illustrated that the mean concentration of IL-8 detected in CF BALF was 320 pg/mL (150-845 pg/mL), with infants infected with a pathogen having higher levels of IL-8 compared to uninfected infants (Sly *et al.*, 2009). Differences are not restricted to CF BALF or sputa and have been shown to exist in faecal samples, with IL-8 concentrations in CF patients being 32,113 pg/g (21,656-178,128) of wet stool, compared to <43.5 pg/g (<22-4079) in healthy controls (Briars *et al.*, 1995).

Whilst CF airway epithelia are large contributors to such high concentrations of IL-8, neutrophils also secrete IL-8. A study comparing circulating blood neutrophils isolated from children with CF and healthy controls demonstrated that the spontaneous release of IL-8 was

significantly higher in neutrophils isolated from individuals with CF, compared to neutrophils obtained from control subjects. Furthermore, the spontaneous release of IL-8 was higher for CF airway neutrophils than CF circulating neutrophils. In both instances, challenge with LPS failed to increase the production of IL-8 further (Corvol *et al.*, 2003). Such values reported clinically are up to one thousand times higher than those seen *in vitro* and arise due to many different factors, including the simplistic nature of the assay, the concentration of the bacterial cell-free supernatants used, the airway epithelial cell density, the absence of whole live or heat-killed bacteria, the absence of host neutrophils, as well as the absence of other CF pathogens to name just a few.

Of all the dual challenge studies, only co-stimulation with *S. aureus* and PAO1 exoproducts and *S aureus* and CF isolate 7 exoproducts significantly increased IL-8 production by CF epithelia, compared to baseline ($P<0.05$) (Figure 50). Furthermore, there appeared to be a decrease in the amount of IL-8 produced compared to PAO1 alone, although this was not significant. None of the co-stimulation combinations tested in CF epithelia exhibited an additive effect with regards to the IL-8 response upon comparison to *S. aureus* and *P. aeruginosa* alone. This is unlikely to be due to saturation of pro-inflammatory signalling pathways however, with *E. coli* LPS inducing an IL-8 response nearly three times higher than those induced by co-infections with *S. aureus* and the CF isolates. Whilst Cigana *et al.* employed a non-CF mouse model of infection, the authors reported that co-infection with *S. aureus* and *P. aeruginosa* did not lead to an additive effect upon the production of the murine IL-8 analogues (Cigana *et al.*, 2017). However, these results are in contrast to a study assessing co-infection in young children, which demonstrated that the presence of *S. aureus* and *P. aeruginosa* exerted an additive effect upon the concentration of IL-8 detected in BALF (Sagel *et al.*, 2009a).

These results are also in conflict with the only study to date addressing *S. aureus*-*P. aeruginosa* co-infection *in vitro*, which reported that *S. aureus* exoproducts inhibited the release of IL-8 release in both Beas-2B (non-CF) and CFBE41o− (CF) airway epithelia, following co-stimulation with *P. aeruginosa* exoproducts. This finding was shown to be due to the ability of *S. aureus* exoproducts to repress NF-κB activity and subsequent IL-8 transcription (Chekabab *et al.*, 2015). This ability of *S. aureus* cell-free supernatant to dampen the IL-8 response has also been reported previously in human umbilical vein endothelial cells (HUVEC) after the cells were co-challenged with TNF-α (Tajima *et al.*, 2007). Whilst *S. aureus* has also been reported to inhibit IL-8 (Zurek *et al.*, 2015), other bacteria also downregulate IL-8 production, including lethal toxin from *Bacillus anthracis* which destabilises IL-8 mRNA (Mozaffarian *et al.*, 2000).

Despite this, it is appreciated that the effects of bacterial co-infection upon the host's immune response is likely to be complex, which is further influenced by the wide phenotypic diversity seen across *P. aeruginosa* CF clinical isolates. Additionally, Chekabab *et al.* (2015) reported that *S. aureus* inhibited the NF-κB induction of IL-8 via TLR1/2 only but not TLR4 and -5, despite these TLRs all signalling via NF-κB. Thus, the activation or deactivation of intracellular signalling mechanisms during polymicrobial infection is complicated and requires further study, as the early models used to study polymicrobial infection and the airway inflammatory response also begin to develop.

Unlike CF epithelia, all *S. aureus-P. aeruginosa* combinations (except CF isolate 6) significantly increased the IL-8 response in non-CF epithelia compared to *S. aureus* and *P. aeruginosa* alone. Co-stimulation also appeared to exert an additive effect compared to the IL-8 response induced by *S. aureus* and *P. aeruginosa* (Figure 51). Why co-infection exerts this additive effect upon IL-8 production in healthy airways across isolates but not in CF epithelia requires further study. It may be due to the dysregulated hyper-inflammatory phenotype of CF epithelia both at baseline and following challenges with microbial products.

The levels of IL-6 secreted at baseline were significantly higher in CF epithelia compared to non-CF epithelia (Figure 53 and Figure 54), a finding supported by Berube *et al.* (Berube *et al.*, 2010). Previous research using primary submucosal bronchial epithelia demonstrated that the levels of IL-6 secreted under resting conditions were 8-fold higher in CF bronchial cells than non-CF (Escotte *et al.*, 2003), whilst another demonstrated that the baseline secretion of IL-6 by IB3-1 airway epithelia was six-fold higher compared to C38 epithelia (Blau *et al.*, 2007). Berube *et al.* demonstrated that the IL-6 mRNA turnover rate was slower than the mRNA in CF epithelia, thus allowing IL-6 to be synthesised more quickly in CF epithelia (Berube *et al.*, 2010). These results are in contrast however to a previous study which demonstrated that the baseline secretion of IL-6 in submucosal glands from CF patients homozygous for Phe508del were similar to non-CF submucosal glands (Tabary *et al.*, 1998).

Mono-stimulations with *S. aureus* and the CF isolates of *P. aeruginosa,* including reference strain PAO1, failed to increase the production of IL-6 in CF epithelia (Figure 53). Whilst this contrasts with previous reports *in vitro* (Berube *et al.*, 2010, Jones *et al.*, 2003, Carpagnano *et al.*, 2003, Armstrong *et al.*, 1997, Borgatti *et al.*, 2007, Bonfield *et al.*, 1999), this may be due to the concentration of bacterial filtrates used, which are unable to increase its production above baseline. More likely, it may be due to the CF airway epithelia signalling cascades are saturated and the cells are maximally producing IL-6, which was significantly higher at baseline than that produced by non-CF epithelia ($P<0.001$). The IL-6 response at baseline in CF epithelia was also significantly higher than the IL-6 response induced by *S. aureus-P. aeruginosa* co-stimulation in non-CF airway epithelia.

Challenge with either *S. aureus* or *P. aeruginosa* exoproducts alone also did not significantly increase the concentration of IL-6 above baseline in non-CF epithelia (Figure 54).

Unsurprisingly, the concentration of IL-6 detected in CF patient sputum is highly variable. Some studies have shown that the average levels of IL-6 are low, with one study citing a concentration of approximately 1.7 pg/mL in sputum obtained from children with CF (Sagel *et al.*, 2012), whilst another recorded a mean IL-6 concentration of 2.35 pg/mL in CF sputum obtained from teenage and young adults (Husson *et al.*, 2005). Whilst Columbo *et al.* stated that concentrations of IL-6 were undetectable in CF sputa (Colombo *et al.*, 2005), concentrations of 26 pg/mL (12-110 pg/mL) have been detected, which was shown to be significantly lower than that measured in the sputum of healthy controls (225 pg/mL (200±426 pg/mL)) (Osika *et al.*, 1999). IL-6 concentrations of 40 ± 17 pg/mL have also been reported in freshly isolated CF bronchial epithelia in the absence of detectable infection (Bonfield *et al.*, 1999). Studies assessing the impact of antibiotics upon pulmonary exacerbations measured an IL-6 concentration of 7.28 pg/mL (3.7-14.4) in CF sputum, which was reduced to 2.16 pg/mL (1.3-3.5) following antibiotic treatment (Nixon *et al.*, 1998).

Whilst IL-6 regulates the acute phase response (Morrone *et al.*, 1988), it also induces to a lesser degree, neutrophil infiltration by enhancing neutrophil migration to IL-8 (Wright *et al.*, 2014). Thus, chronically elevated levels of both IL-8 and IL-6 in CF epithelia at baseline may not only impair the effectiveness of airway neutrophils due to early priming, but the secretion of NE is likely to impair other arms of the pulmonary innate immune system, including SP-D (Hirche *et al.*, 2004). Furthermore, co-stimulation with *S. aureus* and *P. aeruginosa* exoproducts significantly increased the production of IL-6 by non-CF epithelia, compared to mono-stimulation with *S. aureus* and *P. aeruginosa* exoproducts alone (Figure 54). This finding was not seen in CF epithelia. Thus, the inability of CF epithelia to upregulate IL-6 production as the size of the bacterial challenge increases and diversified may also impair the effectiveness of the airway immune response.

Exposure of CF and non-CF bronchial epithelia to the TLR4 agonist LPS, induced both IL-8 (Figure 52) and IL-6 (Figure 55) production, a finding shown previously (Kammouni *et al.*, 1997, Chekabab *et al.*, 2015). Whilst TLR4 has been shown to be downregulated in primary CF airway epithelia compared to healthy non-CF airway epithelia (Hauber *et al.*, 2005, John *et al.*, 2010), this has not been determined in IB3-1 and C38 cell lines. CF airway epithelia produced significantly greater levels of the two pro-inflammatory cytokines IL-8 and IL-6 following challenge with LPS, compared to non-CF epithelia, where *S. aureus* exoproducts did not elicit any effect upon its production **(**Figure 52 and Figure 55). These findings are supported by a previous study which demonstrated that *S. aureus* exoproducts did not exert any effect upon

the release of IL-8 following stimulation with LPS in both CF and non-CF airway epithelia (Chekabab *et al.*, 2015).

No IL-10 was detected in the CF and non-CF airway culture supernatants following challenges with *S. aureus* and/or *P. aeruginosa* exoproducts, or with purified LPS. As with IL-6, it is believed that this is the first study to address the impact of *S. aureus* and *P. aeruginosa* co-stimulation upon IL-10 release in CF and non-CF airway epithelia *in vitro* and thus makes comparisons to the literature difficult. However, previous studies have demonstrated that very low to absent levels of IL-10 were produced by CF epithelia, compared to non-CF airway epithelia (Becker *et al.*, 2004, Bonfield *et al.*, 1999, Bonfield *et al.*, 1995a). This phenomenon is not limited to airway epithelia however, where T cells expressing mutant CFTR are accompanied by a reduction in the secretion of IL-10 (Moss *et al.*, 1996). As mentioned previously, it is possible that the concentrations of filtrates used were too low to allow this anti-inflammatory cytokine to be detected, or that IB3-1 epithelia possibly do not make IL-10. As C38 is an isogenic cell line of IB3-1, it is expected to be genetically identical, except in regard to CFTR expression. Thus, if IB3-1 do not synthesise IL-10, it is expected that C38 will also not produce this pro-resolving cytokine. This absence of IL-10 is likely to be involved in the inability to downregulate the pro-inflammatory response, leading to chronic (Chmiel *et al.*, 2002, Moss *et al.*, 1996). In CF patient sputum, levels of IL-10 are often recorded as undetectable (Colombo *et al.*, 2005, Watt *et al.*, 2005, Balfour-Lynn *et al.*, 1997), or at very low levels, i.e. 24 pg/mL (±0-228 pg/mL) (Osika *et al.*, 1999).

Transforming growth factor-β (TGF-β) is another pro-resolving cytokine which has been shown to inhibit the production of IL-8 by airway epithelia through the inhibition of NF-κB (Kelley *et al.*, 2001, Jagels and Hugli, 2000), as well as reduce IL-8 mediated neutrophil trans-endothelial migration (Smith *et al.*, 1996). TGF-β has also been shown to increase ASL absorption and modulate CFTR function (Manzanares *et al.*, 2015, Sun *et al.*, 2014). Thus, its production by CF and non-CF epithelia in response to challenges with bacterial exoproducts warrants future study.

Subsequent results from assessing cell metabolism confirmed that changes within IL-8 and IL-6 production were not due to changes in airway epithelial cell viability, following mono- and co-stimulation (Figure 56), which could otherwise affect meaningful interpretation of results. The results from this study demonstrate that the IL-8 and IL-6 stimulatory activity of *P. aeruginosa* filtrates are extracellular, heat-resistant and are not driven by bacterial proteases alone.

Finally, the ability of *S. aureus* and *P. aeruginosa* untreated exoproducts (not subjected to heat treatment) to change airway epithelial cell morphology was determined. *S. aureus* and PAO1 induced changes to cell morphology in both CF and non-CF bronchial epithelia, causing cell rounding, whilst the CF isolates of *P. aeruginosa* did not (Figure 57 and Figure 58). Cell

rounding and sloughing as a result of *S. aureus* exoproducts in particular may play a role in disease progression, with *P. aeruginosa* being shown to adhere preferably to damaged airway epithelia (Lingner *et al.*, 2017, de Bentzmann *et al.*, 1996b). The differences seen across the *P. aeruginosa* CF isolates in relation to their ability to induce changes to cell morphology may arise due to differences within final bacterial density, as well as the arsenal diversity and concentrations of extracellular virulence properties contained within each of the bacterial supernatants used – as reflected in results chapter 3. It is likely that numerous virulence factors together impair cell viability (Campodonico *et al.*, 2008).

When drawing the data together, the impact of polymicrobial infection upon the CF airway inflammatory response and immune dysregulation is likely to be complex. A lack of enhancement to the IL-8 response in the majority of co-infections in CF epithelia, suggests that *S. aureus-P. aeruginosa* co-colonisation may not always exacerbate inflammation in CF airways; a finding which may be due to the heightened secretion of pro-inflammatory markers both at baseline and following infection with a single pathogen. Conversely, non-CF epithelia secrete very low concentrations of IL-8 and IL-6 at baseline, with co-infection appearing to exert an additive effect upon the pro-inflammatory response. Thus, an altered inflammatory response of CF epithelia early at baseline and during an active polymicrobial infection may not only facilitate damage to the airways, but also impair airway innate immunity and facilitate the development of chronic bacterial infections.

5.7 Limitations

The work presented in this chapter has a number of limitations. Firstly, the bacterial filtrates were heat-inactivated to inactivate protease activity and to minimise airway epithelial cell toxicity, a methodology that has been used to study host inflammatory response to CF pathogens (Chekabab *et al.*, 2015, Fink *et al.*, 2003, Wu *et al.*, 2005, Beaudoin *et al.*, 2013). A potential drawback to using heat-inactivated supernatants however is the abolition of a major key virulence property utilised by *P. aeruginosa*, proteases, with LasB for example, having been detected CF sputum at levels of 100 µg/mL (Jaffar-Bandjee *et al.*, 1995). Additionally, proteases have been shown to degrade IL-8 and IL-6 *in vitro* to undetectable levels (LaFayette *et al.*, 2015). Whilst this ability to degrade pro-inflammatory cytokines is important, its overall impact upon the inflammatory response in CF airways requires further study. *P. aeruginosa* LasR mutants with abolished protease activity are associated with heightened airway inflammation (LaFayette *et al.*, 2015, Smith *et al.*, 2006a, Hoffman *et al.*, 2009).

CF airway inflammation is also driven by other stimuli not included here, such as the presence of large actively respiring polymicrobial communities, the excessive influx of neutrophils and the release of NE (De Rose, 2002). The use of submerged epithelial monolayers also means that the impact of bacterial filtrates upon the polarised release of inflammatory mediators is

unknown. For example, do *S. aureus* and *P. aeruginosa* exoproducts encourage higher concentrations of IL-8 to be released upon the apical cell surface, consequently encouraging neutrophil migration from the basal cell surface and into the airway lumen?

5.8 Future work

As the *in vitro* models used to date to study polymicrobial infection in CF are in their infancy, future work would seek to expose polarised airway epithelia grown on transwells®, to *S. aureus* and *P. aeruginosa* exoproducts. The impact upon the polarised release of IL-6, IL-8 and IL-10 into the apical and basolateral compartments would be determined, along with the release of other important inflammatory mediators in CF, including IL-1β and TNF-α which primes neutrophils, increases chemotaxis and increases adhesion to the respiratory endothelium (Courtney *et al.*, 2004). As mentioned in the discussion, the production of the pro-resolving cytokine TGF-β also warrants investigation.

The use of artificial CF sputum to culture both *S. aureus* and *P. aeruginosa* would also more closely mimic the *in vivo* growth environment of the CF lung (Kirchner *et al.*, 2012). Additionally, repeating experiments using of size exclusion filters would allow comparisons to be made between the whole culture supernatants of *S. aureus P. aeruginosa,* with the >3 and <3 kDa fractions. This would help to determine the size of the major factor(s) driving the pro-inflammatory response in CF and non-CF epithelia during mono- and co-stimulation.

Lastly, anoxia has previously been shown to exert a minimal effect upon the intracellular metabolism and proliferation of IB3-1 and C38 airway epithelia compared to normoxia (Shahriary *et al.*, 2012). Thus, as a continuation of chapter 4, future work would seek to culture both airway epithelia and bacterial strains under anoxic conditions to determine the impact of normoxia and anoxia upon airway inflammation, in response to co-infection. A previous study has reported that IB3-1 exhibited a ten-fold increase in the release of IL-8, compared to C38 under anoxic conditions (Shahriary *et al.*, 2012).

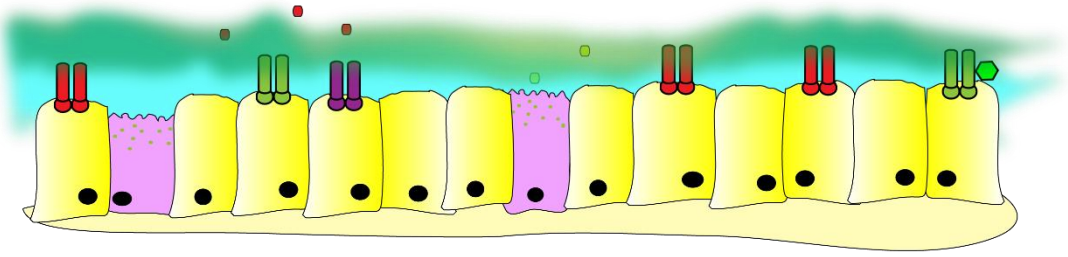
5.9 Conclusion

The data contained within this chapter supports the highly contested notion that CF airway epithelia display a heightened inflammatory response at baseline, in the absence of detectable infection. Conflicting with Chekabab *et al.* (2015), *S. aureus* did not influence the inflammatory response in CF and non-CF epithelia compared to baseline, whilst only PAO1 was shown to be more pro-inflammatory than baseline and *S. aureus* in both CF and non-CF epithelia.

Unlike CF epithelia, co-stimulation combinations increased the production of IL-8 and IL-6 in non-CF epithelia compared to single species challenges and exerted an additive effect upon inflammation. This additive effect contrasts with Chekabab *et al*. who reported that *S. aureus* inhibits *P. aeruginosa* induced inflammation. *S. aureus* exoproducts also failed to elicit any effect upon the concentrations of IL-8 and IL-6 released following exposure to purified LPS from *E. coli*. The pro-resolving cytokine IL-10 was not detected in either cell line following single and dual challenges with *S. aureus* and *P. aeruginosa* extracellular products.

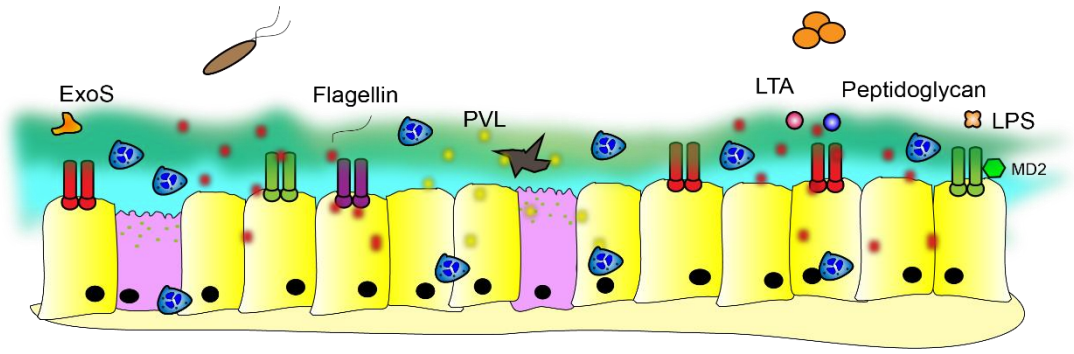
The elevated secretion of the major pro-inflammatory mediators IL-8 and IL-6 by CF airway epithelia at baseline, coupled with a failure to increase the inflammatory response as the bacterial burden increases and diversifies, may cause extensive damage to CF airways, impair aspects of airway innate immunity and facilitate bacterial colonisation. This proposed mechanism relating to the differences in inflammation in CF and non-CF airway epithelia is summarised in Figure 59 and Figure 60.

Baseline inflammation in non-CF airways



- Non-CF airway epithelia secrete very little pro-inflammatory mediators in the absence of detectable infection
- Neutrophils remain within the circulation
- Functional mucociliary escalator

Infection in non-CF airways



- Airway epithelia stimulated by shed and secreted bacterial products
- Airway epithelia respond to an increased and diversified bacterial challenge
- Inflammation facilitates in the influx of neutrophils into the airway lumen

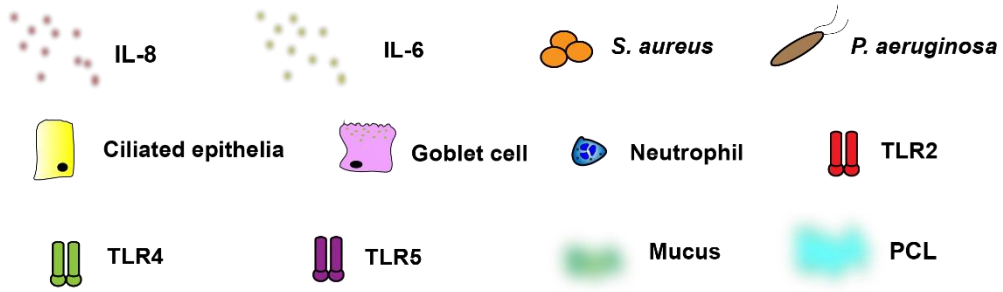
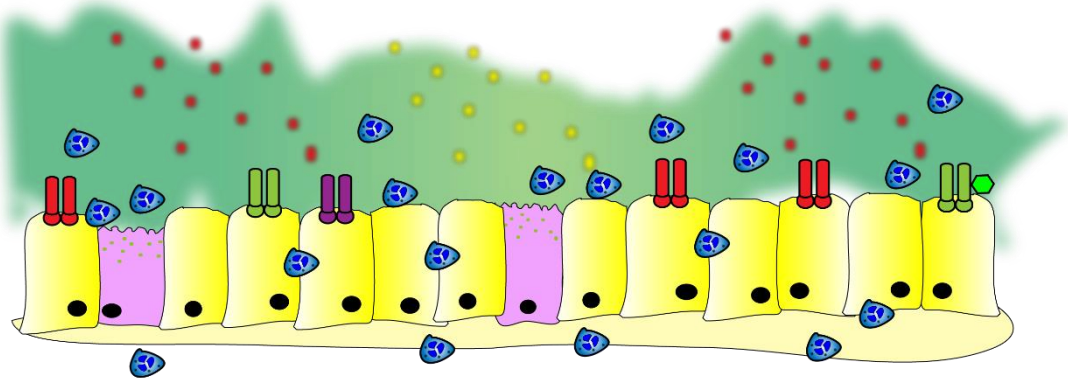


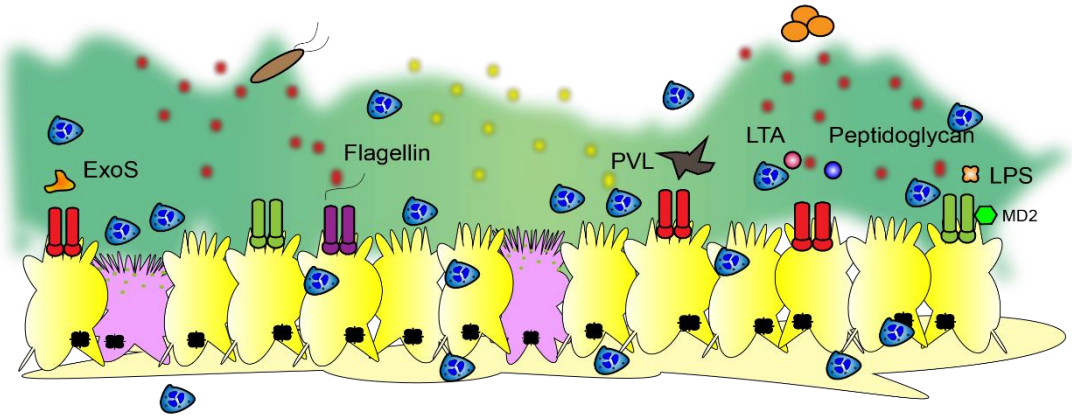
Figure 59. Proposed model of inflammation in non-CF airways. Non-CF airway epithelia produce very little pro-inflammatory IL-8 and IL-6 in the absence of detectable infection. Only during an active infection do airway epithelia mount an increase in IL-8 and IL-6, which is increased during *S. aureus-P. aeruginosa* co-infection. The influx of neutrophils into the airway lumen are effective at killing the colonising pathogens.

Elevated baseline inflammation in CF airways



- Hyperinflammatory phenotype (elevated IL-8 and IL-6 release) in the absence of detectable infection
- Large neutrophil influx
- Mucus plugging

Infection in CF airways



- Bacterial burden increases and diversifies
- Airway epithelial surface receptors are stimulated by shed and secreted bacterial exoproducts
- Failure to respond appropriately to single and polymicrobial infection
- Excessive tissue damage

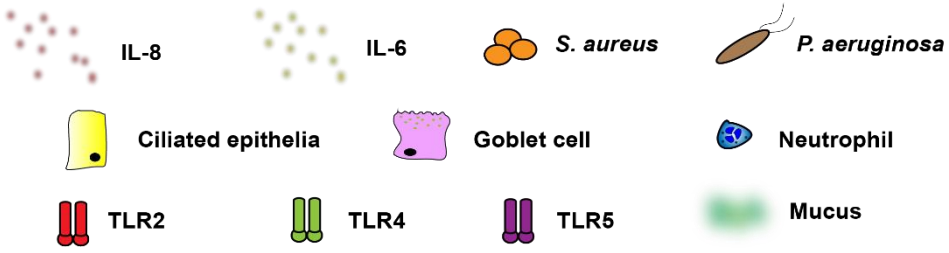


Figure 60. Proposed model of inflammation in CF airways. CF airway epithelia exhibit a hyper-inflammatory phenotype at baseline compared to healthy non-CF airway epithelia. Elevated pro-inflammatory mediators favour the excessive influx of neutrophils in the absence of infection and facilities their premature priming, consequently damaging the airways. Unlike non-CF airways, increases in the bacterial burden or species diversity fail to elicit a suitable increase in the airway inflammatory response.

6 Impact of *S. aureus* upon the colonisation of CF airways by *P. aeruginosa*

6.1 Chapter transition

The data presented in chapter 5 suggests that shed and secreted *S. aureus* exoproducts exert a negligible impact upon the CF airway inflammatory response. Despite this, the role of *S. aureus* within CF airway pathology and disease progression remains highly contested, particularly in regard to whether earlier *S. aureus* infection impacts upon subsequent *P. aeruginosa* infection. Just as the *in vitro* models to study the impact of polymicrobial infection upon airway inflammation are in their infancy, so are the models to determine the impact of polymicrobial infection upon CF airway colonisation.

6.2 Introduction

CF airways are colonised by a highly diverse community of pathogens, ranging from bacteria such as *H. influenzae, Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex, viruses such as rhinoviruses and fungi, including *Aspergillus fumigatus* (Delhaes *et al.*, 2012, Etherington *et al.*, 2014, de Almeida *et al.*, 2010, LiPuma *et al.*, 1999, Goss *et al.*, 2004, Rayner *et al.*, 1990, Boutin *et al.*, 2015). Despite this wide microbial diversity, CF airway infection is dominated by two bacterial species, *S. aureus* and *P. aeruginosa,* with infections occurring in a highly sequential, age-dependent order (Stressmann *et al.*, 2011a, Stressmann *et al.*, 2012, Cystic Fibrosis Trust, 2018).

The Gram-positive bacterium *S. aureus* is often detected in the lungs from three months of life and predominates throughout the first decade of life (Cystic Fibrosis Trust, 2016b). *S. aureus* is believed to colonise the nasal mucosa prior to tracking towards the lower airways (Schlichting *et al.*, 1993), where it has been documented to cause a decrease in paediatric lung function (Hudson *et al.*, 1993, Junge *et al.*, 2016). One study assessing *S. aureus* persistence in individuals with CF reported that mean persistence of the same clone in 29 individuals was 8.25 years (Hirschhausen *et al.*, 2013), whilst another reported that CF patients are colonised by a single *S. aureus* strain for many years, but can temporarily become colonised with two or more strains (Kahl *et al.*, 2003).

Progression into adulthood is typically associated with a transition to *P. aeruginosa* dominance (FitzSimmons, 1993, Cystic Fibrosis Trust, 2016b), with early *P. aeruginosa* acquisition having been associated with a worse prognosis (Emerson *et al.*, 2002). Once colonised, *P. aeruginosa* is almost impossible to eradicate (Langton Hewer and Smyth, 2017) and is associated with elevated inflammation, decreases in lung function and increased mortality (Kerem *et al.*, 1990,

Nixon *et al.*, 2001, Li *et al.*, 2005). Despite this transition to *P. aeruginosa* dominance, the prevalence of *S. aureus* remains relatively high (Limoli *et al.*, 2016, Cystic Fibrosis Trust, 2018). This ability for *S. aureus* and *P. aeruginosa* to co-exist and co-colonise CF airways is known to influence disease severity and lead to shortened survival, as outlined previously. The highly sequential nature of CF airway infection is demonstrated in Figure 61 below (a repeat of Figure 4).

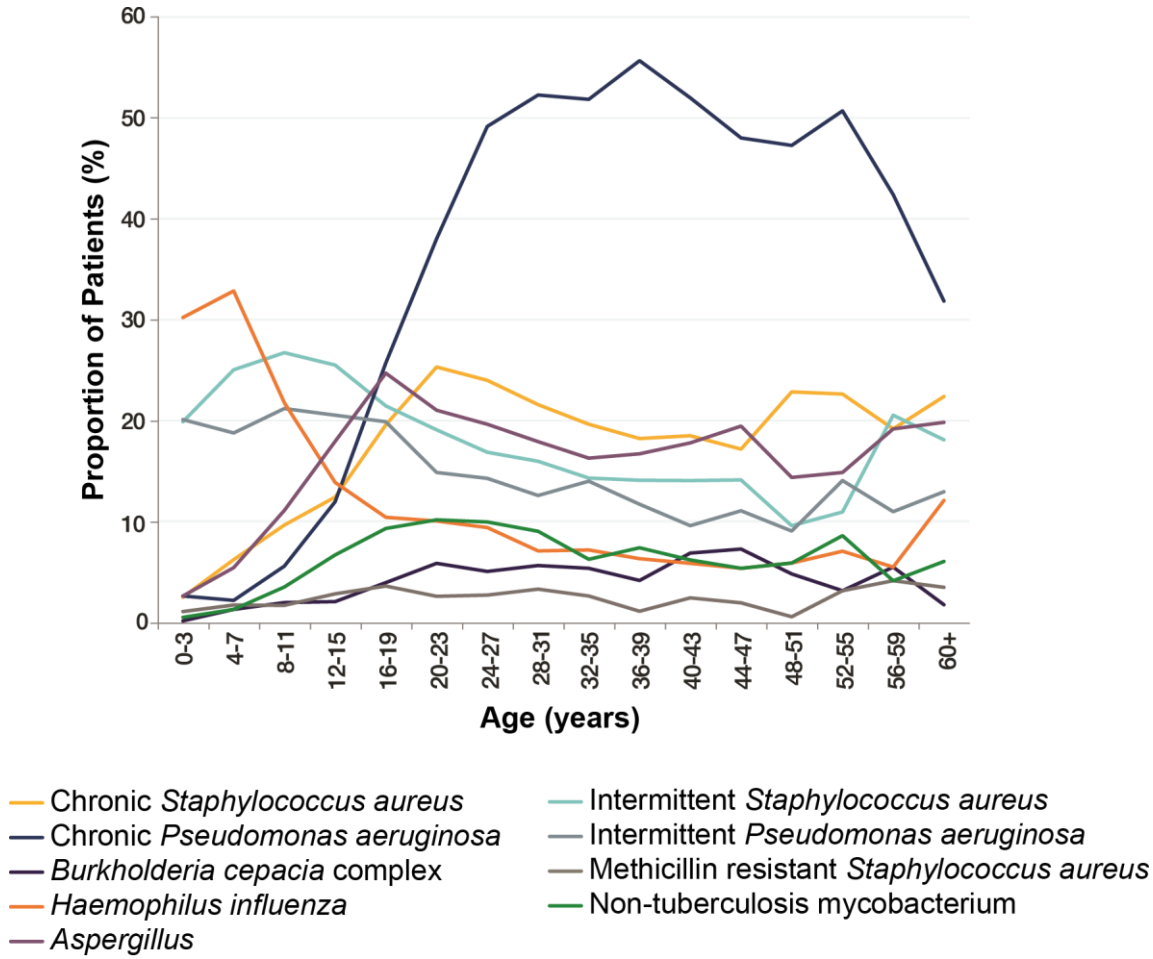


Figure 61. Respiratory infections by age. Infection of CF airways is highly sequential and age dependent. Whilst intermittent (light blue) and chronic (orange) *S. aureus* infections predominate in the first decade of life, chronic *P. aeruginosa* infections (dark blue) predominate in adulthood.

The highly sequential, age-dependent order of infection has led to the hypothesis that earlier colonisation with *S. aureus* primes CF airways to subsequent infection with *P. aeruginosa* (Folkesson *et al.*, 2012, Lyczak *et al.*, 2000, Govan and Nelson, 1992). *S. aureus* has been identified as a risk factor for earlier acquisition by *P. aeruginosa* (Kosorok *et al.*, 1998, Maselli *et al.*, 2003), with continuous prophylactic treatment of *S. aureus* having been shown to facilitate earlier and enhanced *P. aeruginosa* colonisation (Ratjen *et al.*, 2001, Stutman *et al.*, 2002). Narrow spectrum flucloxacillin prescribed in the UK has also been associated with earlier first acquisition of *P. aeruginosa* compared to those not receiving the antibiotic (Hurley *et al.*, 2018).

Most studies to date assessing the key host-pathogen interactions in CF airways employ well characterised laboratory strains such as *P. aeruginosa* PAO1 and PA14, with *in vitro* experiments typically using bronchial and alveolar cultures grown under submerged culture (Jarry and Cheung, 2006, Pompilio *et al.*, 2010, Van Ewijk *et al.*, 2007, Saiman *et al.*, 1992, Chi *et al.*, 1991, Saiman *et al.*, 1990b, Schaible *et al.*, 2013, Plotkowski *et al.*, 1991, Bajolet-Laudinat *et al.*, 1994, de Courcey *et al.*, 2012). When studying direct host-pathogen interactions, such models are restricted by the fact they form single cell sheets (monolayers), lack cell polarity, do not include additional cell types (such as fibroblasts) and lack the formation of tight junctions. Those studies performed at ALI to date typically employ a single culture model of epithelial cells (Bucior *et al.*, 2014, Bucior *et al.*, 2012, Starner *et al.*, 2006, Escotte *et al.*, 2006, Plotkowski *et al.*, 1999).

Models to study polymicrobial infection are in their infancy, with only a handful of studies having determined the impact of polymicrobial infection upon airway colonisation. One study demonstrated that respiratory syncytial virus (RSV) enhanced *P. aeruginosa* binding in submerged IB3-1 epithelia and non-CF epithelia (Van Ewijk *et al.*, 2007), whilst another demonstrated that RSV enhanced the adhesion of *Streptococcus pneumoniae* to HEP-2 and A549 submerged monolayers (Hament *et al.*, 2004). The bacterium *Stenotrophomonas maltophilia* has also been shown to enhance *P. aeruginosa* binding to submerged IB3-1 monolayers (Pompilio *et al.*, 2010), whilst another demonstrated the ability of *P. aeruginosa* to enhance *Burkholderia cepacia* adhesion (Saiman *et al.*, 1990a). The sequential infection of human CF airway epithelia with *S. aureus* and *P. aeruginosa* had yet to be investigated.

6.3 Aims

This chapter sought to combine the use of select CF clinical isolates of *P. aeruginosa,* alongside well characterised laboratory strains of *P. aeruginosa* and *S. aureus,* with a novel *in vitro* multicellular co-culture model of CF and non-CF airways grown at ALI. Focusing upon bacterial adhesion (the initiating stage of airway colonisation), this research aimed to elucidate whether prior infection with *S. aureus* enhanced the binding of *P. aeruginosa* to CF airways. Control experiments were performed by performing mono-infections using either *S. aureus* or *P. aeruginosa,* reversing the order of infection (inoculating with *P. aeruginosa* first followed by *S. aureus*) and by repeating all experiments in the non-CF airway model.

6.4 Methods

Bacterial strains and growth conditions. *S. aureus* ATCC 6538, *P. aeruginosa* PAO1 and *P. aeruginosa* CF clinical isolates 5 and 6 were used. CF isolates 5 and 6 were used due to their wide variations in virulence. CF isolate 5 produced pyocyanin, proteases, exerted staphylolytic activity, outcompeted *S. aureus* in mixed species biofilms and exhibited swimming and swarming motility, whilst CF isolate 6 exhibited none of these phenotypic traits.

Single colonies of each isolate grown routinely on LB agar were inoculated into 10 mL of LB broth and were grown for approximately 16 h under normoxia and static conditions at 37 °C.

Cell culture. IB3-1 (CF) and C38 (non-CF) bronchial epithelia were cultured in DMEM/F12 containing 10% (v/v) FBS and 1x anti-mycotic-antibiotic at 37 °C and 5% CO_2. MRC-5 fibroblasts were routinely grown in EMEM containing 10% (v/v) FBS, 2 mM L-glutamine and 1x anti-mycotic-antibiotic at 37 °C and 5% CO_2.

Submerged cell culture. IB3-1 and C38 cells were seeded into 24-well polystyrene tissue culture treated plates at $1.5x10^5$ cells/mL and incubated overnight at 37 °C with 5% CO_2. The following day at confluence, cell culture media was aspirated and replaced with 1 mL/well of antibiotic-free DMEM/F12 supplemented with 1% (v/v) for a minimum of 16 h.

Culturing polarised airway epithelia on transwell® filter supports. Sterile cell culture transwell® inserts (0.3 cm^2 surface area, 0.4 µm pore size) were coated with 10 $µg/cm^2$ human placental collagen type IV, prior to the apical addition of fibroblasts at a cell density $3x10^4$ cells/well. After culturing for four days, apical medium was removed and IB3-1 or C38 cells were seeded on top of the fibroblasts at $5x10^4$ cells/well in DMEM/F12. The co-cultures were then left under submerged conditions for a further four days, allowing the epithelial cells to form confluent monolayers on top of the fibroblasts. After this period, the apical cell culture medium was removed and not refreshed, introducing the cells to an ALI and inducing cell differentiation. Medium in the basolateral compartment was refreshed every 3-4 days for a minimum of 14 days from ALI formation.

Bacterial binding to submerged airway epithelia monolayers. Sixteen h prior to infection, cell culture media was aspirated from each well and replaced with antibiotic free DMEM/F12 containing 1% (v/v) FBS. Overnight cultures of *S. aureus* or *P. aeruginosa* were centrifuged at 4,000 x *g* for 15 min at 4 °C and the pellet washed in 10 mL of PBS, a process repeated twice. Monolayers were infected with either *S. aureus* or *P. aeruginosa* at a MOI of 5 (5 bacteria to 1 epithelial cell), suspended in infection medium (DMEM/F12 only). Plates were incubated for 2 h at 37 °C and 5% CO_2, prior to being washed twice with PBS to remove unbound bacteria. 100 µL of trypsin-EDTA (0.25%) was added to each well to detach the monolayer and left to incubate for 15 min at 37 °C and 5% CO_2. Nine hundred µL of infection media was then added

to inactivate the trypsin. 100 µL of the suspension was then vortexed for 2 min, serially diluted and placed onto MSA or PIA. Plates were then incubated at 37 °C for approximately 18 h, prior to the enumeration of Log_{10}CFU/mL.

Apical infections of polarised co-cultures grown on transwells® at ALI. 24 h prior to infection, cell culture media was aspirated from the basolateral compartment of transwells® and replaced with 600 µL of antibiotic-free DMEM/F12 containing 1% (v/v) FBS. The following day, overnight cultures of *S. aureus* and/ or *P. aeruginosa* were centrifuged at 4,000 x *g* for 15 min at 4 °C and the pellet washed in 10 mL of PBS, a process repeated twice. Following the final wash, the bacterial pellet was resuspended in infection media (DMEM/F12 only, without FBS and antibiotics) to an OD_{470} of 1.0 and further diluted to give a final MOI of approximately 10 (10 bacteria to 1 epithelial cell).

100 µL of the bacterial inoculum was then added to the apical surface of each transwell®. The plates were incubated for 2 h at 37 °C and 5% CO_2. For co-infection studies, after the initial 2 h incubation period with the first bacterium, the transwells® were washed once with 200 µL of PBS and the second bacterium was added at MOI of approximately 10, prior to the plates being incubated for a further 2 h at 37 °C and 5% CO_2.

Bacterial binding to polarised co-cultures grown on transwells®. After the end of each mono- or co-infection, transwells® were washed once with 200 µL of sterile PBS, prior to the addition of 200 µL of ice-cold sterile 0.25% (v/v) Triton X-100 to lyse the airway epithelia and fibroblasts. This concentration of Triton-X100 has been used previously to determine *P. aeruginosa* adhesion to epithelia grown on Transwells® at ALI (Bucior *et al.*, 2014, Bucior *et al.*, 2010). Plates were incubated on ice for 30 min. 100 µL of the lysed suspension was then vortexed for 2 min, serially diluted and placed onto MSA or PIA. Plates were incubated at 37 °C for approximately 18 h, prior to the enumeration of CFU/mL. Due to the well-established variability regarding bacterial adhesion experiments, bacterial adhesion to the transwell® is expressed as a percentage of the bacterial inoculum (quantified by dilution plating). This is summarised in the equation below.

$$\% \text{ Adhesion} = \frac{\text{Number of bacteria recovered from the transwell}}{\text{Number of bacteria in the inoculum}} \times 100$$

The methodology for mono-infection of transwells® airway models is illustrated in Figure 62 below.

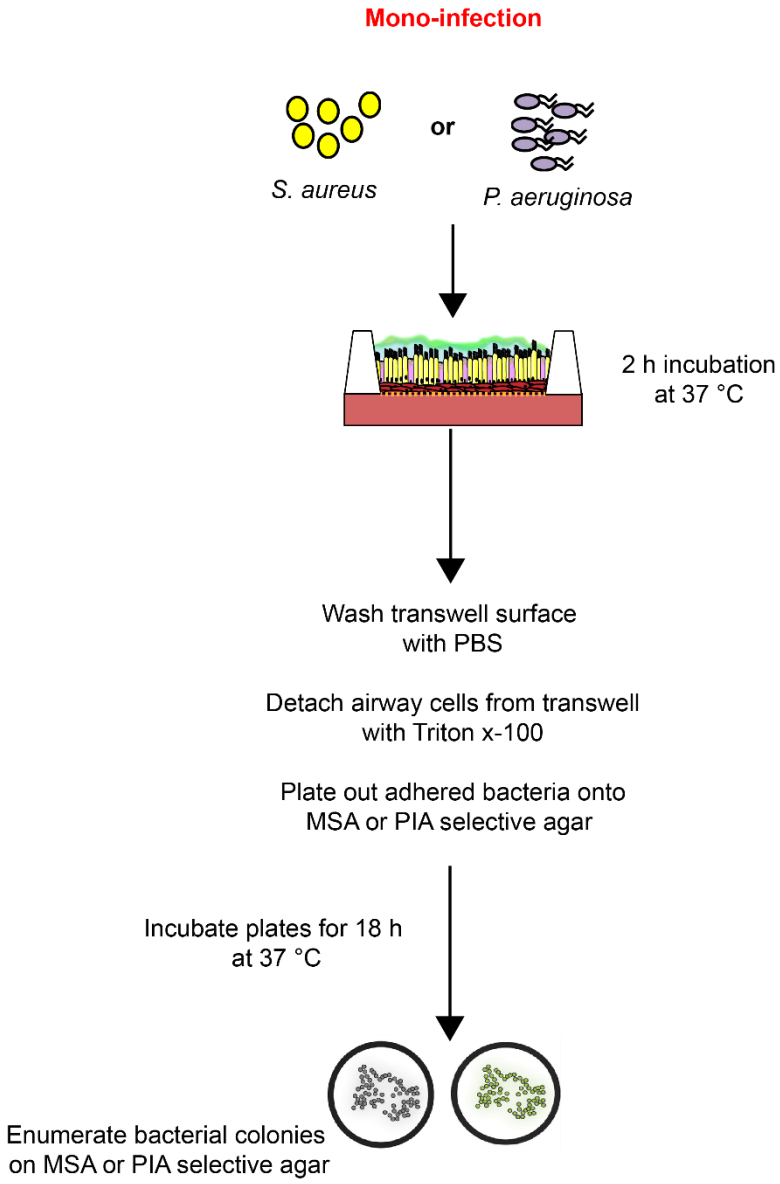


Figure 62. Mono-infection of transwell® airway models. During mono-infection, CF and non-CF airway models grown on transwells® were infected with either *S. aureus* or *P. aeruginosa* for 2 h and incubated at 37 °C. The transwells® were subsequently washed with PBS to remove any unbound bacteria and the airway cells detached using Triton X-100. The bacterial suspensions were serially diluted and subsequently plated out for enumeration onto either MSA or PIA selective agar. Plates were incubated for approximately 18 h at 37 °C prior to enumerating the bacterial colonies.

The methodology used to study sequential bacterial infection in transwell® airway models is depicted in Figure 63 below.

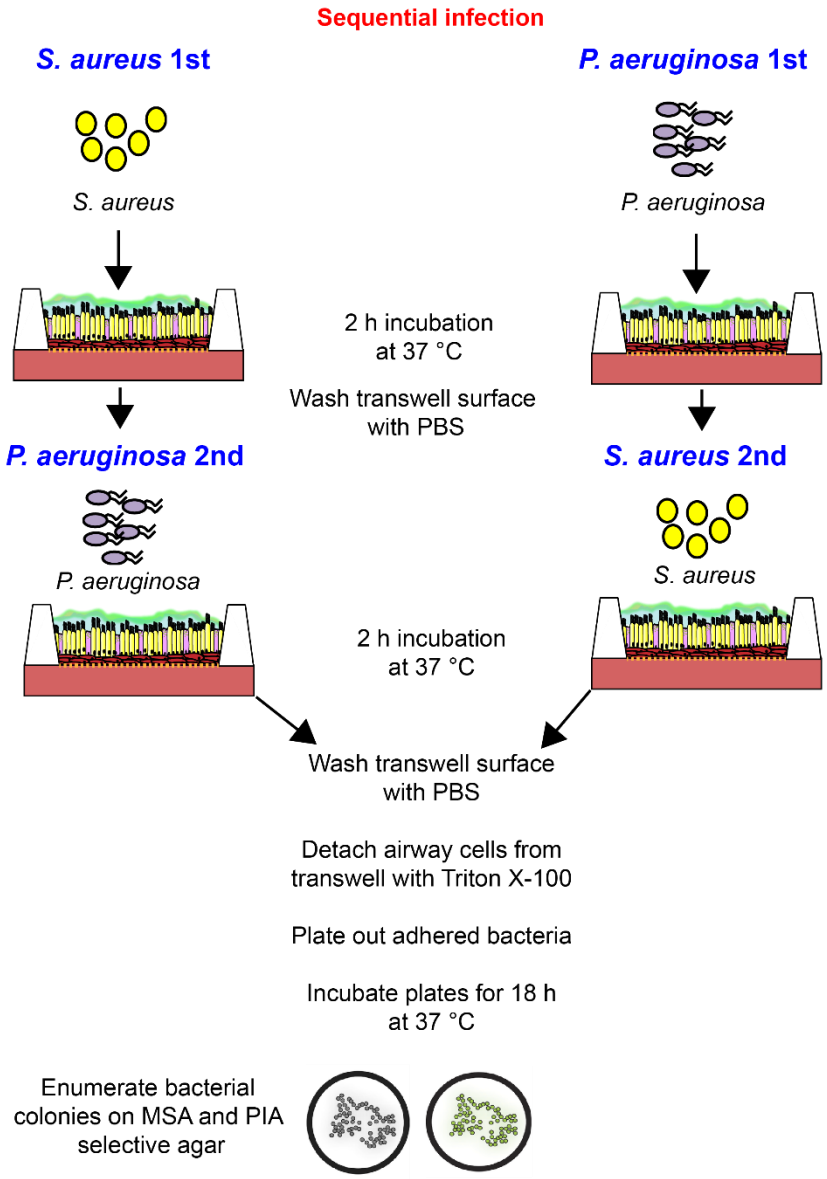


Figure 63. Sequential infection of transwell® airway models. Transwells® were first infected with either *S. aureus* (left flow diagram) or *P. aeruginosa* (right flow diagram) at a MOI of 10 for 2 h and incubated at 37 °C, prior to being washed with PBS. If transwells® were initially infected with *S. aureus*, then *P. aeruginosa* was subsequently added to the transwells® at a MOI of 10, for a further 2 h and incubated at 37 °C. If transwells® were initially infected with *P. aeruginosa*, then *S. aureus* was subsequently added to the transwells®. Following this, the airway models were washed with PBS to remove any unbound bacteria and the airway cells lysed using Triton X-100. The bacteria were serially diluted and plated out onto both MSA and PIA selective agar. Plates were incubated for approximately 18 h at 37 °C prior to enumerating the bacterial colonies.

Statistical analysis. All results unless otherwise specified are expressed as mean ±S.E.M. Data for each experiment were collected from three independent experiments (N=3), each performed in triplicate. All statistical analyses were performed using GraphPad Prism 6 software (Graphpad, La Jolla, CA, USA) with significance being set to P<0.05. The specific tests and *post-hoc* test used for each experiment are described in the figure legends.

6.5 Results

6.5.1 Binding of *S. aureus* ATCC 6538 to submerged monolayers of CF and non-CF airway epithelia

With colonisation being the initiating step in CF airway infection, primary experiments determined whether CF epithelia grown as submerged monolayers were more susceptible to *S. aureus* binding. As shown in Figure 64**,** equal numbers of *S. aureus* ATCC 6538 bound to both IB3-1 (CF) and C38 (non-CF) monolayers after 2 h of infection.

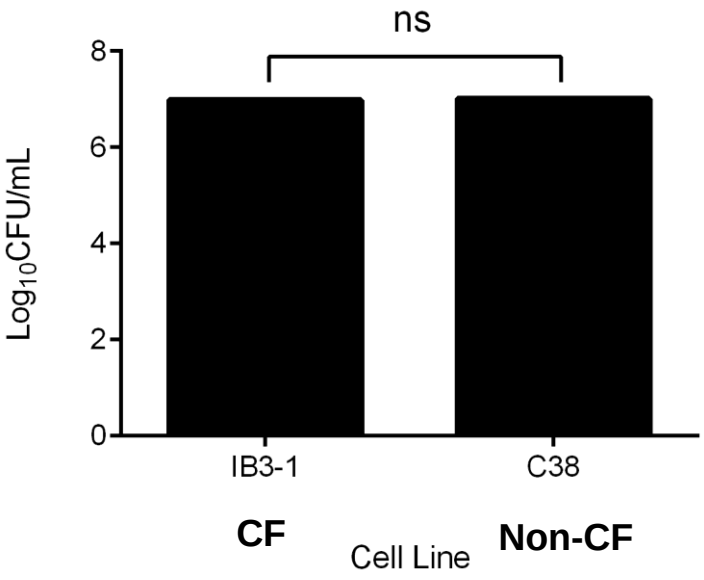


Figure 64. *S. aureus* binds equally to submerged CF and non-CF epithelial monolayers. Submerged monolayers of CF and non-CF airway epithelia were incubated with *S. aureus* (MOI of approximately 5) for 2 h, prior to being washed with PBS to remove any unbound bacteria. Airway cells were detached using trypsin-EDTA (0.25%) and the number of Log_{10}CFU/mL was determined after plating onto MSA plates. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. An unpaired, two-tailed *t*-test was performed to determine statistical significance.

6.5.2 Binding of *P. aeruginosa* PAO1 to submerged monolayers of CF and non-CF airway epithelia

In order to determine whether CF bronchial epithelia were more susceptible to binding by *P. aeruginosa* PAO1, submerged epithelial monolayers were infected with PAO1 for 2 h, prior to the number of adhered bacteria being enumerated. As shown in Figure 65, PAO1 adhered in significantly higher numbers to C38 (non-CF) submerged airway epithelia, compared to IB3-1 (CF) epithelia (*P*<0.01).

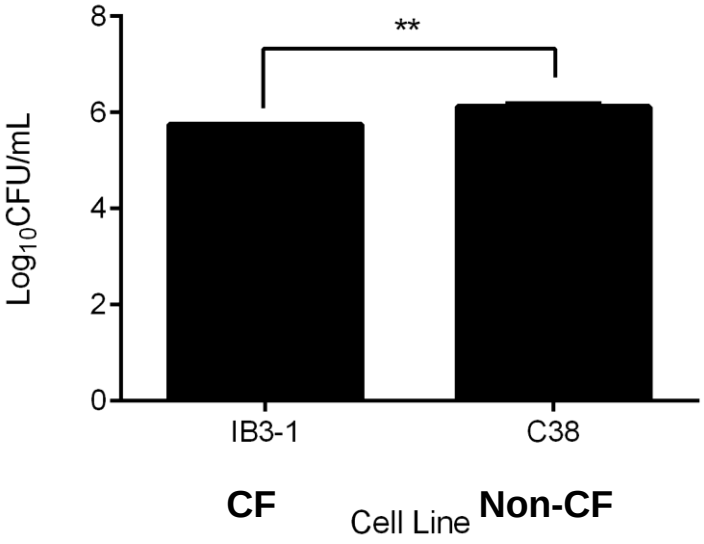


Figure 65. *P. aeruginosa* PAO1 binds in higher numbers to submerged non-CF airway epithelial monolayers. Submerged monolayers of IB3-1 (CF) and C38 (non-CF) cells were incubated with *P. aeruginosa* PAO1 at a MOI of approximately 5 for 2 h, prior to being washed with PBS to remove any unbound bacteria. Airway epithelia were detached using trypsin-EDTA (0.25%) and the number of Log_{10}CFU/mL was determined after plating onto PIA plates. Results are expressed as the mean ± S.E.M from three independent experiments (*N*=3), each performed in triplicate. An unpaired, two-tailed *t*-test was performed to determine statistical significance (***P*<0.01).

6.5.3 Effect of prior infection with *S. aureus* upon *P. aeruginosa* PAO1 adhesion to CF and non-CF airway epithelia grown at ALI

To assess whether previous *S. aureus* infection enhances *P. aeruginosa* binding, CF and non-CF epithelia grown as co-cultures with fibroblasts at ALI were sequentially infected, first with *S. aureus* for 2 h, followed by *P. aeruginosa* for a further 2 h. *S. aureus* and *P. aeruginosa* mono-infections were also performed in both airway models (*S. aureus* only and *P. aeruginosa* only) to allow comparisons to be made.

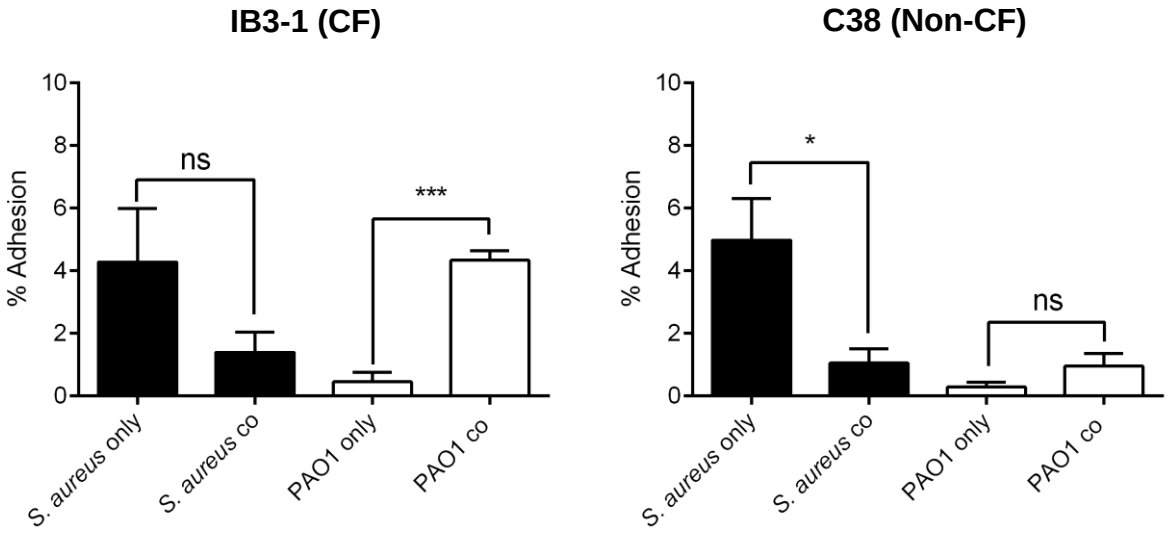


Figure 66. Prior infection with *S. aureus* enhances PAO1 adhesion to CF epithelia. Transwells® were mono-infected with either *S. aureus* (*S. aureus* only) or PAO1 (PAO1 only). For sequential infection, transwells® were first infected with *S. aureus* for 2 h (*S. aureus* co), followed by PAO1 for a further 2 h (PAO1 co). Airway cells were detached using Triton-X100 and the number of adhered bacteria enumerated on MSA and PIA selective agar, to easily discriminate between the two species. Adhesion is expressed as a percentage of the bacterial inoculum originally added to the transwells®. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate. Statistical differences were determined using an unpaired *t*-test (**P*<0.05, ****P*<0.001).

As shown in Figure 66, during mono-infection, approximately 4.3% of the *S. aureus* inoculum bound to the CF airway model (*S. aureus* only). During sequential infection with *P. aeruginosa,* the amount of *S. aureus* bound to the CF airway model decreased (*S. aureus* co), but this was not significant compared to *S. aureus* only. Approximately 0.5% of the PAO1 inoculum bound to the CF airway model during mono-infection (PAO1 only), where prior infection with *S. aureus* significantly increased subsequent PAO1 binding (PAO1 co) (*P*<0.001).

In the non-CF airway model, approximately 4.9% of the *S. aureus* inoculum bound during mono-infection (*S. aureus* only). During sequential infection with *P. aeruginosa,* the amount of *S. aureus* adhering to the non-CF model (*S. aureus* co) was significantly decreased compared to *S. aureus* only (*P*<0.05). Approximately 0.3% of the PAO1 inoculum bound to the non-CF

model during mono-infection (PAO1 only), where prior exposure to *S. aureus* did not significantly enhance subsequent PAO1 binding (PAO1 co).

6.5.4 Effect of prior infection with *P. aeruginosa* PAO1 upon *S. aureus* adhesion to CF and non-CF airway epithelia grown at ALI

To determine whether the specific order of infection influences bacterial binding, transwells® were first infected with *P. aeruginosa* PAO1 for 2 h, followed by subsequent infection with *S. aureus* for a further 2h. As shown in Figure 67, the amount of PAO1 bound to the CF airway model during sequential infection (PAO1 co) was not significantly different compared to PAO1 binding during mono-infection (PAO1 only). Previous infection with PAO1 significantly increased *S. aureus* binding however (*S. aureus* co), compared to *S. aureus* only ($P<0.05$). In the non-CF airway model, the number of bound PAO1 was not significantly different during sequential infection (PAO1 co) compared to mono-infection (PAO1 only). Unlike the CF airway model, previous infection with PAO1 did not significantly increase *S. aureus* binding (*S. aureus* co), compared to *S. aureus* mono-infection (*S. aureus* only).

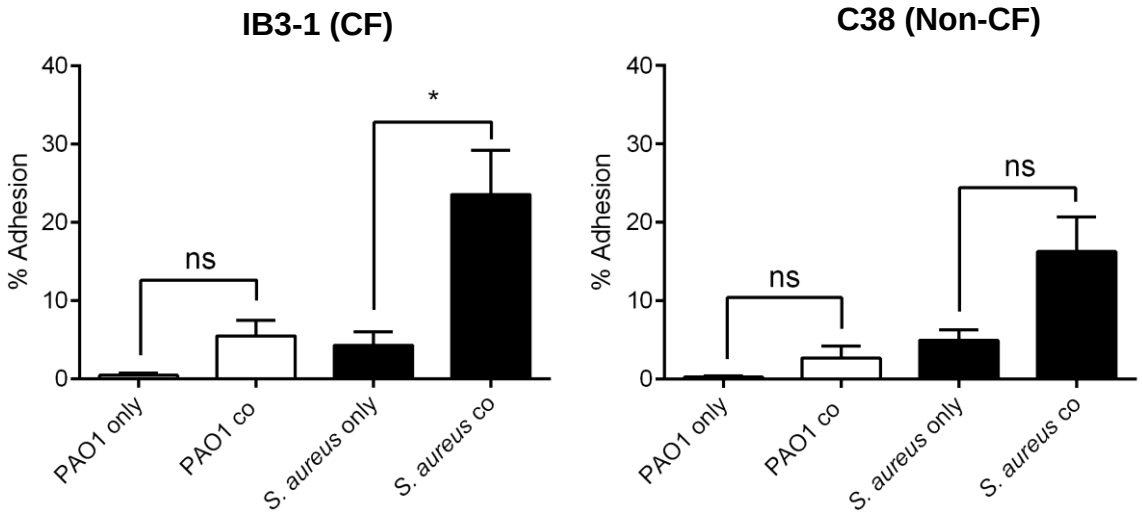


Figure 67. Prior infection with PAO1 enhances *S. aureus* adhesion in CF airways. Transwells® were first infected with PAO1 for 2 h (PAO1 co), followed by *S. aureus* (*S. aureus* co) for a further 2 h. Mono-infections with *S. aureus* (*S. aureus* only) and PAO1 (PAO1 only) served as controls. Airway cells were detached using Triton-X100 and the bacteria enumerated on MSA and PIA selective agar to easily discriminate between the two bacterial species. Adhesion is expressed as a percentage of the inoculum. Data represents the mean ± S.E.M. of three independent experiments ($N=3$) each performed in triplicate. Statistical differences were determined using an unpaired *t*-test (*$P<0.05$).

6.5.5 Effect of prior infection with *S. aureus* upon *P. aeruginosa* CF isolate 5 binding to CF and non-CF airway epithelia grown at ALI

Having established the model system using laboratory strains, the system was used to test the effects of pre-infection with *S. aureus* upon subsequent binding of CF clinical isolates of *P. aeruginosa*. As shown below in

Figure **68**, *S. aureus* binding was not significantly different comparing *S. aureus* mono-infection (*S. aureus* only), to sequential infection with *P. aeruginosa (S. aureus* co) in either airway model. Additionally, prior infection with *S. aureus* did not enhance the adhesion of *P. aeruginosa* CF clinical isolate 5 in either airway model (5 co) compared to mono-infection (5 only).

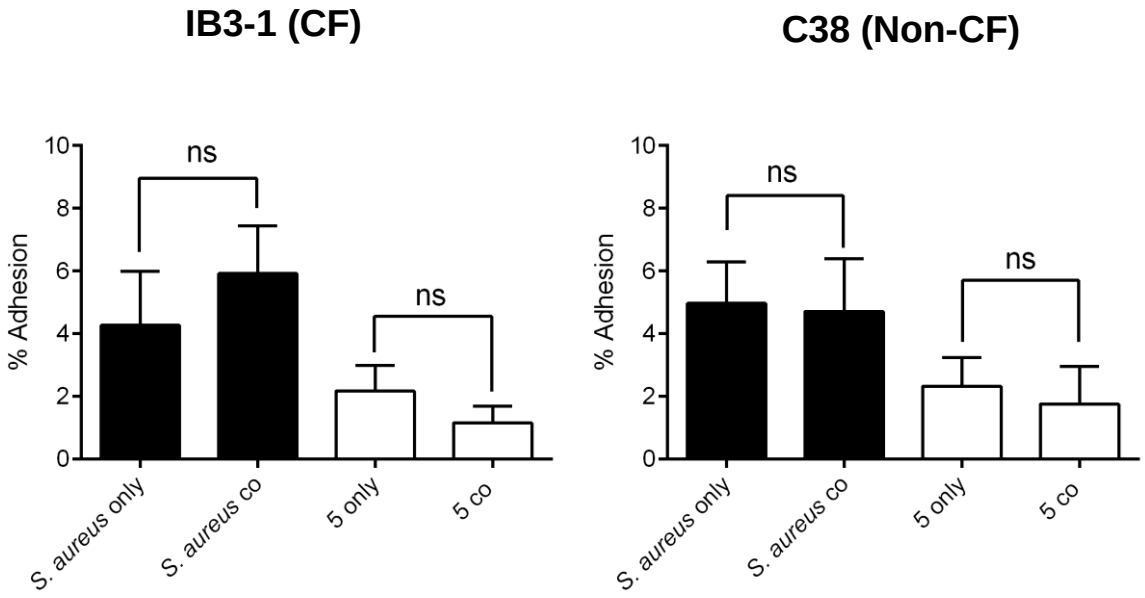


Figure 68. Prior infection with *S. aureus* does not enhance the binding of *P. aeruginosa* CF isolate 5. Transwells® were first infected with *S. aureus* for 2 h (*S. aureus* co), followed by *P. aeruginosa* CF isolate 5 (5 co) for a further 2 h. Mono-infections with *S. aureus* (*S. aureus* only) and *P. aeruginosa* CF isolate 5 (5 only) served as controls. Airway cells were detached using Triton-X100 and the bacteria enumerated on MSA and PIA selective agar to easily discriminate between the two bacterial species. Adhesion is expressed as a percentage of the inoculum. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate.

6.5.6 Effect of prior infection with *P. aeruginosa* CF isolate 5 upon *S. aureus* adhesion to CF and non-CF airway epithelia

The order of infection was subsequently reversed to determine whether prior infection with *P. aeruginosa* CF clinical isolate 5 was able to enhance the binding of *S. aureus.* To assess this, transwells® were first infected with *P. aeruginosa* CF isolate 5 for 2h, followed by *S. aureus* for a further 2 h.

As shown in Figure 69, the amount of *P. aeruginosa* CF isolate 5 bound to the CF and non-CF airway model during sequential infection (5 co) was not significantly different compared to CF isolate 5 bound during mono-infection (5 only). Furthermore, prior infection with *P. aeruginosa* CF isolate 5 did not enhance *S. aureus* adhesion in either airway model (*S. aureus* co) compared to *S. aureus* during mono-infection (*S. aureus* only).

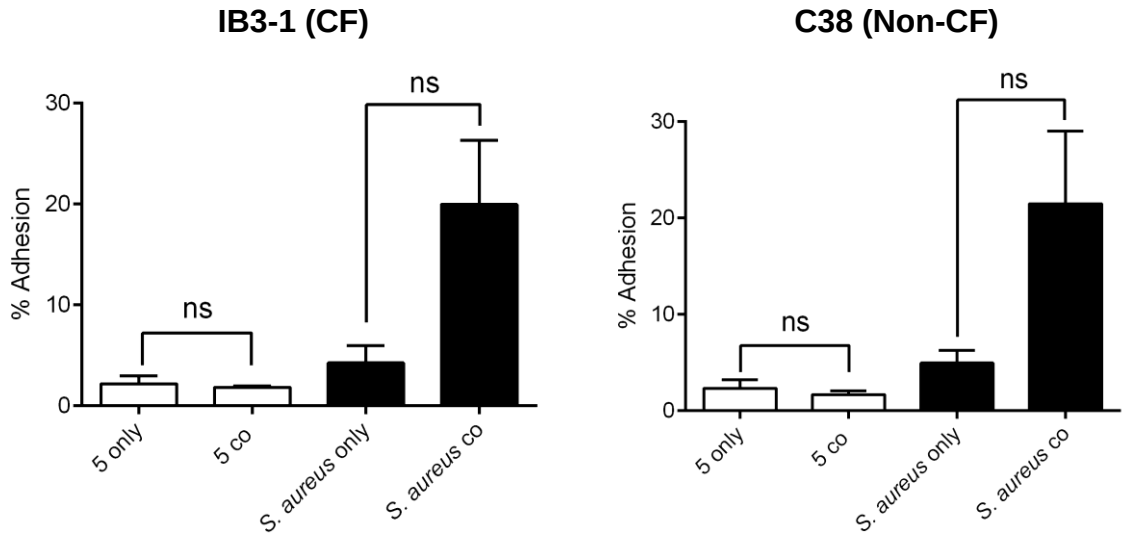


Figure 69. Prior infection with *P. aeruginosa* CF isolate 5 does not enhance *S. aureus* binding. Transwells® were first infected with *P. aeruginosa* CF isolate 5 (5 co) for 2 h, followed by *S. aureus* (*S. aureus* co) for a further 2 h. Mono-infections with *S. aureus* (*S. aureus* only) and *P. aeruginosa* CF isolate 5 (5 only) served as controls. Airway cells were detached using Triton-X100 and the bacteria enumerated on MSA and PIA selective agar to easily discriminate between the two bacterial species. Adhesion is expressed as a percentage of the inoculum. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate.

6.5.7 Effect of prior infection with *S. aureus* upon *P. aeruginosa* CF isolate 6 binding to CF and non-CF airway epithelia grown at ALI

The impact of prior colonisation with *S. aureus* upon *P. aeruginosa* CF isolate 6 binding was determined. Transwells® were first infected with *S. aureus* for 2h, followed by *P. aeruginosa* CF isolate 6.

As shown in Figure 70, in the CF airway model, *S. aureus* binding during sequential infection with *P. aeruginosa* (*S. aureus* co) was not significantly lower than *S. aureus* binding during mono-infection (*S. aureus* only). Prior infection with *S. aureus* however, significantly increased the binding of *P. aeruginosa* CF isolate 6 (6 co), compared to *P. aeruginosa* CF isolate 6 during mono-infection (6 only).

In the non-CF airway model, the amount of *S. aureus* bound during sequential infection with *P. aeruginosa* (*S. aureus* co) was significantly lower than *S. aureus* only mono-infection (*P*<0.05). Moreover, prior infection with *S. aureus* did not enhance the binding of *P. aeruginosa* CF isolate 6 (6 co), compared to CF isolate 6 alone (6 only).

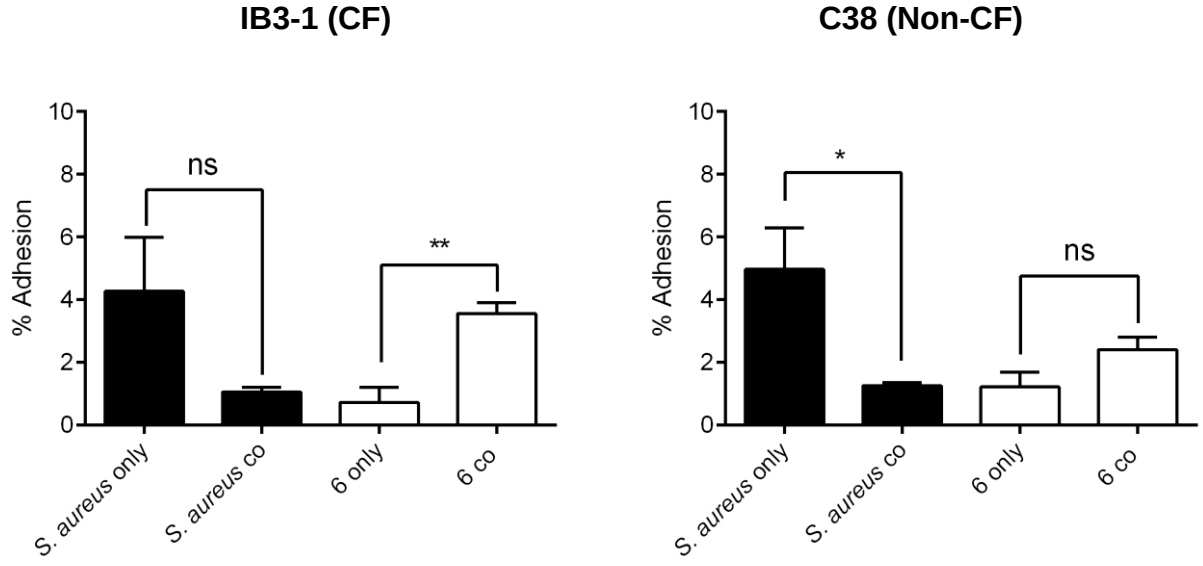


Figure 70. Prior infection with *S. aureus* enhances the adhesion of CF isolate 6 to CF airways. Transwells® were first infected with *S. aureus* (*S. aureus* co) for 2 h, followed by *P. aeruginosa* CF isolate 6 (6 co) for a further 2 h. Mono-infections with *S. aureus* (*S. aureus* only) and *P. aeruginosa* CF isolate 6 (6 only) served as controls. Airway cells were detached and bacteria enumerated on MSA and PIA selective agar to easily discriminate between the two bacterial species. Adhesion is expressed as a percentage of the inoculum. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate. Statistical differences were determined using an unpaired *t*-test (**P*<0.05, ***P*<0.01).

6.5.8 Effect of prior infection with *P. aeruginosa* CF isolate 6 upon *S. aureus* adhesion to CF and non-CF airway epithelia grown at ALI

Lastly, the order of infection was reversed and the impact of prior infection with *P. aeruginosa* CF isolate 6 upon *S. aureus* binding was determined.

As shown in Figure 71 below, the amount of *P. aeruginosa* CF isolate 6 bound during sequential infection (6 co), was significantly higher than I6 during mono-infection (6 only) (*P*<0.001). However, prior infection with CF isolate 6 did not enhance *S. aureus* adhesion (*S. aureus* co) compared to *S. aureus* during mono-infection (*S. aureus* only).

In the non-CF model, the amount of *P. aeruginosa* CF isolate 6 bound during sequential infection (6 co) was significantly higher than CF isolate 6 mono-infection (6 only) (*P*<0.01). As seen in the CF airway model, prior infection with *P. aeruginosa* CF isolate 6 did not enhance *S. aureus* binding (*S. aureus* co) compared to *S. aureus* during mono-infection (*S. aureus* only).

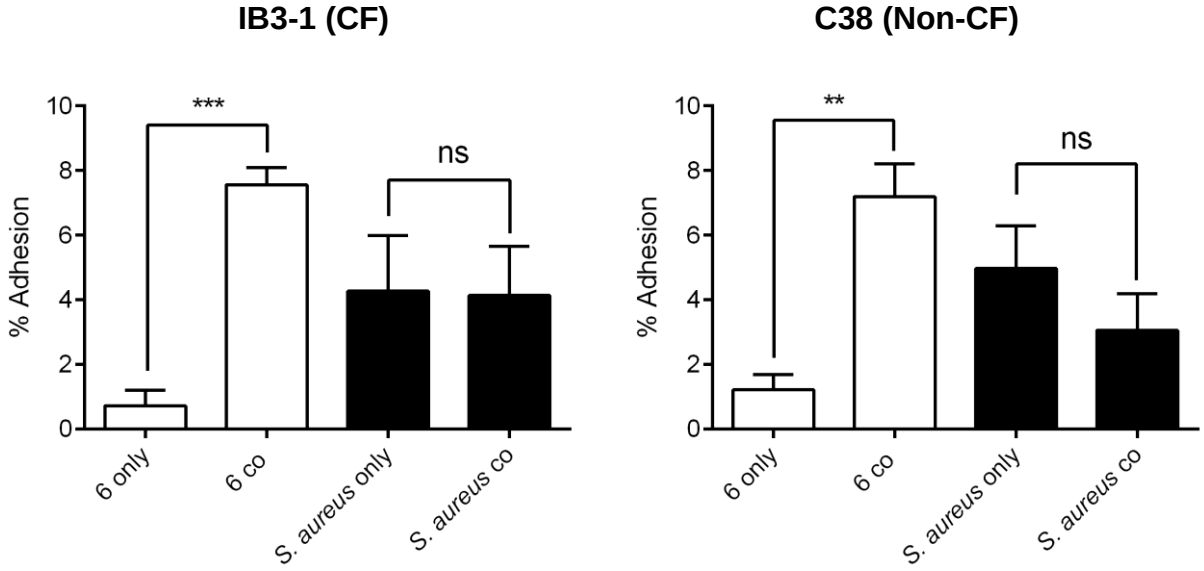


Figure 71. Prior infection with *P. aeruginosa* CF isolate 6 does not enhance *S. aureus* adhesion. Transwells® were first infected with *P. aeruginosa* CF isolate 6 (6 co) for 2 h, followed by *S. aureus* (*S. aureus* co) for a further 2 h. Mono-infections with *S. aureus* (*S. aureus* only) and *P. aeruginosa* CF isolate 6 (6 only) served as controls. Airway cells were detached using Triton X-100 and bacteria enumerated on MSA and PIA selective agar to easily discriminate between the two bacterial species. Adhesion is expressed as a percentage of the inoculum. Data represents the mean ± S.E.M. of three independent experiments (*N*=3) each performed in triplicate. Statistical differences were determined using an unpaired *t*-test (***P*<0.01, ****P*<0.001).

6.6 Discussion

The early host-pathogen interactions that occur within the CF lung are poorly understood, particularly in relation to *S. aureus* pathophysiology and the sequential nature of CF airway infection. Part of this reason is due to the infancy of suitable model systems in which to study polymicrobial infection.

The highly ordered and age-dependent sequence of infection in CF airways has led to the hypothesis that prior infection with *S. aureus* primes the lungs to subsequent colonisation with *P. aeruginosa* (Lyczak *et al.*, 2000, Kosorok *et al.*, 1998). Furthermore, the prophylactic treatment of *S. aureus* has been shown to be potentially facilitate earlier and enhanced *P. aeruginosa* colonisation (Stutman *et al.*, 2002, Ratjen *et al.*, 2001, Goss and Muhlebach, 2011). This chapter subsequently aimed to use previously characterised and established *in vitro* co-culture models of CF and non-CF airways (Bielemeier, 2012b) to determine the impact of polymicrobial infection upon airway colonisation and whether earlier *S. aureus* infection enhances the binding of *P. aeruginosa* laboratory and CF isolates. Bacterial adhesion was the subject of the study due to it being the first step in facilitating acute airway colonisation and chronic airway infections.

Initial experiments infected submerged monolayers of IB3-1 and C38 epithelia with the laboratory strain of *S. aureus* ATCC 6538. As shown in Figure 64, *S. aureus* bound equally to CF and non-CF submerged epithelia *in vitro.* This supports a previous report where *S. aureus* was shown to adhere equally to the CF cell line CFT-1 and the non-CF cell line LCFSN under submerged conditions (Jarry and Cheung, 2006). A study using lab strains and CF isolates of *S. aureus* also demonstrated that *S. aureus* bound in equal numbers to freshly isolated primary CF and non-CF epithelia grown as submerged monolayers (Schwab *et al.*, 1993). This finding was also seen following *S. aureus* infection of CF and non-CF epithelial-fibroblast co-cultures at ALI (Figure 66). This equal binding of *S. aureus* to CF and non-CF respiratory epithelia suggests that the CFTR is not playing a direct role in mediating *S. aureus* binding. If this was the case, IB3-1 CF epithelia would be expected to exhibit reduced bacterial adhesion due to reduced CFTR expression.

Previous authors have identified a number of *S. aureus* adhesion ligands that may facilitate airway colonisation. This includes the ability of *S. aureus* to bind to airway surface asialo-GM1 (Krivan *et al.*, 1988), which has previously been reported to be increased upon the surface of primary CF epithelia (Saiman and Prince, 1993). Once more, it would expected that this may facilitate enhanced *S. aureus* binding, a finding not seen in this chapter. Fibronectin binding protein upon the surface of *S. aureus* has also been shown to facilitate binding to host epithelia β1-integrin (Fowler *et al.*, 2000, Sinha *et al.*, 1999). Additonally, *S. aureus* has been shown to adhere to airway mucins (Sanford *et al.*, 1989).

Compared to *P. aeruginosa,* little is known about *S. aureus* pathophysiology within CF airways and the mechanisms it uses to colonise the lungs. Future work would seek to determine these mechanisms, such as through the introduction of exogenous asialo-GM1 to determine whether it competitively inhibits *S. aureus* binding, as was shown for *P. aeruginosa* (Saiman and Prince, 1993). Blocking or silencing fibronectin binding protein expression upon the surface of *S. aureus* or silencing the expression of asialo-GM_1 or β-1 integrin upon the surface of polarised airway epithelia would also provide a strategic approach to greater understand these host-pathogen interactions.

Unlike *S. aureus, P. aeruginosa* PAO1 adhered in higher numbers to submerged non-CF airway epithelia, than to submerged CF epithelia (Figure 65)**.** Infection of the *in vitro* co-culture models following growth at ALI however, demonstrated that *P. aeruginosa* PAO1 and all the *P. aeruginosa* CF isolates tested bound to the CF and non-CF models equally during mono-infection (Figure 66). Previous research has highlighted the role of cell polarity in the study of host-pathogen interactions, where *P. aeruginosa* has been shown to adhere in significantly higher numbers to A549 submerged monolayers compared to A549 epithelia grown at ALI (Carterson *et al.*, 2005), with differences in cell polarity potentially explaining this finding.

The equal binding of *P. aeruginosa* PAO1 and CF isolates to CF and non-CF airway epithelia grown at ALI during mono-infection (Figure 66,

Figure **68** and Figure 70) is supported by Darling *et al.* who demonstrated that PAO1 and two mucoid CF isolates of *P. aeruginosa* adhered in equal numbers to polarised CFBE41o- (CF) and 16HBE14o- (non-CF) respiratory epithelia (Darling *et al.*, 2004). Although conducted under submerged conditions, Plotowski *et al.* (1992) demonstrated that a non-mucoid CF isolate of *P. aeruginosa* adhered equally to primary CF and non-CF respiratory epithelia obtained nasal polyps (Plotkowski *et al.*, 1992a). This finding is supported by Bryan *et al.* (1998) who demonstrated that *P. aeruginosa* at 10^6 CFU bound equally to CF and non-CF airway submerged epithelial cell lines (Bryan *et al.*, 1998). Such findings contrast with another study by Imundo *et al.* (1995), who demonstrated that *P. aeruginosa* PAO1 bound to polarised IB3-1 in greater numbers than non-CF C38 epithelia (Imundo *et al.*, 1995).

Several studies have suggested that wildtype CFTR in healthy non-CF airways is involved in the receptor mediated uptake of *P. aeruginosa* via LPS binding, consequently removing the bacterium from the airway lumen and preventing airway colonisation (Pier *et al.*, 1996, Pier *et al.*, 1997, DiMango *et al.*, 1998). Thus, as CF epithelia are unable to carry out this innate defence mechanism due to the absence or impaired functioning of the CFTR, CF airways become hypersusceptible to developing chronic airway infections. Thus, the susceptibility of CF airways to *P. aeruginosa* infection may not due to inherent differences in the ability of

Gram-negative bacteria to adhere, but rather the inability to become internalised into host cells and expelled from the airway lumen.

Studies presented in this chapter have revealed that during mono-infection, *S. aureus* bound to submerged IB3-1 and C38 monolayers in significantly higher numbers than PAO1 ($P<0.01$). However, at ALI, *S. aureus* adhesion was only significantly higher than PAO1 in the C38 (non-CF) airway model ($P<0.05$) (Figure 66). Cigana *et al.* (2017) reported that *S. aureus* embedded in agar beads was associated with a higher bacterial load compared to *P. aeruginosa*, independent of the bacterial strains used (Cigana *et al.*, 2017). This could be due to the fact *S. aureus* is considered a "true" pathogen, whilst *P. aeruginosa* is an opportunistic pathogen. Thus, *S. aureus* may be better equipped to colonise the airways and adhere in higher numbers. Further work is required to address this however.

Results from this study show that prior infection with *S. aureus* enhances *P. aeruginosa* adhesion, a phenomenon seen only with lab strain PAO1 and CF isolate 6 and a finding restricted to the CF airway model of infection (Figure 66 and Figure 72). The ability for one bacterium to enhance the adhesion of another is not a newly-discovered phenomenon. Prior exposure to the motile Gram-negative bacterium *Stenotrophomonas maltophilia* has been shown to enhance *P. aeruginosa* binding to submerged monolayers of IB3-1 CF epithelia (Pompilio *et al.*, 2010), whilst prior incubation of submerged non-CF 16HBE14o- cells with *P. aeruginosa* did not enhance the binding of *S. maltophilia* (De Vidipo *et al.*, 2001). This synergy between pathogens has been reported more extensively with respiratory viruses, which have been shown to enhance the binding of *S. aureus* (Saadi *et al.*, 1993), *H. influenzae* (Jiang *et al.*, 1999), *Streptococcus pneumoniae* (Ishizuka *et al.*, 2003) and *P. aeruginosa* (Van Ewijk *et al.*, 2007) in numerous *in vitro* submerged airway epithelial cell models. However, these studies were conducted using epithelia grown under submerged conditions and were not influenced by the potential role of cell polarity (Plotkowski *et al.*, 1999), the presence of other airway cell types or the role that mucus has to play in governing host-pathogen interactions. Currently no known studies have focused upon bacterial co-infections using epithelial cultures grown at ALI.

Why *S. aureus* was able to enhance the adhesion of PAO1 and CF isolate 6 in the CF co-culture model (Figure 66 and Figure 72) despite *S. aureus* binding in equal numbers to the non-CF co-culture model warrants further study. Although the pathophysiological responses that occur in the airway models during the 4 h incubation period require characterisation, including measurement of cell viability (e.g. lactate dehydrogenase release), it is possible that the CF model exhibited a heightened susceptibility to *S. aureus* infection, which was further compromised following the addition of *P. aeruginosa.* CF epithelia have previously been shown to exhibit elevated rates of apoptosis and increased oxidative stress (Rottner *et al.*, 2011, Soleti *et al.*, 2013) along with defects in apoptotic cell clearance (Vandivier *et al.*, 2002a, Vandivier

et al., 2009). During development and characterisation of the established *in vitro* ALI models used in this study, Bielemeier (2012) reported larger decreases in cell viability following *S. aureus* and *P. aeruginosa* infection in the CF model compared to the non-CF model, albeit after a 24 h infection (Bielemeier, 2012b). A study by Shahriary *et al.* demonstrated that IB3-1 and C38 cell lines did not exhibit differences in cell proliferation under normoxia or anoxia (Shahriary *et al.*, 2012). Whilst this was conducted under submerged culture and not in the presence of fibroblasts, it seems unlikely that varying rates of cell proliferation or cell number across the two models post-establishing the airway transwells® could contribute to such differences in bacterial binding.

Why *S. aureus* was only able to enhance the adhesion of PAO1 and *P. aeruginosa* CF isolate 6 in the CF co-culture model (Figure 66 and Figure 70) and not *P. aeruginosa* CF isolate 5 (

Figure **68**) also warrants further study. A study conducted by Cigana *et al.* (2017) introduced laboratory strains of *S. aureus* embedded in agar beads into the lungs of non-CF B6 mice to evaluate the effect of subsequent infection of agar beads embedded with the highly virulent *P. aeruginosa* strain PA14 and two CF clinical isolates. Whilst co-infection was associated with increased mortality, Cigana *et al.* (2017) reported that the bacterial load of *P. aeruginosa* in the lungs of mice pre-colonised with *S. aureus* was not significantly different to mice which were not pre-infected with *S. aureus.* This finding was also seen for CF isolate 5, therefore it is entirely possible that the enhanced adhesion of PAO1 and CF isolate 6 is an isolate-specific phenomenon.

Perhaps changes to the airway epithelium by *S. aureus* colonisation may provide access to additional airway ligands which facilitate *P. aeruginosa* binding. This could either be a result of the upregulation of *P. aeruginosa* specific epithelial surface ligands on damaged or repairing epithelia (Bucior *et al.*, 2010) (which could be assessed using qPCR during longer incubation periods), or through access to the underlying subepithelial fibroblasts and components of the extracellular matrix. A study by Tirouvanziam *et al.* demonstrated how *P. aeruginosa* infection of CF tracheal grafts into immunocompromised mice led to losses in airway cell integrity and rapid cell exfoliation, where bacteria then bind to underlying cells of the airways and the basal lamina (Tirouvanziam *et al.*, 2000). It is possible that the expression and abundance of *P. aeruginosa* adhesins to ligands upon epithelia, fibroblasts and the extracellular matrix varies across the *P. aeruginosa* isolates during microevolution and adaptation to the CF lung.

Yang *et al.* (2011) demonstrated that *P. aeruginosa* strains producing type IV pili were able to aggregate with *S. aureus* to form microcolonies (Yang *et al.*, 2011). The presence or absence of type IV pili or variations in its expression across the CF isolates may lead to differences in microcolony formation, allowing *S. aureus* to act as a "bridge" between the airway cell surface and *P. aeruginosa.* However, whether these interactions occur in these *in vitro* models, or in the CF lung place is unknown.

Perhaps PAO1 and CF isolate 6 are just better equipped than CF isolate 5 at displacing *S. aureus* from its binding sites, as PAO1 and CF isolate 6 both caused decreases in *S. aureus* binding during co-infection, whilst CF isolate 5 did not. Both *S. aureus* and *P. aeruginosa* have been shown to bind to asialo-GM1 (Davies *et al.*, 1999, Imundo *et al.*, 1995) and thus maybe the species compete for asialo-GM1 receptor availability (Bucior *et al.*, 2012, Simpson *et al.*, 1992, Bucior *et al.*, 2010, Tirouvanziam *et al.*, 2000, Davies *et al.*, 1999, Imundo *et al.*, 1995).

The impact of differences in bacterial growth upon enumerated CFU during these studies is likely to be minimal, as stationary phase cultures were used. Planktonic growth studies shown in Figure 17 demonstrate that following dilution of a stationary phase culture, PAO1 and the CF isolates exhibit a lengthy lag phase, which exceeded the 4 h incubation time. Moreover, the use of DMEM/F12 as the infection medium for both *S. aureus* and *P. aeruginosa*, rather than nutrient-rich LB broth may prolong this further. Furthermore, the ability of one bacteria to inhibit the growth of another during sequential infection and thus influence binding is also likely to be minimal, due to the use of stationary phase cultures and the washing of the bacterial pellets prior to inoculation of the transwells®, which removed pre-formed virulence factors.

Future work would seek to include PA14 used by Cigana *et al.* (2017), as well as determine whether *S. aureus* can enhance the binding of several early and late CF isolates of *P. aeruginosa.* The histology of infected transwells® would also provide insights into the localisation of bacterial binding in both mono- and sequential infections. If the two bacteria occupy identical areas as reported *in vivo* (Hogan *et al.*, 2016, Wakeman *et al.*, 2016), this may suggest more of a direct interaction between the two organisms. It is possible that the use of agar embedded beads in the Cigana *et al.* (2017) study encourages the pathogen to remain restricted to growth within the bead, preventing them from forming more direct interactions.

One of the limitations of previous studies addressing the sequence of bacterial infection is that the reverse sequence is not performed to determine whether the actual order of pathogen addition is important. To act as a control in this study, transwells® were first infected with *P. aeruginosa* for 2 h, prior to subsequent infection with *S. aureus.* Interestingly prior infection with PAO1 significantly increased *S. aureus* adhesion only in the CF airway model (Figure 67), whilst the CF isolates of *P. aeruginosa* did not exert this effect in either model.

Firstly, this finding may be due to the fact that PAO1 is a widely used reference strain in CF research and not a CF isolate. Previous chapters in this study have shown how CF isolates differ not only to each other, but also to laboratory strain PAO1. A study addressing bacterial adhesion demonstrated how asialo-GM1 treatment of polarised Madin-Darby canine kidney cells (MDCK) enhanced the binding of *P. aeruginosa* laboratory strain PA103, but not any of the corneal or respiratory isolates, including those obtained from CF patients (Schroeder *et al.*, 2001b). Thus, this brings in to question the relevance of PAO1 and emphasises the need to evaluate clinical CF isolates of *P. aeruginosa* in future experiments addressing CF pathology. The impact of prior infection with *P. aeruginosa* upon the transcriptome of IB3-1 and C38 epithelia could be determined in future experiments to decipher whether there are any variations in the expression of ligands known to be specific for *S. aureus* adhesion.

The traditional dogma of direct bacteria-host cell interactions within CF airways has been challenged within the CF microbiology community. Compared to other airway diseases such as pneumonia, CF is characterised by the presence of excess dehydrated mucus. Previous authors have cited that *S. aureus* and *P. aeruginosa* are localised within static mucus plugs and therefore do not interact directly with airway epithelia (Worlitzsch *et al.*, 2002, Baltimore *et al.*, 1989, Ulrich *et al.*, 1998). Despite this, *P. aeruginosa* has been shown to form intracellular clusters within primary airway epithelia following growth at ALI (Garcia-Medina *et al.*, 2005), with another study showing that mutations in the CFTR enhanced *P. aeruginosa* uptake in polarised airway epithelia (Darling *et al.*, 2004). As previously mentioned, a study infecting CF and non-CF airway grafts with *P. aeruginosa* demonstrated that whilst the bacterium first adhered to the mucus within the lumen, losses in cell integrity and cell sloughing caused the bacteria to then bind to the underlying cells of the airways and the basal lamina (Tirouvanziam *et al.*, 2000), which are richer in high affinity receptors such as asialoGM1 (Saiman and Prince, 1993) and fibronectin (Roger *et al.*, 1999).

Whilst such findings may be a result of the chosen methodology and models systems used, intriguingly, surface protein neuraminidase is known to be one of the most highly expressed genes in CF isolates of *P. aeruginosa* (Lanotte *et al.*, 2004), which is involved in exposing the asialoGM1 receptor (Saiman and Prince, 1993). Moreover, wild type CFTR has been shown to bind to the outer core of *P. aeruginosa* LPS, facilitating bacterial endocytosis and its removal from the airway lumen (Pier *et al.*, 1997, Pier *et al.*, 1996, Schroeder *et al.*, 2002) suggesting that this mechanism may play a role in airway defence. In addition to this, the concentrations of the two major airway mucins MUC5AC and MUC5B have been shown to be decreased in CF sputum by approximately 80% compared to heathy controls during stable disease (Henke *et al.*, 2004, Rubin, 2007). Mucin degradation by the abundant levels of NE and its metabolism by anaerobes in the CF lung are likely to be involved in further modifying airway mucin

concentration and rheology, which may influence direct and indirect host-pathogen interactions (Henke *et al.*, 2011, Flynn *et al.*, 2016, Hampton *et al.*, 2014, Madan *et al.*, 2012).

Drawing all of these findings together, perhaps during the early stages of *P. aeruginosa* airway colonisation, *P. aeruginosa* uses the CFTR to bind to CF epithelia. The inability of mutant CFTR to internalise *P. aeruginosa* facilitates its ability to remain within the airway lumen of individuals with CF and colonise. This process may be further aided by the secretion of *P. aeruginosa* exoproducts, such as pyocyanin, which at physiologically relevant concentrations, has been shown to reduce the expression and trafficking of CFTR in lung and primary nasal epithelia (Kong *et al.*, 2006). This interaction between *P. aeruginosa* and mutant CFTR may also help explain why *P. aeruginosa* dominates in CF adults and yet is not the dominant pathogen in other conditions where mucociliary clearance is impaired but the CFTR is functional, such as the genetic condition primary ciliary dyskinesia (PCD) (Noone *et al.*, 2004, Chen *et al.*, 2008). In PCD where airway cilia are dysfunctional, 32% of adults were culture positive for *P. aeruginosa,* whilst this was 59% for CF (Chang *et al.*, 2015). Perhaps the presence of static mucus alone does not facilitate *P. aeruginosa* colonisation and could be aided by direct bacterial adhesion to respiratory epithelia.

P. aeruginosa colonisation could also be further promoted due damage induced by prior *S. aureus* colonisation, which allows early *P. aeruginosa* infection to exploit the injured airway epithelia and losses in cell polarity. *P. aeruginosa* has been shown to preferably bind to the basolateral side of damaged airway epithelia in polarised *in vitro* models (Bucior *et al.*, 2010, Bucior *et al.*, 2012, Fleiszig *et al.*, 1998). This finding has also been reported in primary cell outgrowth model cultures (de Bentzmann *et al.*, 1996a), organ cultures (Tsang *et al.*, 1994), buccal epithelial cells (Lingner *et al.*, 2017), murine trachea (Ramphal and Pyle, 1983) and rat tracheal surfaces (Yamaguchi and Yamada, 1991). Moreover, decreased cell polarity has been shown to facilitate enhanced *P. aeruginosa* binding, compared to fully polarised epithelia (Plotkowski *et al.*, 1999, Carterson *et al.*, 2005, Lee *et al.*, 1999). Injured airway epithelia express higher amounts of basolateral receptors upon their apical surface (Fleiszig *et al.*, 1998, Heiniger *et al.*, 2010), which in turn facilitates enhanced *P. aeruginosa* uptake following actin polymerisation involving Rho-GTPases (Kazmierczak *et al.*, 2004, Kazmierczak *et al.*, 2001) and tyrosine kinases (Esen *et al.*, 2001). The expression of asialoGM_1 and fibronectin upon the surface of regenerating airway epithelia may facilitate the enhanced binding of *P. aeruginosa* to injured epithelia (de Bentzmann *et al.*, 1996a, Roger *et al.*, 1999, Plotkowski *et al.*, 1992b), coupled with the binding of type IV pili to N-glycoproteins upon the apical surface of respiratory epithelia (Doig *et al.*, 1988, Bucior *et al.*, 2010, Bucior *et al.*, 2012).

The apical presence of *P. aeruginosa* also reportedly upregulates the presence of basolateral receptors at the apical surface of epithelia (Tran *et al.*, 2014), mediating changes in phosphoinositol-3-kinase-protein (PI3K) and facilitating *P. aeruginosa* invasion into host cells (Kierbel *et al.*, 2007, Kierbel *et al.*, 2005). As pyocyanin has also been shown to induce the expression of the tetracarbohydrate moiety sialyl-lewis^x in IB3-1 CF epithelia (Jeffries *et al.*, 2016), this may facilitate additional binding of *P. aeruginosa* to CF airways (Scharfman *et al.*, 1999).

Flagella-mediated swimming motility is common in early CF isolates (Bragonzi *et al.*, 2009) and allows *P. aeruginosa* to preferably colonise hypoxic regions of CF mucus (Worlitzsch *et al.*, 2002). This form of motility may allow *P. aeruginosa* to migrate to injured tissue (Schwarzer *et al.*, 2016) which has been damaged by chronic *S. aureus* infection. Whilst *Helicobacter pylori* has been shown to migrate to urea secreting epithelia within the stomach (Huang *et al.*, 2015), it has been suggested that the release of amino acids from damaged respiratory epithelia may drive *P. aeruginosa* migration to these sites (Schwarzer *et al.*, 2016). Damage to the CF respiratory tissue may also provide access to the underlying mucosa, allowing *P. aeruginosa* to adhere to sub-epithelial fibroblasts (Azghani *et al.*, 1992) in the *in vitro* airway co-culture model, as well as *in vivo*.

The presence of *P. aeruginosa* and secretion of its extracellular virulence factors such as pyocyanin are known to induce goblet cell hyperplasia, mucus hypersecretion and LPS-induced MUC2 production (Hao *et al.*, 2012, Li *et al.*, 1997). Furthermore, pulmonary exacerbations induced by chronic airway infection with *P. aeruginosa* have been shown to increase the levels of mucins relative to healthy individuals (Henke *et al.*, 2007). Thus, perhaps over the course of chronic infection *P. aeruginosa* persistence in the airway lumen subsequently facilitates *P. aeruginosa* adhesion to airway mucins (Arora *et al.*, 1998, Vishwanath and Ramphal, 1984, Sajjan *et al.*, 1992, Ramphal *et al.*, 1996), reflecting the localisation of *P. aeruginosa* seen in autopsy samples obtained from CF patients (Schwab *et al.*, 2014, Baltimore *et al.*, 1989).

Whilst advances are being made to the understanding of the complex *S. aureus-P. aeruginosa* interspecies interactions in CF airways, due to the obvious ethical implications, the exact localisation of CF pathogens *in vivo* remains unknown, particularly during early infection in paediatric CF lungs. Can the bacteria interact directly with CF epithelia and utilise the mechanisms demonstrated in previous studies to escape the host immune response? Or alternatively, do CF pathogens simply bind to airway mucus with defects occurring in CF innate immunity facilitating *P. aeruginosa* colonisation.

6.7 Limitations

One of the limitations of this study is the length of bacterial incubation used to infect airway epithelial cultures grown under submerged conditions and at ALI. Whilst mono-infections to measure bacterial adherence are routinely conducted between a 30 min and 2 h incubation period (Carterson *et al.*, 2005, Pier *et al.*, 1996, Pier *et al.*, 1997, Bucior *et al.*, 2014, Bucior *et al.*, 2012, Bucior *et al.*, 2010, Schwarzer *et al.*, 2016, Van Ewijk *et al.*, 2007), this provides only a snapshot and may not be a long enough to discern greater differences in *S. aureus* and *P. aeruginosa* adhesion during co-infection.

Bacterial cultures were also pelleted and washed in PBS prior to being added to *in vitro* models, thus removing any bacterial exoproducts. This method has been widely employed in several previous studies assessing *P. aeruginosa* mono- and co-infection (Van Ewijk *et al.*, 2007, Pompilio *et al.*, 2010, Saiman *et al.*, 1990a, DiMango *et al.*, 1998, Plotkowski *et al.*, 1992a, Darling *et al.*, 2004, Kato *et al.*, 2010, Schwarzer *et al.*, 2016), whilst another study inoculated bacterial colonies directly into PBS from the agar plate and adjusted the culture density (Pier *et al.*, 1996). This is a step away from the *in vivo* environment however, where an arsenal of exoproducts contributes to airway damage during the course of infection and may play a role in airway colonisation. For example, losses in alpha toxin by *S. aureus* has been shown to significantly increase integrin-mediated adhesion and internalisation of *S. aureus* into A459 alveolar epithelial cells (Liang and Ji, 2006).

Both the chosen length of incubation and the removal of extracellular virulence factors were important in this study, particularly when focusing upon bacterial enumeration (rather than studies addressing mechanisms of infection and airway viability). Extensive damage to both models could cause significant losses in respiratory epithelia, causing them to slough off during the wash step, thus complicating bacterial enumeration. The wide diversity of extracellular virulence factors secreted and their varying concentrations across the two bacterial species and the *P. aeruginosa* CF isolates would also likely complicate the interpretation of the results and the extent to which exoproducts play in promoting bacterial colonisation.

Another limitation is that further characterisation of the *in vitro* co-culture models of CF and non-CF airways is required. This includes determining the concentration and depth of mucus produced by each model, the production of antimicrobial peptides such as HBD's, as well as conducting experiments to investigate ciliary beating frequency.

Finally, as bacterial binding was reliant upon enumerating CFU, questions remain as to the relevance of CFU, as previous research has demonstrated that *P. aeruginosa* can clump together (Imundo *et al.*, 1995, Plotkowski *et al.*, 1992a). Would bacterial clumps still give rise to a single CFU, as expected for a single bacterium? To control for this however, samples were

vortexed and mixed by pipetting, prior to plating on selective agar, to reduce the likelihood of bacterial clumps.

6.8 Future work

Future research would seek to address the mechanisms underlying the findings presented in this chapter, such as determining whether each CF pathogen becomes internalised by polarised non-CF epithelia and not by CF airway epithelia during mono- and co-infection. The classical gentamicin protection assay could be performed for this study, where the antibiotic is incubated for a given time period to kill externally bound bacteria, prior to then being washed off and the epithelial cells lysed, to enumerate the live bacteria which have become internalised (Chi *et al.*, 1991, Darling and Evans, 2003). The cell-associated virulence factors of the *P. aeruginosa* CF isolates could also be determined, such as their ability to bind to purified asialo-GM_1 and wtCFTR.

As the airway epithelia needed to be detached and lysed in order to enumerate the bacteria which had adhered, the terminal nature of such an experiment consequently prevented data from being obtained in relation to the impact of mono- and co-infection upon airway cell viability. Thus, future studies would seek to address this, through measuring the release of intracellular lactate dehydrogenase (LDH) (Bucior *et al.*, 2010) and determining if cells were apoptotic or necrotic using flow cytometry by propidium iodide and annexin V staining. It would also provide answers as to whether co-infection significantly decreases viability compared to mono-infection and whether this is also influenced by the sequence of infection.

Performing histology on transwell® cultures would provide information concerning the type of damage caused by both bacterial species. Cigana *et al.* (2017) reported that *S. aureus* was able to induce abscess-like lesions in the lungs of B6 mice following mono-infection, with a central necrotic core of *S. aureus* surrounded by macrophages and fibrin. Conversely, *P. aeruginosa* remained embedded within the agar beads (Cigana *et al.*, 2017). If *S. aureus* is able to inflict more damage or induce lesions which vary to those produced by *P. aeruginosa,* this would be of particular interest for future studies. Histology would also provide insights into the actual sites of pathogen binding and whether *S. aureus* and *P. aeruginosa* bind primarily to airway epithelia or alternative sites.

As a continuation of chapter 4, the impact of low to absent levels of oxygen must also be determined to understand how this microenvironment influences bacterial adhesion to the CF lung. Previous work has shown that hypoxia increases the expression of PA-I lectin in *P. aeruginosa,* increasing adhesion to the basolateral membrane of intestinal epithelia (Kohler *et al.*, 2005). Furthermore, hypoxia has been shown to decrease *P. aeruginosa* internalisation into CF and non-CF airway epithelia (Schaible *et al.*, 2013) and is also known to downregulate

CFTR expression in respiratory and intestinal epithelia (Zheng *et al.*, 2009, Guimbellot *et al.*, 2008).

Future experiments would seek to use CF isolates of *S. aureus,* a number of which have been co-isolated with *P. aeruginosa* to determine their impact upon binding to CF and non-CF airways. As SCV's of *S. aureus* are becoming increasingly isolated from CF airways and display an increased ability to adhere and internalise within airway epithelia (Mitchell *et al.*, 2011, Tuchscherr *et al.*, 2010, Vaudaux *et al.*, 2002, Kahl *et al.*, 1998), their impact upon airway colonisation also requires investigation. This is also the case for MRSA isolates which are becoming increasingly isolated from CF airways internationally, including in the United States (Cystic Fibrosis Foundation, 2008) and the UK (Thomas *et al.*, 1998, Solis *et al.*, 2003, Miall *et al.*, 2001).

As *P. aeruginosa* extracellular products such as pyocyanin are known to induce ciliary dysfunction (Hao *et al.*, 2012), the impact of *P. aeruginosa* cell-free supernatants upon cell viability, mucus secretion and polarised secretion of inflammatory mediators by the CF and non-CF airway co-culture models must also be investigated. Deciphering whether pre-treatment of airway epithelia with cell-free supernatants of *S. aureus* also enhances *P. aeruginosa* adhesion requires further study.

6.9 Conclusion

The originality of this research is that it firstly demonstrates the impact of *S. aureus-P. aeruginosa* co-infection upon bacterial adhesion to CF and non-CF airways and uses a novel *in vitro* co-culture model of CF and non-CF airways grown at ALI. The relevance of *S. aureus* within CF airway microbiology is highly contested. The evidence for chronically prescribing anti-staphylococcal antibiotics is lacking, whilst there is a suggestion that *S. aureus* primes CF airways to earlier *P. aeruginosa* colonisation. This study demonstrates that *S. aureus* infection appears to enhance *P. aeruginosa* adhesion to CF airway epithelia in an isolate-specific manner, as summarised in Figure 72.

Whilst these findings are novel, they are in their infancy and need to be supported by a comprehensive range of future studies as described above. If *S. aureus* does induce irreversible patho-physiological changes to CF lungs, even eradication of *S. aureus* may not provide therapeutic benefits, as *P. aeruginosa* is still able to colonise the airways. Larger research questions also remain, such as why *S. aureus* can dominate in CF airways for many years prior to *P. aeruginosa* becoming the predominant organism later in life.

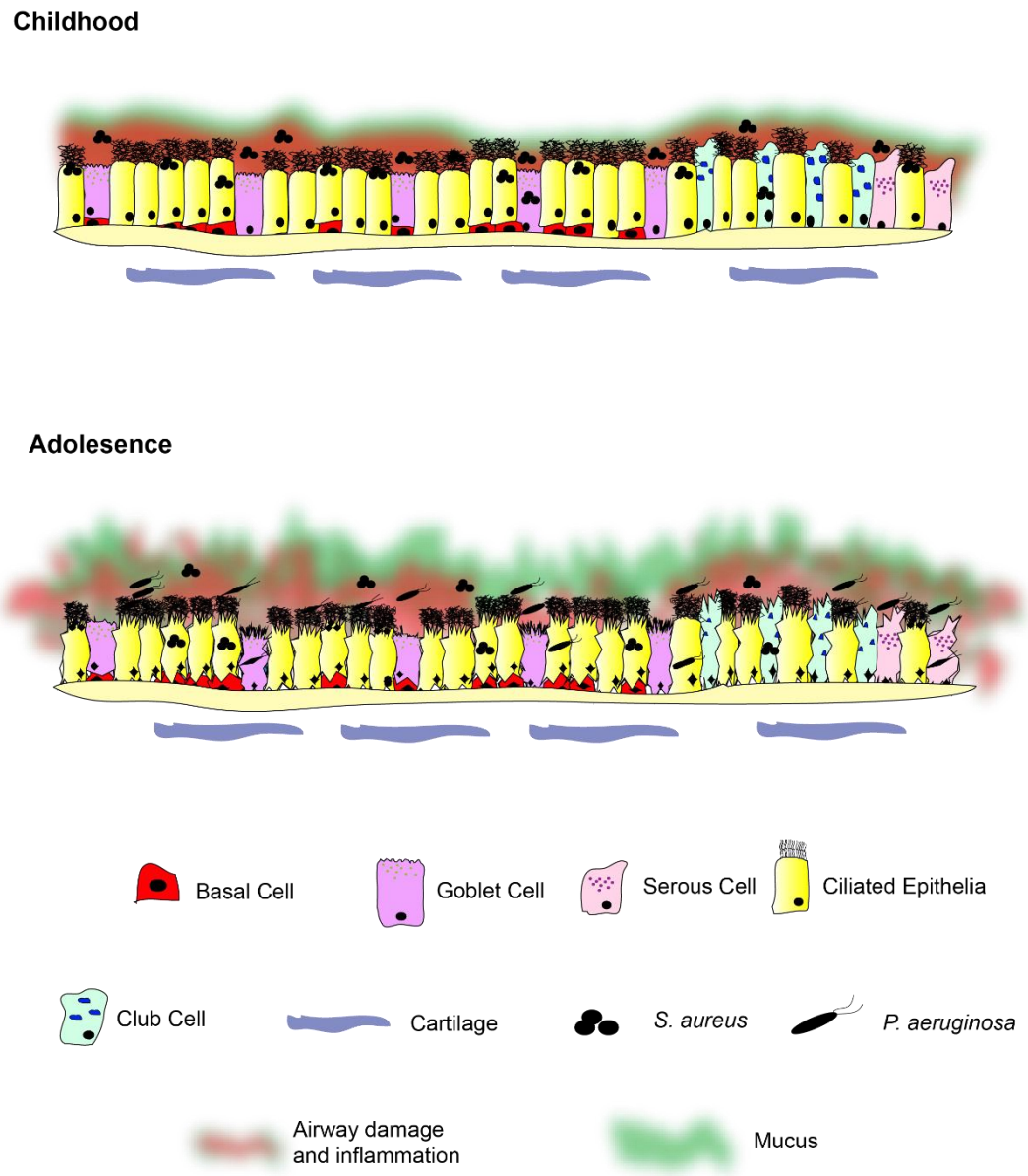


Figure 72. Priming of CF airways by *S. aureus*, to subsequent *P. aeruginosa* colonisation. During childhood, CF lungs are colonised predominantly by *S. aureus*, which through a series of direct and indirect host-pathogen interactions creates a CF lung environment more favourable to *P. aeruginosa* colonisation. *P. aeruginosa* is eventually successful in colonising the lung and through numerous mechanisms is able replace *S. aureus* as the predominant CF pathogen.

7 Final discussion

7.1 Results summary

Whilst extensive research over the last six decades has aided to greatly increase the median life expectancy of individuals with CF to 47 years of age (MacKenzie *et al.*, 2014, Dodge *et al.*, 2007, Keogh *et al.*, 2018, Cystic Fibrosis Trust, 2018), respiratory infections continue to be the main cause of morbidity and mortality from CF (Lyczak *et al.*, 2002, Ciofu *et al.*, 2013). Major obstacles to the development of more effective treatments is the need to obtain a greater understanding of the polymicrobial nature of CF airway infection and how pathogen-pathogen and host-pathogen interactions influence disease progression and health outcomes. Such advances are also further compounded by a lack of relevant model systems in which to study such interactions.

In spite of this, research conducted over recent years has already demonstrated how interspecies interactions between *S. aureus* and *P. aeruginosa* impacts upon the antibiotic susceptibilities of both *S. aureus* (Orazi and O'Toole*,* 2017) and *P. aeruginosa* (Beaudoin et al., 2017) compared to mono-infection, whilst *S. aureus-P. aeruginosa* co-infection is associated with a worsening of pulmonary function and decreased survival (Limoli *et al.*, 2016).

The overall aims of this thesis outlined at the end of the introduction were to:

1. **Phenotypically characterise CF clinical isolates of *P. aeruginosa***
2. **Explore the effects of static growth and anoxia upon *S. aureus-P. aeruginosa* interactions**
3. **Elucidate the effects of *S. aureus-P. aeruginosa* co-stimulation upon the airway inflammatory response**
4. **Determine whether prior *S. aureus* infection influences *P. aeruginosa* airway colonisation**

Evaluation of Aim 1: Phenotypically characterise CF clinical isolates of *P. aeruginosa*

Chapter 3 was concerned with characterising eight novel CF clinical isolates of *P. aeruginosa* obtained from Birmingham Children's Hospital, focusing upon a number of phenotypic traits previously summarised as being important in establishing and maintaining CF airway infection (Ballok and O'Toole, 2013, van 't Wout *et al.*, 2015).

The data presented in chapter 3 are in agreement with the well-documented wide phenotypic diversity that is known to exist across CF clinical isolates of *P. aeruginosa* (Mowat *et al.*, 2011, Clark *et al.*, 2015b, Tingpej *et al.*, 2007, Winstanley *et al.*, 2016, Mayer-Hamblett *et al.*, 2014b, Workentine *et al.*, 2013, Ashish *et al.*, 2013). The eight CF isolates exhibited 6 distinct colony morphological characteristics (Table 6), as well as a lack of correlation between the production of one virulence factor with another. Adaptation to the CF lung environment is typically associated with losses in motility (swimming, swarming and twitching) and the secretion of extracellular virulence factors (Workentine *et al.*, 2013), in favour of acquiring a mucoid phenotype (Sousa and Pereira, 2014). Despite this, non-mucoid *P. aeruginosa* CF isolate 8 failed to secrete detectable proteases (Figure 18) and yet exhibited swarming motility (Figure 23), whilst mucoid *P. aeruginosa* CF isolate 3 exhibited protease activity (Figure 18) and swarming motility (Figure 23). Thus, specific CF isolates exhibited a co-occurrence of phenotypes typically associated with acute and chronic infection, a finding reported previously in a longitudinal genotypic and phenotypic study of a *P. aeruginosa* strain from an adult with CF (Clark *et al.*, 2015b). Phenotyping the eight CF clinical isolates in this study enabled isolates to be selected for further study.

One of the main impacts of the wide phenotypic diversity across *P. aeruginosa* CF isolates is its effect upon antimicrobial susceptibility testing. Current antibiotic sensitivity tests in diagnostic microbiology laboratories rely on selecting the most common morphotypes from cultured sputum and exposing them to a series of antibiotic concentrations (Kahlmeter *et al.*, 2003). However, colonies of the same morphotype isolated from CF sputum have already been shown to exhibit different antibiotic susceptibilities (Foweraker *et al.*, 2009, Foweraker *et al.*, 2005) and the results are known to be a poor predictor of clinical outcomes (Hurley *et al.*, 2012). The CF lung environment is a unique environmental niche and one that restricts antibiotic delivery and potency. Biofilm versus planktonic growth and variations in oxygen availability have all been shown to influence the effectiveness of antibiotics (Hill *et al.*, 2005). Whilst mixed morphotype testing from specimen plates have previously been shown to be a more efficient way to determine antimicrobial susceptibilities of common CF pathogens (Wolter *et al.*, 1995, Van Horn, 1993), such practices have yet to enter routine practice in diagnostic laboratories. Moreover, future studies must continue to determine the influence of

polymicrobial infections upon antibiotic efficacies (Orazi and O'Toole, 2017, Beaudoin *et al.*, 2017).

Poor clinical responses relating to antibiotic susceptibility testing has led to the suggestion of using alternative treatments to combat *P. aeruginosa* infection. This includes developing compounds to target *P. aeruginosa* protease production to limit damage to the airways, as well as producing compounds to target LasR quorum sensing and thus reduce bacterial virulence (Cathcart *et al.*, 2011, Jakobsen *et al.*, 2013). However, as *P. aeruginosa* proteases have been shown to effectively degrade the pro-inflammatory mediators IL-8 and IL-6 (LaFayette *et al.*, 2015), subsequent losses in protease activity through targeting proteases or quorum sensing may heighten the airway inflammatory response and thus worsen pulmonary function and overall health.

A “trojan horse” approach to treating bacterial infections has also been suggested. Coupling siderophores to antibiotics would consequently facilitate their intracellular uptake by *P. aeruginosa,* mediating bacterial killing (Schalk and Mislin, 2017). However, delivering these to the sites of infection are likely to face the same challenges as conventional antibiotic treatments, such as mucus plugging, bacterial biofilms and immune infiltrates. The introduction of social cheats into bacterial populations has also been suggested, including cheats which do not express virulence factors, but are able to outcompete the resident strains and exhibit susceptibility to antibiotics. Thus, once the less virulent social cheats have dominated, they can be cleared with an appropriate antibiotic (Brown *et al.*, 2009). Such a strategy also has drawbacks however, including successful delivery of the cheats to the sites of infection within the CF lung, as well as unintended co-evolution of the social cheat with the wildtype bacterial population.

Evaluation of Aim 2: Explore the effects of static growth and anoxia upon *S. aureus-P. aeruginosa* interactions

The characterisation of the novel *P. aeruginosa* CF clinical isolates conducted in chapter 3 also provided a basis for selecting CF isolates for further study, to determine the impact of normoxia and anoxia upon *S. aureus-P. aeruginosa* interspecies interactions within planktonic co-culture and mixed species biofilms. This is in light of studies providing evidence that the CF lung environment contains regions of anoxia (Worlitzsch *et al.*, 2002, Tunney *et al.*, 2008, Rogers *et al.*, 2003). *S. aureus* and *P. aeruginosa* co-colonisation has previously been associated with a worsening of pulmonary function compared to colonisation with *S. aureus* and *P. aeruginosa* alone (Rosenbluth *et al.*, 2004), along with increased frequency of pulmonary exacerbations (Limoli *et al.*, 2016). Furthermore, two additional studies reported that the pulmonary function in individuals co-infected with *S. aureus-P. aeruginosa* was worse compared to those infected with *S. aureus* alone (Hudson *et al.*, 1993, Hubert *et al.*, 2013).

Interspecies interactions between *S. aureus* and *P. aeruginosa* not only influence disease progression (Sibley *et al.*, 2009) but have also been shown to modulate bacterial virulence (Fugere *et al.*, 2014a, Korgaonkar *et al.*, 2013) and the host's immune response (Pernet *et al.*, 2014). Understanding such interactions is essential for understanding CF airway infections, which in turn could provide new treatment strategies and therapeutics to influence bacterial community composition.

As discussed in chapter 4, under normoxia all *P. aeruginosa* CF isolates tested were able to dominate over *S. aureus* in planktonic co-culture (Figure 27) and mixed species biofilms (Figure 28), which is in agreement to a number of previous studies conducted under normoxia (Kessler *et al.*, 1993a, Baldan *et al.*, 2014b, Biswas *et al.*, 2009b, Fugere *et al.*, 2014b, Filkins *et al.*, 2015, Fothergill *et al.*, 2007a, Mashburn *et al.*, 2005a). This finding is not limited to the use of traditional culture broths such as LB, as *P. aeruginosa* has also been shown to reduce *S. aureus* viability and dominate following growth in artificial CF sputum (Haley *et al.*, 2012). Such a finding explains in part the inverse relationship between the two major CF pathogens, where the advent of adolescence is associated with the transition from *S. aureus* to *P. aeruginosa* dominance (Cystic Fibrosis Trust, 2018).

Anoxia was shown to attenuate the ability of select *P. aeruginosa* isolates to dominate in mixed culture in both planktonic culture (Figure 27) and mixed species biofilms (Figure 28), thus providing *S. aureus* with a survival advantage. Spatial segregation of *S. aureus* and *P. aeruginosa* has previously been reported to promote bacterial co-existence in wound biopsies and *in vitro* wound models, despite pathogens retaining their virulence (Fazli *et al.*, 2009, Dalton *et al.*, 2011). *P. aeruginosa* alginate overproduction has also been associated with facilitating *P. aeruginosa* co-existence with *S. aureus,* where the production of alginate was associated with a reduction in the production of other extracellular virulence factors such as siderophores and rhamnolipids (Limoli *et al.*, 2016). Results presented in chapter 4 suggest that anoxia is an additional mechanism which may facilitate *S. aureus-P. aeruginosa* co-existence in an isolate dependent manner. This is likely to have implications upon patient health, as *S. aureus-P. aeruginosa* co-existence has previously been shown to worsen pulmonary function and overall survival (Limoli *et al.*, 2016, Maliniak *et al.*, 2016, Hudson *et al.*, 1993). Interestingly, not all *P. aeruginosa* CF isolates lost their ability to reduce *S. aureus* viability under anoxia and were still able to dominate in planktonic co-culture and mixed species biofilms. Such findings re-emphasise the highly complex nature of CF airway infections and that environmental factors such as oxygen availability are likely to exert isolate specific changes to community composition and bacterial dominance.

In addition to influencing bacterial community dynamics, the retention of *P. aeruginosa* virulence properties under anoxia is also likely to impact upon the host. The ability to secrete proteases under anoxia may facilitate *P. aeruginosa* damage to airways despite dynamic changes in oxygen availability over the course of infection due to mucus plugging, biofilm formation and consumption of oxygen by infiltrating neutrophils. Conversely, oxygen dependent losses in bacterial virulence may also facilitate disease progression, with losses in protease production preventing the degradation of IL-8, thus heightening airway inflammation and neutrophil chemotaxis (LaFayette *et al.*, 2015).

Subsequent experiments sought to determine the anti-staphylococcal compound(s) which are likely to mediate *P. aeruginosa* dominance under both normoxia and anoxia. Heat and size exclusion treatment of cell-free *P. aeruginosa* culture supernatants suggest that the compound facilitating *P. aeruginosa* dominance over *S. aureus* is extracellular, heat stable and >3 kDa in size (Figure 42). Future work would seek to construct a series of genetic mutants of *P. aeruginosa* using siRNA (small interfering RNA), to sequentially knock out known virulence factors that are >3 kDa in size. The addition of cell-free culture supernatants obtained from these mutants could then be added to *S. aureus* as performed previously in this study, to assess whether the mutant loses its ability to antagonise the viability of live *S. aureus*. Purified virulence factor be added to the mutant culture supernatant exogenously, to determine whether this restores the ability of *P. aeruginosa* to kill *S. aureus.*

Perhaps eventual identification of the anti-staphylococcal compound(s) mediating *P. aeruginosa* dominance may reveal the mechanisms which facilitate the transition to *P. aeruginosa* dominance within CF airways and in turn open new therapeutic avenues to potentially interfere with disease progression. However, it has been previously reported that *P. aeruginosa* dominance is dependent upon the production of multiple virulence factors, as deletion of single virulence factors has been shown reduce *S. aureus* antagonism (Limoli *et al.*, 2017). Thus, it is possible that more than one extracellular virulence factor retained within the >3 kDa fraction facilitates *P. aeruginosa* dominance.

S. aureus exoproducts were also shown to positively modulate *P. aeruginosa* motility (Figure 46) in an isolate dependent manner, either restoring motility to detectable levels, or significantly enhancing it. As this activity was exhibited in both fractions (>3 and <3 kDa fractions) and was not sensitive to heat treatment, deciphering the compounds which facilitate this species synergism may ultimately aid in being able to develop targeted treatments to modulate these interspecies interactions. The ability of *S. aureus* exoproducts to enhance *P. aeruginosa* motility may exert a detrimental effect in the CF lung, allowing *P. aeruginosa* to migrate to areas of improved nutrient availability and away from host defence mechanisms. Furthermore,

flagella has previously been reported to be involved in biofilm formation (O'Toole and Kolter, 1998).

Another attractive approach in the treatment of *S. aureus-P. aeruginosa* co-infection is to target co-expressed virulence genes. Despite differences in genome size, a previous study demonstrated how both bacterial species expressed a gene involved in survival in environments high in antibiotics, which could be targeted to control bacterial pathogenicity (Hosseinkhan *et al.*, 2018).

Evaluation of Aim 3: Elucidate the effects of *S. aureus-P. aeruginosa* co-stimulation upon the airway inflammatory response

As well as influencing bacterial community composition, the interspecies interactions that occur between the two major CF pathogens *S. aureus* and *P. aeruginosa* are likely to also govern the host's innate immune response. *P. aeruginosa* has previously been shown to induce the production of type-IIA-secreted phospholipase A2 by CF epithelia, a bactericidal enzyme that is able to kill *S. aureus*, but exhibit minimal bactericidal activity upon *P. aeruginosa* (Pernet et al., 2014). Furthermore, a clinical study focusing upon *S. aureus-P. aeruginosa* co-infection reported heightened airway inflammation in individuals co-infected with *S. aureus-P. aeruginosa*, compared to those infected with either pathogen (Sagel *et al.*, 2009a).

As airway epithelia are likely to sense diffusible bacterial exoproducts (Klinger *et al.*, 1978, Ericsson *et al.*, 1986, Hollsing *et al.*, 1987b), chapter 5 sought to determine the impact of secreted and shed extracellular products from *S. aureus* and/or *P. aeruginosa* upon the inflammatory response of submerged monolayers of CF and non-CF airway epithelia. How CF and non-CF airway epithelia detect and respond to multiple bacterial stimuli during a polymicrobial infection is poorly understood, with only one current study to date addressing *S. aureus-P. aeruginosa* co-stimulation upon airway inflammation *in vitro* (Chekabab et al., 2015).

Whilst the use of differentiated polarised ALI cultures would increase the physiological relevance of the findings and provide data regarding the directional release of inflammatory mediators, submerged epithelial monolayers remained useful. As well as being high a widely used methodology and high throughput (Chekabab *et al.*, 2015, Becker *et al.*, 2004, Massion *et al.*, 1994, Palfreyman *et al.*, 1997, Beaudoin *et al.*, 2013), they represent the most superficial cell layer within the airways, which is most likely to be in contact with microbial products.

Unlike previous work, this study employed the use of static bacterial cultures, rather than those grown under vigorous culture aeration (Chekabab *et al.*, 2015). As published previously, CF bacteria grow statically under varying oxygen tensions, which is likely to induce physiological changes and influence the production of virulence factors (Gaines *et al.*, 2005). Whilst static growth has been shown to select for motile variants of *P. aeruginosa,* switching to shaking

culture reversed this finding, giving rise to non-motile variants (Wyckoff et al., 2002*)*. Gaines *et al.* reported that static growth increased the expression of endotoxin A by *P. aeruginosa* PAO1 (Gaines *et al.*, 2005).

The results presented in chapter 5 demonstrate how immortalised CF bronchial epithelia secrete elevated concentrations of both IL-8 and IL-6 at baseline, compared to non-CF airway epithelia (Figure 50, Figure 51, Figure 53 and Figure 54). The origin of inflammation within CF airways is highly contested within the CF community. Some reports have reported that the baseline secretion of inflammatory cytokines is equal in CF and non-CF epithelia (Scheid *et al.*, 2001, Kube *et al.*, 2001, Black *et al.*, 1998, Becker *et al.*, 2004), whilst others have demonstrated that mRNA and protein expression levels of IL-8 from primary CF epithelia and gland cells are constitutively upregulated compared to non-CF cells (Tabary *et al.*, 1998, Carrabino *et al.*, 2006, Kammouni *et al.*, 1997, Bonfield *et al.*, 1999). BALF analysis of infants with CF who were culture-negative for common CF pathogens reported increased neutrophil counts, free NE and IL-8 compared to control subjects (Khan *et al.*, 1995), whilst the continuous inhibition of CFTR has been shown to significantly increase in IL-8 secretion both at baseline and following exposure to *P. aeruginosa* (Perez *et al.*, 2007). Such findings are not restricted to airway epithelia, where silencing of the CFTR in macrophages has been shown to induce a pro-inflammatory phenotype (Xu *et al.*, 2010).

Results presented in chapter 5 also demonstrated that CF epithelia exhibited a significantly higher IL-8 response compared to non-CF epithelia, following challenges with exoproducts from PAO1 and most of the *P. aeruginosa* CF clinical isolates (Figure 50 and Figure 53). It is likely that the IL-8 produced at baseline will contribute to the overall inflammatory response induced by *P. aeruginosa* products. IL-6 production in CF epithelia was heightened and unaffected by single or dual challenges with *S. aureus* and/or *P. aeruginosa* exoproducts (Figure 53).

Furthermore, of all the dual challenge studies, only co-stimulation with *S. aureus* and PAO1 exoproducts significantly increased IL-8 production by CF epithelia, compared to baseline and *S. aureus* or *P. aeruginosa* alone (Figure 50). IL-6 production in CF epithelia was heightened and unaffected by single or dual challenges with *S. aureus* and/or *P. aeruginosa* exoproducts (Figure 53). Conversely, non-CF epithelia exhibited a low inflammatory phenotype at baseline, where only single challenges with PAO1 (and not *S. aureus* or the CF clinical isolates) significantly increased the IL-8 response compared to baseline (Figure 51). Unlike CF epithelia, co-stimulation with all *S. aureus-P. aeruginosa* isolate combinations increased the IL-8 and IL-6 response, compared to both baseline and single microbial challenges (Figure 51 and Figure 54). A lack of enhancement to the IL-8 and IL-6 response in the majority of co-infections with *S. aureus-P. aeruginosa* in CF airways suggests that *S. aureus-P. aeruginosa*

may not always exacerbate inflammation. Such a finding was reported by Reece *et al.* who demonstrated that only specific combinations of whole-live *Aspergillus fumigatus* with *P. aeruginosa* increased IL-6 and IL-8 production by CFBE41o− bronchial epithelial cells, compared to *Aspergillus fumigatus* and *P. aeruginosa* alone (Reece *et al.*, 2018). The authors demonstrated that the mechanisms as to why *A. fumigatus* with *P. aeruginosa* did not exert an additive effect upon inflammation was due to saturation of the ERK and p38 MAPK signalling pathways (Reece *et al.*, 2018).

These findings seen in both CF and non-CF epithelia are in contrast to a study by Chekabab *et al.* who reported that *S. aureus* exoproducts inhibited the release of IL-8 in both Beas-2B (non-CF) and CFBE41o- (CF) airway epithelia induced by exposure to *P. aeruginosa* exoproducts (Chekabab *et al.*, 2015). *S. aureus* exoproducts have also been shown to inhibit IL-8 gene expression and protein production by human umbilical vein endothelial cells (HUVEC), although this inhibition was shown to be *S. aureus* strain specific (Tajima *et al.*, 2007). As neither Tajima *et al.* or Chekabab *et al.* employed the *S. aureus* strain ATCC 6538, the inability for *S. aureus* to dampen the pro-inflammatory response in this study may be a strain-specific phenomenon, due to differences in bacterial physiology and virulence. Such findings may also be influenced by the bacterial growth conditions employed, following bacterial culture in LB broth, under static conditions. Chekabab *et al.* reported differences in the ability of *S. aureus* to induce an IL-8 response following growth in LB broth and tryptic soy broth (TSB). Whilst *S. aureus* growth in LB broth elicited a minimal IL-8 response, *S. aureus* growth in TSB elicited a dose-dependent inflammatory response in airway epithelia (Chekabab *et al.*, 2015).

It is also entirely possible that *S. aureus* does not dampen the pro-inflammatory response induced by *P. aeruginosa. P. aeruginosa* has previously been shown to influence the majority of the airway inflammation in a non-CF mouse model of infection, where co-infection with *S. aureus* exerted more of a follow-on effect, rather than an additive or inhibitory effect upon the inflammatory response to *P. aeruginosa* (Cigana *et al.*, 2017). Moreover, bacterial co-infection in paediatric CF airways has also been associated with increased IL-8 and neutrophil counts in BALF compared to mono-infection (Sagel *et al.*, 2009a).

It is also important to highlight that the choice of immortalised airway epithelia may also give rise to the results obtained. The work presented here employed the use of IB3-1 CF epithelia and the isogenic cell line C38, a CF corrected phenotype with WT CFTR in an adeno-associated viral vector. The Chekabab *et al.* study which reported an inhibitory effect of *S. aureus* upon the inflammatory response induced by *P. aeruginosa* employed the CF epithelial cell line CFBE41o-, as well as the unmatched healthy bronchial Beas-2B epithelial cell line (Chekabab *et al.*, 2015). There are numerous cell lines which are used in respiratory research

(Fulcher *et al.*, 2009) and the findings could potentially be influenced by a number of factors, such as the CFTR mutation severity, differences in NF-κB activity and the location from which the cells were obtained in the airways. Although immortalised cell lines in respiratory research provide advantages over primary airway epithelia due to their increased availability and reduced in patient variability, they also exhibit limitations. As IB3-1 epithelia were obtained from one CF patient prior to immortalisation, there is an argument that they only represent a "*N*" of 1 and thus lack variability and patient diversity. In the context of these inflammatory studies, the use of isogenic genetically matched cell lines however (which are only expected to differ in their CFTR expression) provide evidence that the presence or absence of the CFTR plays a key role in governing airway inflammation.

S. aureus exoproducts did not elicit a dampening effect upon the release of IL-8 and IL-6 following exposure to purified LPS from *E. coli*, a finding reported previously (Chekabab *et al.*, 2015). This finding is unsurprising as the previously reported modulatory effect of *S. aureus* is restricted to TLR1/2 induced NF-κB signalling (Chekabab *et al.*, 2015), with LPS being known to signal through TLR4 (Chow *et al.*, 1999). Additional work is required to assess the signalling mechanisms of CF and non-CF epithelia in response to *S. aureus* and *P. aeruginosa* using inhibitors targeting NF-κB and MAPK pathways.

It is possible that the IB31-1 CF cell line failed to produce IL-10 as this anti-inflammatory cytokine has previously been shown to be below the limit of detection in studies using immortalised and primary CF epithelia (Massengale *et al.*, 1999, Becker *et al.*, 2004, Bonfield *et al.*, 1995a, Bonfield *et al.*, 1999). As the non-CF cell line C38 is derived from IB3-1 airway epithelia, this inability to produce detectable levels of IL-10 is likely to be conserved.

Heat-inactivated bacterial cell-free supernatants were used to minimise airway epithelia toxicity, which could otherwise complicate the interpretation of results across cell lines and following single and dual challenges. Whilst one of the findings of this methodological approach is that airway inflammation can be induced by exoproducts which are not sensitive to heat-treatment, it abolishes the activity of *P. aeruginosa* proteases in particular, which are important in CF airway pathology and are known to degrade both IL-8 and IL-6 (Saint-Criq *et al.*, 2018, Okuda *et al.*, 2011, LaFayette *et al.*, 2015). The extent to which *P. aeruginosa* proteases dampen the inflammatory response in the airways of individuals with CF requires further study.

The effects of polymicrobial infection upon the airway inflammatory response is likely to complex. The work presented in chapter 5 suggests that the IL-8 and IL-6 response in CF epithelia in the absence of microbial infection is likely to impair neutrophil function due to premature priming (Taggart *et al.*, 2000), as well as lead to extensive airway damage due to the secretion of NE (Wagner *et al.*, 2016). Coupled with an altered inflammatory response during *S. aureus-P. aeruginosa* co-infection compared to non-CF epithelia, this may aid in the

ability of these two predominant CF pathogens to evade the host immune response and chronically colonise the airways.

Evaluation of Aim 4: Determine whether prior *S. aureus* infection influences *P. aeruginosa* airway colonisation

There is a lack of consensus regarding the role of *S. aureus* in CF airway infection, from its effect upon disease pathology, to whether it facilitates earlier *P. aeruginosa* colonisation (Lyczak *et al.*, 2002). In turn there is no standardised global approach regarding the treatment of *S. aureus* infection, where flucloxacillin prophylaxis is recommended for the first three years of life in the UK, whilst this is recommended against in the USA (Stutman *et al.*, 2002, Ratjen *et al.*, 2001, Wong *et al.*, 2013, Smyth and Rosenfeld, 2017). Continuous anti-staphylococcal prophylaxis has been associated with earlier *P. aeruginosa* acquisition (Ratjen *et al.*, 2001, Hurley *et al.*, 2018).

As previously mentioned, models to study polymicrobial respiratory infections upon the host response are in their infancy. The handful of previous studies addressing polymicrobial airway infection in CF have employed the use of submerged CF epithelial cell monolayers, where *S. maltophilia* and RSV have both been shown to enhance the binding of *P. aeruginosa* (Pompilio *et al.*, 2010, Van Ewijk *et al.*, 2007). Currently only one *in vivo* study using a non-CF mouse model has sought to investigate the impact of *S. aureus* infection upon *P. aeruginosa* airway colonisation (Cigana *et al.*, 2017). Whilst several mouse models have been developed over the last two decades and have provided important insights into CF disease pathology, they lack many hallmarks characteristic of CF airway infection. This includes their inability to develop spontaneous lung infections (Grubb and Boucher, 1999), along with their lack of IL-8, instead expressing the homologs; macrophage inflammatory protein-2α (MIP-2α) and keratinocyte chemoattractant (KC) (Tarrant, 2010, Zhang *et al.*, 2001). As IL-8 is the major chemokine secreted in CF airways, *in vivo* comparisons to the CF population can be difficult. Retaining bacterial populations in the airway lumen of murine models requires bacteria to be embedded in agar beads (Cigana *et al.*, 2017, Stotland *et al.*, 2000, Cigana *et al.*, 2016). Such an approach is an exaggerated mode of infection which does not mimic that seen in humans. Additionally, he microaerophilic environment of the beads themselves (Bragonzi *et al.*, 2005), their ability to retain the bacteria within the bead and variations in bead size are all likely to impact upon experimental findings.

This study employed previously established and characterised *in vitro* co-culture models of human CF and non-CF airways (Bielemeier, 2012b), consisting of CF or non-CF epithelia grown on a layer of subepithelial fibroblasts. Human fibroblasts were seeded onto human collagen type IV coated transwells®, as collagen IV is known to underlie the airway epithelium *in vivo* (Sage, 1982).Whilst type IV collagen is only one component of the ECM, fibroblasts

have previously been shown to produce ECM components, which have been shown to better facilitate the growth of bronchial epithelia (Skibinski *et al.*, 2007). Sub-epithelia primary fibroblasts have been shown to play a role in epithelial cell differentiation, the formation of cilia and the production of cytokines (Bielemeier, 2012b, Costea *et al.*, 2003, Sacco *et al.*, 2004). CF or non-CF airway epithelia were subsequently seeded onto the layer of fibroblasts and differentiated for a minimum of 21 days at ALI to permit polarisation (Pezzulo *et al.*, 2011) and the cells fed basolaterally with fresh culture media (Whitcutt *et al.*, 1988). This model was shown to express the tight junction protein ZO-1, express cilia and microvilli, as well as produce the mucin MUC5AC (Bielemeier, 2012b). The models were used to mimic the sequence of infection seen in CF airways, to determine whether prior *S. aureus* infection influences *P. aeruginosa* colonisation.

Both *S. aureus* and the *P. aeruginosa* CF isolates were shown to bind equally to CF and non-CF co-culture models grown at ALI following mono-infection (Figure 66), a finding reported previously using submerged CF and non-CF epithelial monolayers (Jarry and Cheung, 2006, Cervin *et al.*, 1994, Plotkowski *et al.*, 1996). Questions remain however as to the exact sites of bacterial binding within CF airways. Biopsies obtained from individuals with CF have shown how *S. aureus* and *P. aeruginosa* are predominantly localised in airway mucus (Ulrich *et al.*, 1998, Worlitzsch *et al.*, 2002, Baltimore *et al.*, 1989) and both have been shown to bind to mucins *in vitro* (Carnoy *et al.*, 1994, Devaraj *et al.*, 1994, Ramphal *et al.*, 1987, Ulrich *et al.*, 1998). However, CF epithelia have also been reported to exhibit enhanced *P. aeruginosa* binding due to increased apical expression of the cell surface receptor asialo-GM_1 (Saiman *et al.*, 1992, Saiman and Prince, 1993, Zar *et al.*, 1995, Imundo *et al.*, 1995). Mutations in the CFTR have been associated with defects in *P. aeruginosa* internalisation (Pier *et al.*, 1996, Pier *et al.*, 1997), allowing the bacterium to remain within the airway lumen and colonise. Thus, perhaps impaired bacterial internalisation plays a more significant role than bacterial adhesion in facilitating the development of chronic infection.

There is no denying that impairments in mucociliary clearance and mucus plugging plays an enormous role in facilitating bacterial colonisation of the CF airways. However, in isolation it fails to explain why the incidence of *S. aureus* and *P. aeruginosa* in children with primary ciliary dyskinesia (PCD) is half that of those isolated from individuals with CF (Tracy *et al.*, 2016). Whilst both diseases result in impaired mucociliary clearance the CFTR is functional in PCD. Moreover, another study demonstrated that *P. aeruginosa* was prevalent in 59% of individuals with CF compared to 32% for PCD, with *S. aureus* being detected in 74% of those with CF, compared to 24% in PCD (Chang *et al.*, 2015). If mucus alone plays a major role in facilitating bacterial clearance, why do other CF pathogens not dominate? The specificity of wtCFTR to bind to *P. aeruginosa* and not to other CF pathogens potentially explains in part why *P. aeruginosa* is the dominant pathogen in CF airways. Mucoid isolates of *P. aeruginosa* have

been shown to lose their expression of the LPS ligand which binds to the CFTR (Schroeder *et al.*, 2001a, Schroeder *et al.*, 2002). Furthermore, surface protein neuraminidase is known to be one of the most highly expressed genes in CF isolates of *P. aeruginosa* (Lanotte *et al.*, 2004), which is involved in exposing the asialoGM_1 receptor (Saiman and Prince, 1993) known to be present upon the surface of airway epithelia.

Another major CF pathogen *H. influenzae* has previously been shown *in vitro* to bind initially to airway mucins and then to injured respiratory epithelia (Read *et al.*, 1991). Infection of polarised airway epithelia with non-typeable *H. influenzae* has demonstrated how the bacterium preferably bound in clusters to surviving epithelia (Ren *et al.*, 2012). *P. aeruginosa* has also been shown to preferably bind to injured and regenerating airway epithelia, binding to apically enriched proteoglycans, asialoGM_1 and fibronectin (Lingner *et al.*, 2017, Bucior *et al.*, 2010, Bucior *et al.*, 2012, Fleiszig *et al.*, 1998, de Bentzmann *et al.*, 1996b, de Bentzmann *et al.*, 1996a). Thus, it is possible that CF pathogens bind to the static airway mucus in CF airways, as well as to the surface of CF airway epithelia. Together this facilitates damage to the airways through direct and indirect host-pathogen interactions, which further enhances bacterial colonisation.

Co-infection studies demonstrated that *S. aureus* enhanced the binding of *P. aeruginosa* in CF airways only in an isolate-dependent manner, a finding not seen in the non-CF model (Figure 66,

Figure **68** and Figure 70). Furthermore, the sequence of infection also appears to be important, as only prior infection with PAO1 was shown to enhance *S. aureus* binding (Figure 67), whilst the CF isolates did not elicit this priming effect (Figure 69 and Figure 71). Such findings bring into question the relevance of PAO1 as a model organism to study CF microbiology, as well as the approaches that need to be taken to determine the mechanisms which facilitate this synergism between *S. aureus* and *P. aeruginosa* in colonising CF airways. The use of the *in vitro* co-culture models in this study could begin to address this in future studies.

It is appreciated that these results are one of the first to address *S. aureus*-*P. aeruginosa* sequential infection upon the host response in CF, alongside a study published at the end of 2017 using a non-CF B6 murine model (Cigana *et al.*, 2017). Whilst the limitations of CF mice have already been described, there are a number of similar conclusions that can be drawn between the work presented in chapter 6 and the study published by Cigana *et al.* in 2017. Firstly, both studies employed laboratory strains, as well as CF clinical isolates of *P. aeruginosa.* The *P. aeruginosa* bacterial burden during co-infection of the non-CF airway model was similar to that enumerated following mono-infection, a result reported in the non-CF murine model (Cigana *et al.*, 2017) Moreover, the *S. aureus* bacterial burden was significantly higher than *P. aeruginosa* during mono-infection (in both airway models), a

finding also reported by the authors (Cigana *et al.*, 2017). The major distinction between the results presented in Cigana *et al.* and the results from this chapter 6 however is that prior infection with *S. aureus* was shown to enhance *P. aeruginosa* adhesion in CF airways.

It is important to highlight that both airway models display inherent limitations, coupled with drawbacks to the methodological approaches. In Cigana *et al.* this relates to the use of a non-CF murine model to study infection in CF and the need to challenge the lungs with bacteria embedded in agar beads to prevent bacterial clearance. Thus, CF pathogens are thus retained within the microenvironment of the agar bead, which may influence their physiology, virulence and direct and indirect interactions within the airways. As mice do not express IL-8, evaluating the role of this predominant chemokine in CF cannot be studied in the context of mono- and co-infection in the murine model, which relied on assaying its homologs e.g. MCP-1. However, unlike the *in vitro* co-culture transwell® models, the murine model permits much longer-term infection studies to be conducted over many days to weeks. Furthermore, the murine model has a functioning immune system, exhibits microfluidic flow and permits histological and BALF analysis alongside bacterial enumeration to be obtained from a single experiment. Independent experiments would be required to perform histological analysis in this thesis, due to the bacterial adhesion to the transwell® being an endpoint readout, due to the need to detach and vortex the airway epithelia from the transwell® insert. Optimisation experiments are also required if neutrophils and or macrophages are to be added to the *in vitro* model, in order to assess their viability, their ability to adhere to airway epithelia, as well as undergo chemotaxis in response to an apical bacterial challenge. However, current advantages of the *in vitro* airway co-culture models over murine models are that it is composed of human airway cells, allows comparisons to be made across isogenic cell lines, expresses human and CF relevant cytokines and permits bacteria being added as either planktonic or biofilm cultures to the epithelial surface, rather than through the introduction of agar beads into murine airways via the trachea. As it is also not a whole-body system, the *in vitro* airway models provides the user with greater control over experimental variables, with a reductionist approach aiding in the study of host-pathogen interactions in CF airways.

The incubation periods employed in this study mimicked more of an acute airway infection, rather than chronic infection, which is characteristic of CF. However, such methods sought to understand the early infection events of *P. aeruginosa* colonisation and whether the prior presence of *S. aureus* facilitates enhances its ability to adhere to CF airways. Differences in cell viability across the two models could potentially impact upon the total number of bacteria bound, particularly if the epithelia detach and slough off with longer incubation periods and the bacteria bound to those cells are lost. *S. aureus* and *P. aeruginosa* have both been shown to induce apoptosis in epithelial cells (Losa *et al.*, 2014, Rajan *et al.*, 2000, Kahl *et al.*, 2000). Losses in epithelial cells would subsequently complicate the interpretation of adhesion results.

A number of previous studies have reported losses in epithelial cell viability and integrity following 10 h of infection using primary mouse trachea epithelia (Garcia-Medina *et al.*, 2005), within the first couple of hours in the human CF cell line CFT1 (Lee *et al.*, 1999) and 7 hours following infection of 16 HBE14o- epithelia (Plotkowski *et al.*, 1999). The incubation periods used in these mono- and co-infection (2-4 h) studies also allowed comparisons to be made to the published literature, with it being widely used when studying bacterial adhesion, including in CF (Bucior *et al.*, 2010, Bucior *et al.*, 2012, Worlitzsch *et al.*, 2002, Wang *et al.*, 2017, Letourneau *et al.*, 2011, Van Ewijk *et al.*, 2007, Ahmed *et al.*, 2014).

Studies exploring other host-pathogen interactions however, such as the impact of co-infection upon antibiotic susceptibility would employ much longer incubation periods (24-72 h). This would facilitate the development of long term single and mixed species biofilms. A number of studies have already begun to determine the susceptibility of CF pathogens to antibiotics in the presence of respiratory epithelia (Anderson *et al.*, 2008, Moreau-Marquis *et al.*, 2010, Orazi and O'Toole, 2017, Crabbe *et al.*, 2017).

7.2 Future work

Future work to that conducted in chapter 3 would seek to include other important *P. aeruginosa* phenotypes associated with CF airway infection, such as RSCV's, which are associated with increased biofilm production, an increased resistance to antibiotics and poor pulmonary function (Starkey *et al.*, 2009, Haussler *et al.*, 1999, Schneider *et al.*, 2008). Determining their impact in the context of polymicrobial infections, including their interspecies interactions with *S. aureus* and their impact upon airway inflammation and adhesion also warrants further study. *P. aeruginosa* SCV's have been shown to inhibit *A. fumigatus* biofilm formation (Anand *et al.*, 2018), resist neutrophil phagocytosis and induce inflammation in murine macrophages (Pestrak *et al.*, 2018).

Bacterial phenotyping was the focus of chapter 3, as changes in genotype do not necessarily reflect changes in phenotype (Burns *et al.*, 2001, Workentine *et al.*, 2013). However, future work would seek to use pulse field gel electrophoresis (Parkins *et al.*, 2014) to determine whether the *P. aeruginosa* CF isolates obtained from Birmingham Children's Hospital belong to single or multiple clonal groups. Though individuals with CF are known to harbour their own unique strains of *P. aeruginosa* (Mahenthiralingam et al., 1996), clonal strains of *P. aeruginosa* have been reported in siblings (Grothues *et al.*, 1988, Wolz *et al.*, 1989) and CF centres (Anthony *et al.*, 2002, Armstrong *et al.*, 2002).

LBN broth was widely used to grow both *S. aureus* and *P. aeruginosa*. As a nutrient rich broth, it allows both species to grow to high densities of up to 10^8-10^9 CFU/mL, bacterial densities routinely detected in CF patients (Darch et al., 2017) and allows easier comparisons to be

made to the published literature. Growth under static conditions was shown to also facilitate the biofilm-mediated growth of *P. aeruginosa* as suspended microcolonies (visual clumps), a phenotype seen in the CF lung (Sriramulu *et al.*, 2005). Whilst it is difficult to mimic the variations in nutrient availability, oxygen availability and the pressures exerted by host factors and antimicrobials *in vitro,* artificial CF sputum has been developed, rich in amino acids, mucin and free DNA (Kirchner *et al.*, 2012). Using synthetic sputum would be an additional step closer to mimicking the growth conditions and environment found in the CF lung and has been used previously to determine antibiotic susceptibilities of *P. aeruginosa* (Kirchner et al., 2012), *P. aeruginosa* evolutionary diversification (Davies *et al.*, 2017) and its interspecies interactions with *S. aureus* (Haley et al., 2012).

Additionally, all the co-culture assays conducted in this study employed the use of a laboratory strain of *S. aureus.* Future experiments would seek to employ CF isolates of *S. aureus*, including those co-isolated with *P. aeruginosa* from the same sputum sample*.* As *S. aureus* is known to adapt to the presence of *P. aeruginosa* as seen with the *S. aureus* SCV phenotype (Biswas *et al.*, 2009a, Hoffman *et al.*, 2006)*,* the impact of anoxia upon co-isolates and airway colonisation would provide novel insights into the interactions between the two major CF pathogens overtime.

Additional experiments are required to address the impact of *S. aureus-P. aeruginosa* exoproducts upon the airway inflammatory response. This includes studying the mRNA expression levels of IL-8, IL-6 and IL-10 at baseline in both CF and non-CF epithelia and following mono- and co-stimulation with *S. aureus* and or *P. aeruginosa* exoproducts. This extends to the study of other important inflammatory mediators by airway epithelia, including TNF-α which is upregulated in CF sputum (Karpati *et al.*, 2000, Bonfield *et al.*, 1995b, Venkatakrishnan *et al.*, 2000) and increases neutrophil chemotaxis, adhesion to the endothelium and the induction of IL-8 (Black *et al.*, 1998, Stecenko *et al.*, 2001, Smart and Casale, 1994, Ishii *et al.*, 1992, Sun *et al.*, 2014). The impact of *S. aureus* and *P. aeruginosa* co-infection upon intracellular signalling in IB3-1 and C38 epithelia requires further characterisation, which could be assessed through designing a NF-κB reporter assay (LaFayette *et al.*, 2015), or through the use of inhibitors to target p38 MAPK (Reece *et al.*, 2018).

Further characterisation of the ALI *in vitro* co-culture models of CF and non-CF airways is also required. Paraffin-embedded sections would allow staining to be performed to assess for the expression of important cell surface receptors important in governing inflammation and bacterial adhesion. The levels of TLR4 expression upon IB3-1 CF epithelia compared to C38 non-CF epithelia could be performed, as TLR4 involved in the detection of LPS has previously been shown to be reduced in CFBE41o- CF epithelia (John *et al.*, 2010). The levels of asialo-

GM1 could also be compared across the two cell lines following growth at ALI to determine whether this plays a role in facilitating bacterial adhesion to the CF epithelium (Saiman and Prince, 1993). Comparing the surface expression levels of TLRs and adhesins at ALI to cells grown under submerged conditions would further elucidate the importance of the methodologies and *in vitro* models used in CF research, particularly when studying direct and indirect host-pathogen interactions. Being able to quantitively compare the production of the mucins MUC5AC and MUC5B across the two *in vitro* models is also required, as well determine the impact of mono- and co-infection upon their production. This would aid to decipher the exact locations of bacterial adhesion in CF and non-CF epithelia. This could be further assisted by electron microscopy.

Whilst *P. aeruginosa* in particular is considered primarily an extracellular pathogen, numerous authors have demonstrated its ability to internalise within CF and non-CF respiratory epithelia as a means to persist within the airways (Pielage *et al.*, 2008, Darling *et al.*, 2004, Pier *et al.*, 1997, Bajmoczi *et al.*, 2009). Using a gold standard gentamicin-exclusion assay to kill surface associated bacteria, future work would seek to determine whether prior infection with *S. aureus* influences *P. aeruginosa* internalisation into CF bronchial epithelia.

7.3 Conclusion

Promising and exciting advances are being made in correcting mutated CFTR at both the genetic and protein level. The potentiator drug Kalydeco (Ivacaftor®) produced by Vertex Pharmaceuticals is available on the NHS and benefits 5% of the CF community, targeting those individuals over the age of two who harbour a 'gating' mutation i.e. G551D. Improving chloride conductivity by increasing the time the CFTR channel is open, the drug has been shown to improve pulmonary function (FEV_1) from 55.4% to 64.1% (Cystic Fibrosis Trust, 2016b). However, these treatments strategies currently face a series of challenges, from costs and effective reproducibility, to the fact that they will only benefit a certain number of patients.

In the case of Ivacaftor/Lumicafor dual therapy drug (Orkambi®), it is currently prescribed on compassionate grounds due to its high costs and lack of evidence regarding long term impact (Cystic Fibrosis Trust, 2016a). It is to be given to CF patients over the age of 12, who are homozygous for the Phe508del mutation (Cystic Fibrosis Trust, 2016b). Lumacaftor acts as a chaperone facilitating CFTR folding and transport to the cell surface, whilst Ivacaftor corrects the secondary gating defect.

Two Phase III clinical trials 'EVOLVE' and 'EXPAND' by Vertex Pharmaceuticals were recently published determining the safety and efficacy of Symdeko®, a dual-therapy combining ivacaftor with tezacaftor. Tezacaftor facilitates the processing and trafficking of the CFTR to the cell surface. In the EVOLVE study for individuals homozygous for the Phe508del mutation, a 4%

improvement in FEV_1 was measured compared to placebo, along with a 35% decrease in pulmonary exacerbations compared to placebo (Vertex Pharmaceuticals Incorporated, 2018). In the EXPAND study for individuals heterozygous for the Phe508del mutation, a 6.8% improvement in lung function was measured compared to placebo and a 2.1% improvement compared to ivacaftor alone (Vertex Pharmaceuticals Incorporated, 2018).

Even if Orkambi® and Symdeko® become available on the NHS, its planned use in those over the age of 12 means that there is still a decade in which the lungs will become chronically colonised by CF pathogens. Furthermore, whilst Kayldeco® improves pulmonary function, *P. aeruginosa* are infection persists (Hisert *et al.*, 2017). Thus, for the foreseeable future at least, the discipline of microbiology will continue to play an active and essential role within CF research. It is hoped that the results presented and the *in vitro* models used act as a platform for future studies addressing the impact of polymicrobial CF airway infection upon bacterial community dynamics and host-pathogen interactions. In turn this may aid in the identification of new treatment strategies or therapeutics to delay or clear these devastating life limiting infections.

7.4 References

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