DOES ALTERED AIRWAY MUCUS COMPOSITION PROMOTE CHRONIC RESPIRATORY INFECTIONS IN CYSTIC FIBROSIS BY PROVIDING A NUTRIENT SOURCE FOR PATHOGENIC BACTERIA?

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Siân Elizabeth Baldock BSc

(King's College London)

Department of Infection, Immunity, and Inflammation

University of Leicester

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Abstract:

Pseudomonas aeruginosa is an important pathogen in cystic fibrosis (CF) chronically colonising most adult CF patients (McCallum et al, 2001). However, mechanisms by which *P. aeruginosa* thrives in the CF airway remain unclear because its nutritional requirements are not fully understood. Because mucus is in abundance, a potential nutrient source may come from mucin glycoproteins. Therefore, the purpose of this study was to investigate whether *P. aeruginosa* could utilise airway mucin as a nutrient source for growth.

P. aeruginosa was grown in mucins purified from CF sputum. It was found that *P. aeruginosa* was unable to utilise mucin, but following DNase treatment growth of *P. aeruginosa* was significantly promoted. Analysis of gene expression following exposure to DNase treated CF sputum revealed that *codA* which encodes cytosine deaminase and catalyses the conversion of cytosine to uracil and 5-methylcytosine to thymine was significantly upregulated. The gene, *gapA,* involved in carbon metabolism was also upregulated.

Previous studies have demonstrated that uracil promotes biofilm formation and that uracil and thymine can be metabolised by *P. aeruginosa* via the reductive pathway of pyrimidine catabolism (Ueda et al, 2009; Kim and West, 1991). Additionally, DNA is in abundance in the CF airway (Lethem et al, 1990). Therefore, it is likely that following dornase alfa treatment, when degraded DNA is liberated it may alter the CF airway mucus composition so that *P. aeruginosa* growth is promoted. Degraded DNA may have an important role in the pathogenicity of *P. aeruginosa* and CF disease. This PhD thesis demonstrates novel findings that *P. aeruginosa* is unable to utilise purified CF patient mucin as a nutrient source for growth, but degraded DNA originating from CF patient sputum can be utilised by *P. aeruginosa* to produce uracil or thymine which are known to be catabolised via the reductive pathway of pyrimidine catabolism.

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As they say, "Everything happens for a reason."

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List of Abbreviations

% - Percent

^{o}C – Degrees Centigrade

Δ - Delta

μ - Specific growth rate

μg – Microgram

μl – Microlitre

μm – Micrometre

μM – Micromolar

AprA – Alkaline protease released by T1SS

BAB – Blood Agar Base

BAL – Bronchoalveolar lavage

BgA – B-galactosidase A

BHI – Brain Heart Infusion

bp – Base pairs

BRU – Biomedical Research Unit (now the BRC)

BSA – Bovine serum albumin

CCC – Carbon catabolite control

cDNA – Complementary DNA

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator protein

CFU – Colony Forming Unit

CP – Crossover point

CT – Cycle threshold

ddH_2O – Double distilled water

DEPC – Diethyl pyrocarbonate

dH_2O – Distilled water

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleotide triphosphate

DOB – Date of birth

DTT- Dithiothreitol

ED – Entner Doudoroff (pathway)

EddB – Extracellular DNA degradation protein

EDTA – Ethylenediaminetetraacetic acid

EMP – Embden-Meyerhoff-Parnas (pathway)

ENaCs – Epithelial sodium channels

EPA – Environmental protection agency

FDA - Food and Drug Administration

FEV_1 – Forced expiratory volume in 1 second

FU – Fluorescent signal intensity

g – Grams

gDNA – Genomic DNA

GuHCl – Guanidine hydrochloride

(x) g – G-force (Relative Centrifugal Force)

HasAP – Haemophore protein

HKG – Housekeeping gene

ID – Identification

IL – Interleukin

Inc. – Incorporated

IV – Intravenous

K – Kilodalton

Kb – Kilo base

Kbp – Kilo base pair

kDa – Kilodalton

KEGG – Kyoto Encyclopaedia of Genes and Genomes

kPa – Kilopascal

L – Litre

LasB – Elastase in T2SS

LPS – Lipopolysaccharide

LRI – Leicester Royal Infirmary

Ltd – Limited

M – Molar

MDa – Mega Dalton

mg – Milligram

min - Minute

ml – Millilitre

mmol – Millimole

mM – Millimolar

MRC – Medical Research Council

mRNA – Messenger RNA

MUCs – Mucin(s)

MW – Molecular weight

MWCO – Molecular weight cut-off

N – Moles

NanA – Neuraminidase A

NET – Neutrophil extracellular traps
ng – Nanogram
nH_2O – Nanopure water
NHS – National health service
nm – Nanometre
nt – Nucleotide
NTC – Non-template control
OD – Optical density
PAS – Periodic acid - Schiff
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
p - Pico
PGM – Porcine gastric mucin
P. aeruginosa – Pseudomonas aeruginosa
psi – Pounds per square inch
qPCR – Quantitative/real-time PCR
QS – Quorum sensing
RIN – RNA integrity number
RNA – Ribonucleic acid
rpm – Revolutions per minute
SDS – Sodium dodecyl sulfate
SEM – Standard error of the mean
sLe^x – Sialyl-lewis x
S. pneumoniae – Streptococcus pneumoniae
SSC – Saline sodium citrate
StrH – N-acetylhexosaminidase
t – Time
T1SS – Type 1 secretion system
T2SS – Type 2 secretion system
T3SS – Type 3 secretion system
T5a/bSS – Type 5 secretion system a/b
T6SS – Type 6 secretion system
TAE – Tris-acetate-EDTA
TBST – Tris-buffered saline (Tween-20)
TCA – Tricarboxylic acid (cycle)
TR(s) – Tandem repeat(s)
U – Units of enzyme
UK – United Kingdom

V – Volts

vs. – Versus

v/v – Volume/volume

w/v – Weight/volume

X - Concentrated

Chapter 1. Introduction

1.1. Overview

Cystic fibrosis (CF) is the most common autosomal recessive disease in the white population. It has a frequency incidence of 1 in 2500 live births, a carrier frequency of 1:25, and globally affects around 70000 individuals with approximately 10000 residing in the UK (McCormick et al, 2002; Cutting, 2005). Being a genetic disease, it affects multiple organ systems, with affected individuals having defects in the 'CF gene'. This 'CF gene' encodes the ion channel known as the cystic fibrosis transmembrane conductance regulator (CFTR) and functions to regulate normal ion transport across the epithelium (Kerem et al, 1989). Pathology is therefore observed in many organs in the body including the pancreas, liver, and gastro-intestinal tract. However, morbidity and mortality is mainly attributed to the decline in lung function resulting from chronic infection in the respiratory tract (Johansen and Hoiby, 1992; Kreda et al, 2005).

According to the Cystic Fibrosis Trust's UK CF Registry Annual Data Report 2015 (2016), 77.3% of all paediatric patients under the age of 16 were diagnosed before three months of age with the median age of diagnosis at two months. Notably, 92.7% of patients under five years of age were diagnosed between birth and three months. This therefore highlights the improved efficiency of diagnosis due to screening and improved awareness. Because CF affects multiple organs, pathology is often observed in many organs. Pathology in the airway is initially observed due to the occurrence of a chronic productive cough. Sputum analysis can confirm airway colonisation with respiratory pathogens together with chest radiographs which may reveal airway obstruction.

Because CF is caused by CFTR dysfunction, this results in abnormal ion transport across the epithelium which means individuals can be diagnosed via a sweat test (Gibson and Cooke, 1959). Diagnosis can be confirmed when the concentration of chloride ions measured are greater than 60 mmol/L on repeated analysis. This is in addition to genotyping which confirms CFTR mutations (Farrell et al, 2008; Castellani et al, 2009).

1.2. The cystic fibrosis transmembrane conductance regulator (CFTR) protein in cystic fibrosis

1.2.1. Genetics of cystic fibrosis

The 'CF gene' was first discovered in 1989 and encodes the CFTR protein located on the apical surface of epithelial cells. For an individual to have CF, both parents of the individual must possess a single mutation of the 230 kb 'CF gene' located on the long (q) arm of chromosome seven at position 31.2 (Rommens et al, 1989; Riordan et al, 1989; Kerem et al, 1989; Kreda et al, 2005). Single mutations of this CF gene are asymptomatic in the unaffected carrier parents, but for CF to clinically manifest faulty copies of the gene must be co-inherited for the dysfunctional CFTR protein to be expressed and thus elicit CF symptoms. Although many other factors such as modifier genes, environment, treatment, and patient compliance can influence disease severity, it is the genotype which determines the overall severity of the symptoms (Collaco and Cutting, 2008).

CFTR protein function is therefore altered in individuals with two defective CF genes. Mutations can result in codon frame-shift via substitution, addition, or deletion. (Riordan et al, 1989; Kerem et al, 1989). Different mutations, of which over 1500 have been identified, can influence the functioning of CFTR to different extents by affecting CFTR activity to varying degrees. Thus, the type of mutation is a determinant of whether there is clinical manifestation. Mutations have therefore been categorised into six classes based on how they affect CFTR function as illustrated in figure 1.1. However, it must be noted that the most clinically severe symptoms, such as those observed in the airways, are due to class 1 or 2 mutations which cause diminished or abolished CFTR function (Rowe et al, 2005). For example, the deletion of phenylalanine at position 508 is the most common mutation and results in defective protein folding and trafficking of the CFTR channel to the apical surface of the epithelial cell surface membrane (Kerem et al, 1989). Of the 98.1% of patients in the UK that have been genotyped, 90.49% have this specific class 2 mutation (UK CF Registry Annual Data Report 2015, 2016).

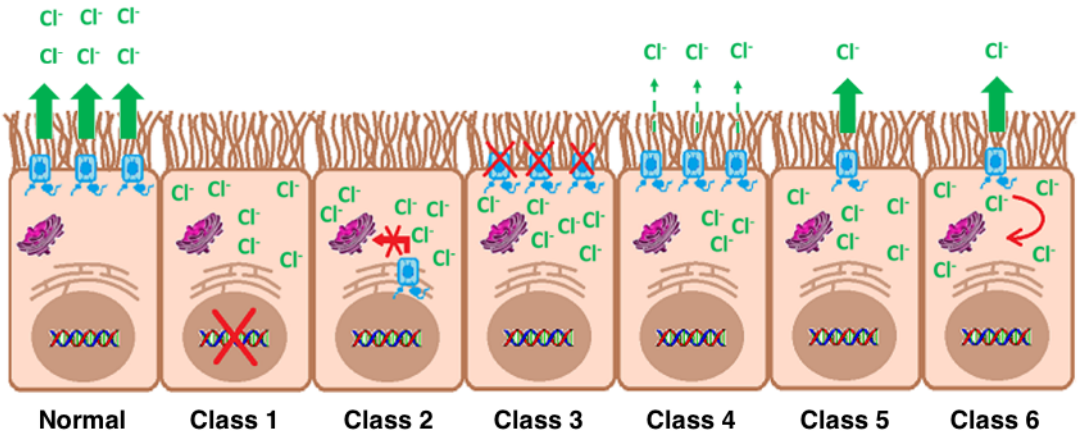

Figure 1.1

Figure 1.1: Mutation classes of CFTR. Schematic representation of the six types of mutation classes of CFTR compared to the normal CFTR protein which allows conductance of chloride ions across the apical surface membrane and prevents overactivation of epithelium sodium channels. CFTR dyfunction classes: class 1 = no synthesis of CFTR resulting in no functional CFTR protein at apical surface, class 2 = defect in CFTR trafficking leads to lack of protein maturation and premature protein degradation, class 3 = defect in channel regulation meaning the channels do not open resulting in no conductance of chloride ions, class 4 = defective channel gating preventing chloride conductance results in reduced conductance of anions, class 5 = abnormal splicing results in a reduced level of CFTR mRNA transcripts and therefore less CFTR channels on the surface membrane, class 6 = reduced CFTR stability causing accelerated turnover of CFTR at the apical surface of the epithelium.

1.2.2. Normal CFTR structure and function in the airway

The CFTR protein is a cAMP-regulated chloride and bicarbonate channel which functions to regulate the movement of chloride, bicarbonate, and water across the apical membrane of ciliated epithelial cells (Rowe et al, 2005). Structurally, it is a 1480 amino acid polypeptide within the ATP-binding cassette (ABC) family of proteins and is composed of two domains (I and II) formed by six alpha helices in each domain which span the apical epithelial membrane to form an inner channel. This central channel possesses specificity for the anions: chloride and bicarbonate. It regulates normal anion and water transport across the epithelium via its two nucleotide binding domains, NBD1 and NBD2, which facilitate channel conductance due to their ability to mediate ATP hydrolysis according to 'the ATP model.' Thus, ATP provides the energy necessary for

opening and closure of the channel, allowing for the passage of anions (Higgins and Linton, 2004; Borst and Elferink, 2002).

This CFTR channel not only conducts anions via ATP channel regulation, but it also inhibits calcium-activated chloride channels and is involved in the transport of intracellular vesicles. Since CFTR is commonly located in cells with a secretory function, such as the sweat glands, the sweat test is therefore a useful diagnostic test for CF in the clinic (Gibson and Cooke, 1959). Furthermore, CFTR activity acts to inhibit overactivation of epithelial sodium channels (ENaC) (Stutts et al, 1997; Rowe et al, 2005). In the respiratory tract CFTR is closely associated with these ENaCs which are crucial for determining the volume of the airway surface liquid (ASL). The ASL normally provides a protective layer surrounding cilia, allowing cilia to freely move (Gaillard et al, 2010). It also prevents contact with potentially harmful stimuli. Due to the rhythmic and constant beating of the cilia via the mucociliary escalator, in addition to the pressure exerted by cough, mucus, which traps foreign particles, oxidative pollutants, and pathogens inhaled from the extracorporeal environment, is forced up the respiratory tract and expelled (Sleigh et al, 1988). Therefore, in a healthy airway infection is mostly avoided.

1.2.3. CFTR dysfunction in cystic fibrosis and effects on the airway

The diminished CFTR activity in the CF airway means that ENaCs are no longer inhibited. Thus, overactivation of the ENaCs leads to hyperabsorption of sodium ions and water absorption from the airway lumen. Consequently, the ASL becomes dehydrated. As explained by the low volume hypothesis, dehydration results in increased mucus viscosity and stasis which makes it difficult for the mucociliary escalator to effectively clear the mucus (Reisin et al, 1994; Com and Clancy, 2009). Additionally, this dysfunction results in the damage of the cilia lining the airways which further promotes poor mucociliary clearance due to the inability to expel mucus via cough (Matsui et al, 2005). Invading pathogens therefore remain trapped in the mucus which provides favourable microaerophilic and anaerobic growth conditions for numerous respiratory pathogens (Knowles and Boucher 2002; Wickstrom et al, 1998). This helps to establish persistent infection by initiating inflammatory responses which

gives rise to increased mucus production and airway obstruction. (Matsui et al, 2005; Laube et al, 2014).

To help prevent respiratory infection and decline in lung function, it is necessary for CF patients to receive intensive physiotherapy to help expel the trapped mucus before infection can be initiated. Additionally, the use of antibiotics has been established for symptomatic treatment of respiratory infections (Lyczak et al, 2002). Treatment and prevention of chronic infection is therefore a goal in CF research.

1.3. Respiratory associated pathogens in cystic fibrosis

1.3.1. Challenges in treating respiratory infections

Due to impaired mucociliary clearance, many pathogens found in the respiratory tract are implicated in causing chronic respiratory infections in CF patients. Although viral pathogens have a role in CF disease and their contribution to respiratory infections should not be ignored, it is the bacterial infections with organisms such as *Pseudomonas aeruginosa* which are responsible for accelerated lung function decline (Johansen and Hoiby, 1992).

Eradicating respiratory infection using antibiotics is difficult due to the vast cocktail of bacteria predominating the CF lung environment. Additionally, the thick mucus secretions which limit bacterial clearance also block the access of inhaled antibiotics. Eventually when the innate host response becomes overwhelmed, this leads to chronic respiratory infection despite established and continued treatment with antibiotics (Lyczak et al, 2002). Evidence of infection despite the use of antibiotics is supported by studies where the levels of T cells, macrophages, and neutrophils were found to be elevated in the alveoli of explanted lungs (Ulrich et al, 2010). Increased immune cells were likewise detected in the lungs from CF patients in comparison to the lungs of healthy individuals (Bjarnsholt et al, 2009).

Therefore, it has become evident that preventing respiratory decline in patients is difficult due to the complexity of the disease. One of the reasons is because the CF microbiome has become well adapted to changing its phenotype and genotype in response to the surrounding lung milieu (Lopes et al, 2014). This enables the formation

of persistent multicellular communities of microbial pathogens which secrete exopolysaccharides known as extracellular polymeric substances into their environment. Consequently, this produces a structural network associated with abundant quantities of extracellular DNA where concentrations can be as high as 20 mg/ml in sputum (Brandt et al, 1995). Notably, extracellular DNA has been shown to originate mostly from the host (Lethem et al, 1990). DNA is released by dying cells and neutrophil extracellular traps (NETs). This innate immune response by neutrophils in response to association with nearby pathogens results in the destruction of virulence factors and helps prevent spread of further infection. However, this is not always effective due to the large quantities of DNA produced. (Brinkmann et al, 2004; Dubois et al, 2012).

In CF, the DNA produced acts a structural adhesive by enabling the cross-linking with the sputum. This in turn promotes bacterial settlement in the form of what is commonly referred to as biofilms (Swords, 2012; Costerton et al, 1987; Whitchurch et al, 2002). The high concentration of DNA in these biofilms is one of the reasons as to why DNase therapy has been developed. Firstly, to break down the structural network in which bacteria such as *P. aeruginosa* thrive, and secondly, to make the mucus less viscous since the amount of DNA correlates with sputum viscosity (Shak et al, 1990). Because secretions become less viscous, the biofilm structure is more permeable to targeted antibiotic therapy (Ranasinha et al, 1993; Ulmer et al, 1996; Tetz et al, 2009). Although DNase treatment has proven to improve lung function, there is still a problem with treating chronic infections (Zahm et al, 1995).

For antibiotic therapy to be successful, it is therefore necessary to understand how the microbiome changes through disease progression. Data collected from sputum cultures have identified that the most prevalent organisms responsible for respiratory infections in early childhood are *Staphylococcus aureus* and *Haemophilus influenzae*. Figure 1.2 produced by the CF Trust for the 'UK Cystic Fibrosis Registry' (2015) demonstrates the proportion of people colonised with certain bacteria in different age groups and alludes to a change in the microbial environment at different stages of life. What can be determined from this figure is that in later life *P. aeruginosa* replaces other organisms as the most prevalent organism cultured in CF sputum. Understanding why there is a

switch of dominant pathogens in early adulthood and what enables *Pseudomonas* survival is necessary for the development of future therapies.

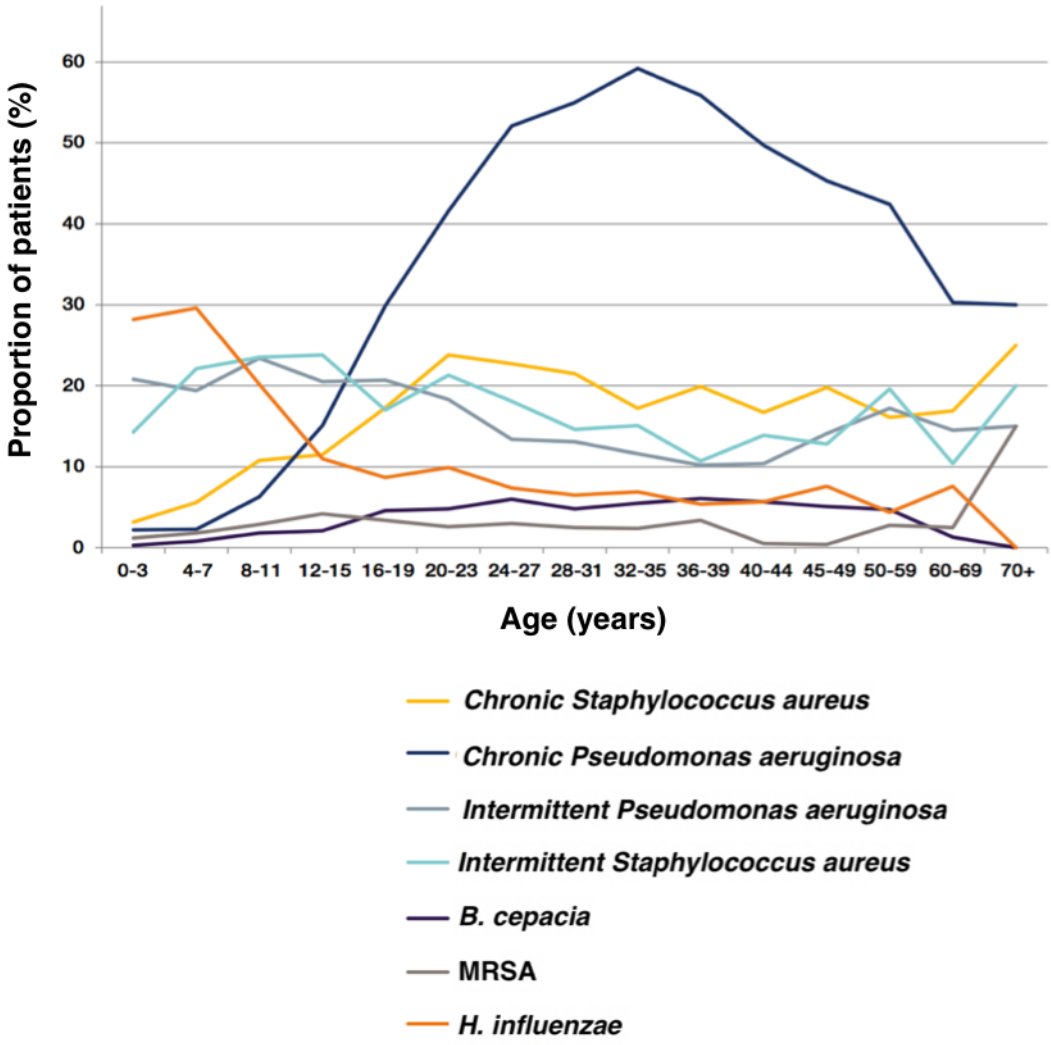

Figure 1.2.

Figure 1.2: Graph illustrating pathogens isolated from respiratory samples at different ages in CF patients (reproduced from: UK CF Registry Annual Data Report 2015, 2016).

1.3.2. *Streptococcus pneumoniae*

One pathogen which may have an underlying involvement in CF is *Streptococcus pneumoniae. S. pneumoniae* is a gram-positive, facultative anaerobe which possesses numerous virulence factors involved in establishing disease (Bergmann and Hammerschmidt, 2006). Its role in infection was first described in 1881 by two independent scientists: Louis Pasteur and George M Sternberg. Both scientists observed pneumococcus in the blood of rabbits following injections of human saliva (Pasteur, 1881; Sternberg, 1881). Because the rabbits died shortly after infection, pneumococcus was attributed to causing disease. However, it wasn't until a few years later that this bacterium was named as 'pneumococcus' due to its association with respiratory disease (Fraenkel, 1886). This soon evolved to it being named *Diplococcus pneumoniae* due to its morphology of cocci pairs in pneumonia. However, it was eventually renamed as *Streptococcus pneumoniae*, nearly one hundred years after its pathogenic ability was first demonstrated (Winslow et al, 1920; Deibel et al, 1974).

Since its discovery, it has been found that *S. pneumoniae* commensally resides asymptomatically in the nasopharynx of healthy individuals (Kadioglu et al, 2008). However, under conditions where the immune system is compromised, such as in young children or the elderly, it can become pathogenic by proliferating in the nasopharynx and eventually spread to the lower airways (Rayner et al, 1995). It is now established that *S. pneumoniae* is responsible for causing numerous diseases including meningitis, pneumonia, bacteraemia, and otitis media (Kadioglu et al, 2002; Hava et al, 2003).

S. pneumoniae is known to establish disease due to the possession of multiple virulence factors. Initially, it can evade clearance via phagocytosis by the innate immune system due to the presence of a capsule with antigens present on the polysaccharide surface (Kim et al, 1999; Tuomanen and Masure, 2000). Based on variations in these surface antigens, capsulated *S. pneumoniae* can therefore be grouped by 'serotyping' which can be determined by the Quellung reaction. It was so named because swelling, known as 'Quellung' in German, was observed following binding of type specific antibody to the polysaccharide capsule (Austrian, 1976). Over 95 distinct serotypes have since been described (Bentley et al, 2006; Calix et al, 2012; Geno et al, 2015). Because the capsule

in *S. pneumoniae* contributes to its pathogenic potential, strains which do not have a capsule are typically avirulent. However, some strains may appear non-encapsulated, but in fact possess a capsule which is non-typeable by the current methods (Marsh et al, 2010). Other important virulence factors produced by *S. pneumoniae* include pneumolysin, enolase, autolysin A, and choline binding proteins, in addition to others described in a review by Kadioglu and colleagues (2008) which are involved in colonisation.

Notably *S. pneumoniae* it is not a prevalent pathogen in CF and therefore not presented in figure 1.2. One of the reasons for this is because it is widely reported to be difficult to culture from CF sputum samples. The reason why it is not so easily detected has been argued to be because *S. pneumoniae* becomes easily overwhelmed by other CF pathogens (del Campo et al, 2005; Stelzer-Braid et al, 2012). However, it is difficult to determine whether detection levels within sputum cultures are lower than other organisms because it is rarely present with no clinical implications, or because clinical microbiologists are not searching for it during culture because it is not regarded as a pathogen of interest in the context of more prevalent CF pathogens such as *P. aeruginosa* and *S. aureus*. It is, however, one such pathogen which may be more significant during the early stages of CF disease. It could therefore be involved in influencing and maintaining a stable microbial ecology by colonising deep within the lung and allowing for the interaction of other CF pathogens in addition to contributing to CF pathology itself (Mitchell, 2003; Maeda et al, 2011). Thus, its role within the CF lung remains to be more clearly elucidated.

1.3.2.1. *S. pneumoniae* metabolism

Despite low detection rates, it could be that *S. pneumoniae* is involved in promoting and supporting growth of other pathogens earlier on in CF disease. As suggested by King (2010), this could be via utilisation of host sugars liberated as a source of carbon for growth. In support of this, pneumococci possess many enzymes and transporter systems involved in the uptake of carbohydrates and amino acids, of which 30% are involved in the uptake of approximately twelve different carbohydrates (Hoskins et al, 2001; Bidossi et al, 2012; Tettelin et al, 2001). Being a facultative anaerobe, *S. pneumoniae*

can catabolise carbohydrates such as glucose to produce ATP in the presence of oxygen, or when oxygen is limited in microaerophilic conditions, as is the case in CF due to mucus stasis (Konings and Otto, 1983; Poolman, 1993; Worlitzsch et al, 2002). This is because the Embden-Meyerhoff pathway (EMP), otherwise known as glycolysis, which yields pyruvate from sugar does not require oxygen. Even if oxygen is present, *S. pneumoniae* maintains a strict state of fermentation. Analysis of its genome has revealed that this is due to the lack of enzymes necessary for aerobic respiration. Therefore, pneumococci do not use the electron transport chain as an electrochemical gradient to produce ATP (Hoskins et al, 2001; Tettelin et al, 2001). NADH is therefore oxidised back to NAD^+ during fermentation and is regenerated for use in essential pathways such as glycolysis. As shown in figure 1.3, lactate, ethanol, acetate, or formate are produced through the reduction of oxidised compounds (Neves et al, 2002; Yesilkaya et al, 2009).

When acetyl-CoA is produced during fermentation, this is via the activation of pyruvate formase lyase or pyruvate dehydrogenase. The product of this reaction, acetyl-CoA, does not enter the TCA cycle as would normally be the case in aerobic respiration. Alternatively, the pyruvate produced by glycolysis instead undergoes homolactic fermentation to yield lactic acid under conditions when oxygen is lacking, or undergoes mixed acid fermentation to produce ethanol, acetate, and formate as shown in figure 1.3 (Neijssel et al, 1997). The latter occurs under aerobic conditions, reduced sugar availability, or the presence of sugars such as galactose which are less preferred than glucose (Melchiorsen et al, 2000; Neijssel et al, 1997; Neves et al, 2002).

Therefore, because *S. pneumoniae* can readily utilise sugars as a source of carbon, it could be that pneumococcus has a role in CF by utilising sugars within the stagnant airway mucus. This is supported by previous studies conducted by Yesilkaya and colleagues (2008) which revealed that *S. pneumoniae* D39 can utilise porcine gastric mucin (PGM) as a nutrient source for growth. More recently it was revealed that sugars within mucin, such as galactose, mannose, and N-acetylglucosamine, are involved in *S. pneumoniae* colonisation by causing a switch from homolactic to mixed acid fermentation (Paixão et al, 2015). This successfully illustrates how pneumococci could survive in the CF lung by using respiratory mucins which are rich in carbohydrate, within mucus as a source of carbon.

If pneumococcus does indeed utilise airway mucins within the CF airway, it is important to consider that it may not be for its own use. Instead, it could be that airway pathogens degrade mucins into breakdown products which are utilised by other pathogens. This has been demonstrated in a recent study where it was shown that anaerobic bacteria originating from the oral cavity were able to catabolise mucins. The breakdown products which were identified as acetate and propionate were then demonstrated to be nutrients for *P. aeruginosa* (Flynn et al, 2016). It would therefore be interesting to reveal whether *S. pneumoniae* can survive in airway sputum. For this reason, *S. pneumoniae* will be the initial focus of this project, but because *P. aeruginosa* is more prevalent, this will be the pathogen of focus in this study.

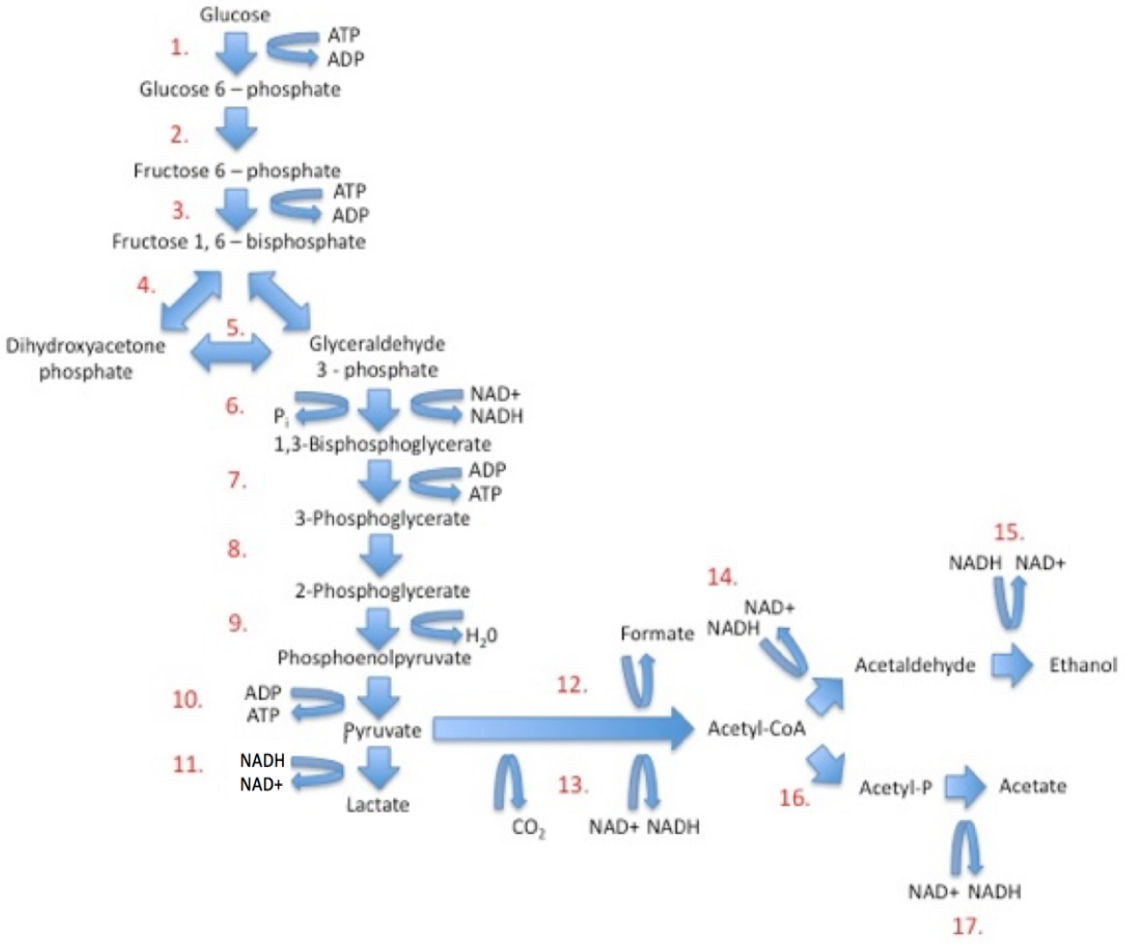

Figure 1.3

Figure 1.3: Embden-Meyerhoff-Parnas (EMP) pathway of carbohydrate metabolism in lactic acid bacteria. Typically, NAD^+ is generated via the conversion of pyruvate to lactate. However, when in the presence of galactose or if there is a limited supply of sugar for bacteria to utilise, then NAD^+ is generated alternatively via the conversion of pyruvate to ethanol and acetate with formate released as a bi-product. This is mediated by the activation of PFL (12), stimulated by microaerophilic and anaerobic conditions. 1 = hexokinase, 2 = phosphoglucoisomerase, 3 = phosphofructokinase, 4 = aldolase, 5 = triosephosphate isomerase, 6 = glyceraldehyde-3-phosphate dehydrogenase, 7 = phosphoglycerate kinase, 8 = phosphoglyceromutase, 9 = enolase, 10 = pyruvate kinase, 11 = lactate dehydrogenase, 12 = pyruvate formate lyase, 13 = pyruvate dehydrogenase, 14 = ADHE, 15 = alcohol dehydrogenase, 16 = phosphotransacetylase, 17 = acetate kinase. Pi = phosphate. (Adapted from Yesilkaya et al, 2009).

1.3.3. *Pseudomonas aeruginosa*

As previously mentioned, another major pathogen which is prevalent in CF is *Pseudomonas aeruginosa*. *P. aeruginosa* is a gram-negative, aerobic bacterium

normally found in a variety of diverse environments; commonly found in damp environmental locations, such as soil, rivers, and oceans (Worlitzsch et al, 2002). It was first described by Carl Gessard in 1882 who noted that upon exposure to UV light, wound dressings appeared blue-green. It was therefore named *Bacillus pyocyaneus* due to the colour of the pigment produced (Gessard, 1882). This pigment is now known to be due to the production of pyocyanin - one of the many virulence factors *Pseudomonas* possesses (El-Fouly et al, 2015). Notably, *P. aeruginosa* can survive in nutrient poor environments and is therefore extremely metabolically versatile as it can utilise a wide range of nutrients due to the expression of many genes which enable the uptake and metabolism of various substrates (Stanier et al, 1966). Therefore, it is even found in areas where it would not be expected. For example, it is frequently found in hospitals despite the use of disinfectants (Rutala, 1997; Higgins et al, 2001). Therefore, many individuals who get colonised with *Pseudomonas* are already hospitalised or are immunosuppressed, such as those with cancer, or acquired immune deficiency syndrome (AIDS) (Fazeli et al, 2012; Steinstraesser et al, 2005; Rolston and Bodey, 1992; Shepp et al, 1994; Quinn, 2003).

Often healthy individuals may encounter this organism but in the airway it is normally efficiently removed via mucociliary clearance together with the innate host response. However, in CF it becomes an opportunistic pathogen, as is the case with other patients with compromised immune systems, injuries, or lesions (Bodey et al, 1983). Its impressive ability to adapt its genotype and phenotype, explained by its large genome (6.3 kb), ensures that once acquired it results in chronic colonisation (Stover et al, 2000). Interestingly, it has been noted that the early clinical CF isolates which are acquired share characteristics with isolates originating from the environment alluding to mutation of strains within the airway (Gibson et al, 2003). Due to these mutations, patients chronically infected with *P. aeruginosa* eventually possess isolates which differ considerably from environmental strains, isolates found earlier on in CF infections, and isolates in non-CF patients with acute infections (Bragonzi et al, 2009).

Johansen and Hoiby (1992) noted that initial infection of the airways with *P. aeruginosa* is seasonal and correlates with the increased incidence of respiratory viral infections. This has been demonstrated in animal models in which mice were infected with

respiratory syncytial virus (de Vrankrijker et al, 2009). Despite the involvement of other CF pathogens such as *H. influenza* and *S. aureus* earlier on in disease, it is *P. aeruginosa* that is widely recognised as being the main cause of morbidity and mortality in CF, where infection leads to prolonged inflammation and severe respiratory lung function decline (Hart and Winstanley, 2002). This consequently causes a diminished quality of life resulting in repeated hospitalisation (Lyczak et al, 2002).

Currently when *P. aeruginosa* is first acquired it is treated with nebulised colomycin in combination with oral ciprofloxacin whilst tobramycin and ceftazidime are administered intravenously (Sagel et al, 2009; Chmiel et al, 2014). This aggressive combinational approach is taken with the aim to prolong the time until chronic infection. This is because a strong correlation has been made between the age of onset of chronic infection and patient life expectancy (Al-Aloul et al, 2004; Hart and Winstanley, 2002; Robinson, 2001). Notably, it is the switch from planktonic growth to biofilm formation that makes *P. aeruginosa* difficult to eradicate despite combinational antibiotic treatment (Drenkard, 2003; Hassett et al, 2010; Lopes et al, 2014). Once chronic infection takes hold it is virtually impossible to eradicate due to the formation of persistent biofilms. Treatment then focuses on suppressing the infection and treating the necessary symptoms as they arise. Unfortunately, repeated antibiotic therapy often results in antibiotic resistance and survival in the host (Drenkard, 2003). Thus, the fact that *P. aeruginosa* prevalence remains so high, as it is detected in approximately 80% of adult CF patients, supports more recent studies demonstrating that current antibiotic treatment is ineffective (Lopes et al, 2014; McCallum et al, 2001; Ciofu et al, 2013). What remains unclear is the exact metabolic requirements of *P. aeruginosa* during infection in the airway and how specifically it thrives. One way in which it thrives and overcomes the assault from antibiotics and lung defence mechanisms is by the production of virulence factors (Langton Hewer and Smyth, 2017).

1.3.3.1. *Pseudomonas* metabolism

P. aeruginosa in contrast to *S. pneumoniae* does not metabolise monosaccharides via the EMP pathway (glycolysis). This is because *P. aeruginosa* lacks the phosphofructokinase enzyme which is necessary for metabolism of sugars in the upper

EMP pathway and other enzymes required for fermentation (figure 1.3, step 3). Therefore, metabolism is not fermentative like pneumococci. Mostly it undergoes aerobic respiration, but when oxygen is absent it can use nitrogen. This demonstrates how *P. aeruginosa* is extremely metabolically versatile by being able to adapt to the different environments in which it resides (Kiewitz and Tummler, 2000).

If sugar is available, as illustrated in figure 1.4, *P. aeruginosa* will instead metabolise sugars via the Entner-Doudoroff (ED) pathway, using alternative enzymes which are not present in the EMP pathway. These enzymes are 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Entner and Doudoroff, 1952). The enzyme 6-phosphogluconate dehydratase catalyses the dehydration of 6-phosphogluconate to form 2-keto-3-deoxy-6-phosphogluconate (KDPG) which is then cleaved by KDPG aldolase to produce pyruvate and glyceraldehyde 3-phosphate. In this pathway glyceraldehyde-3-phosphate is often recycled via the central ED pathway cycle to produce more pyruvate (Phibbs, 1988; Banerjee, 1989). Therefore, pyruvate is still formed but by two different enzymes. Once pyruvate is formed it undergoes oxidative decarboxylation to produce carbon dioxide and acetyl-coA, of which the latter enters the TCA cycle to yield NADH. NADH is then oxidized back to NAD^+ in the electron transport chain so that it can be regenerated once more for central metabolism processes (Jurtshuk, 1996).

In the electron transport chain, a series of redox reactions occur to transfer electrons from electron donors to electron acceptors. These reactions are coupled with the transfer of protons across the membrane to establish an electrochemical gradient. This subsequently drives the formation of ATP (Jurtshuk, 1996). In aerobic respiration, oxygen acts as the terminal electron acceptor in this electron transport chain. However, in the absence of oxygen, it switches to anaerobic respiration, as is the case in CF caused by the stagnant mucus (Worlitzsch et al, 2002). When oxygen is absent, if nitrate, nitrite, or nitric oxide are present, *Pseudomonas* can instead undergo denitrification where nitrate is used as a terminal electron acceptor instead of oxygen (Davies et al, 1989). This, in part explains why pseudomonads are often found in soil and poorly ventilated areas, where oxygen is limited and there is more nitrogen (Worlitzsch et al, 2002).

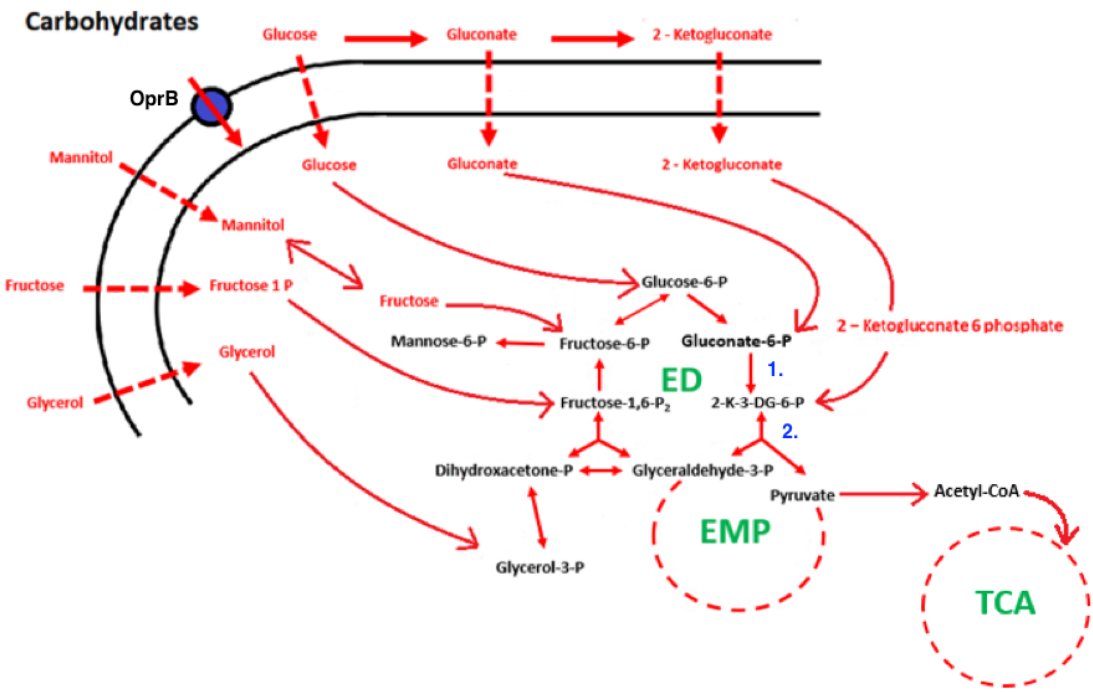

Figure 1.4.

Figure 1.4: Carbohydrate metabolism in *Pseudomonas*. Diagram illustrates uptake of sugars into the bacterial cell via an OprB pore. Sugars can be utilised as a source of carbon for survival by *Pseudomonas aeruginosa* by being fed into the central ED pathway at different points in the pathway. The ED pathway produces 2-keto-3-deoxy-6-phosphogluconate (2-K-3-DG-6-P) due to the 6-phosphogluconate dehydratase (1.) which catalyses the dehydration of 6-phosphogluconate (gluconate-6-P) and eventually pyruvate and acetyl-CoA due to KDPG aldolase (2.). Glyceraldehyde 3-phosphate which is part of both the central Entner-Doudoroff (ED) pathway and lower EMP cycle is recycled in the ED pathway to produce pyruvate (Adapted from Browne et al, 2010).

1.4. Respiratory infection and establishment

1.4.1. Nutrients in the airway

Although it is known that *P. aeruginosa* is extremely metabolically versatile, being able to survive in nutrient poor and hostile environments, currently there is little data explaining how *P. aeruginosa* specifically survives in the airway as its metabolic requirements, especially during infection are extremely complex. Therefore, nutritional preferences remain largely unknown (Kiewitz and Tummler, 2000). Currently it is known that sugars are less preferred substrates for *Pseudomonas* which favours other

nutrients such as organic acids and amino acids (Palmer et al, 2007; Siegel et al, 1977; Wolff et al, 1991). Previously, studies have noted there is a low level of free carbohydrate available in the respiratory tract for bacteria to utilise (Philips et al, 2003). Though recently it has been demonstrated that glucose is elevated in the CF airway in contrast to healthy individuals. In this study, it was argued that elevated paracellular glucose concentration is the main factor driving respiratory growth in cystic fibrosis (Garnett et al, 2013). Figure 1.4 demonstrates how carbohydrates such as glucose and fructose are taken up from the extracellular environment via the OprB porin in the outer membrane of *Pseudomonas* for central carbon metabolism (Wylie and Worobec, 1995).

Although sugars are efficiently metabolised via the ED pathway, if a preferred nutrient of *Pseudomonas* is presented in a mixture along with carbon sources not normally favoured, catabolite repression control regulates the carbon metabolism in an ordered manner so that preferential nutrients are catabolised first (Suh et al, 2002). Such preferred nutrients may come from amino acids and polypeptides. Previous work conducted by Palmer and colleagues (2005, 2007) has demonstrated that *P. aeruginosa* can utilise amino acids which are in abundance in CF sputum as a nutrient source for growth (Barth and Pitt, 1996). A source of amino acids and polypeptides may come from mucus which is also in abundance in the CF airway. Other nutrients present within mucus include mucin sugars such as, N-acetylglucosamine, which has previously been reported as a nutrient source for *P. aeruginosa* by Korgaonkar and Whiteley (2011).

Other nutrient sources include iron which has been frequently detected in CF sputum at concentrations ranging between 0.9 and 63 μM, whereas in healthy sputum iron is absent (Moreau-Marquis et al, 2008; Reid et al, 2002; Stites et al, 1998). Iron is essential for the survival of many bacteria since iron is a key component in the proper functioning of many enzymes present in biological pathways such as the TCA cycle, gene regulation, and DNA biosynthesis (Andrews et al, 2003). However, whilst iron is needed for many processes, its uptake by bacteria must be tightly controlled since iron can be toxic in aerobic conditions due to the formation of damaging reactive oxygen species (ROS) such as hydroxyl and hydroperoxyl radicals (Touati, 2000). For this reason, the most abundant form of iron is ferric iron due to oxidation of ferrous iron. Iron in its ferric form is difficult to acquire due to its poor solubility (Andrews et al,

2003). Therefore, pseudomonads have developed mechanisms by which iron is efficiently acquired in iron-limiting and aerobic conditions to maintain a balance of obtaining the iron they require for cellular processes whilst preventing the occurrence of free iron which can cause toxicity via the Fenton reaction. One method is via the uptake of haem, via two different haem uptake systems: Has and Phu (Ochsner et al, 2000). The other method is via the production of iron chelators known as siderophores. Examples include pyoverdine and pyochelin which have a high affinity for iron and thus sequester insoluble iron from the environment or from other bacterial siderophores to form iron-siderophore complexes which are more soluble (Meyer, 2000; Koster, 2001; Sun et al, 2006). Therefore, iron is important for the survival of *Pseudomonas* in CF since its presence supports many metabolic pathways when using carbon or nitrogen sources as a nutrient for growth (Reid et al, 2002). Finally, other potential nutrients recorded to be in abundance include lactate, DNA, and sugars within mucus (Garnett et al, 2016).

1.4.2. Sputum, mucus, and mucins

1.4.2.1. Sputum

Sputum is the expectorated secretion produced because of airway inflammation in diseases such as bronchitis, asthma, and cystic fibrosis. Sputum not only contains mucus which is formed from mucin together within ions and water, but additionally DNA, filamentous actin, inflammatory cells, lipids, and bacteria (Lethem et al, 1990; Rubin et al, 2007; Matthews et al, 1963; Kater et al, 2007). For this reason, the macromolecular composition of sputum can vary in different disease states (Voynow and Rubin, 2009).

1.4.2.2. Mucus and its function

Mucus is a thick and adherent, viscous gel-like secretion which is present at numerous locations within the body. Being a boundary lubricant, it has an important role in protecting epithelial surfaces in the reproductive, gastrointestinal, and respiratory tracts. In the respiratory tract, it is produced by goblet cells and submucosal glands, and coats the non-keratinised epithelial surfaces of the nasopharynx. Thus, it serves as a semipermeable barrier to inhaled noxious stimuli such as harmful gases, foreign

particles, and pathogens which have potential to damage the airway (Wickstrom et al, 1998; Knowles and Boucher, 2002; Houtmeyers et al, 1999).

Although mucus mainly consists of water (~95%), enormous glycoproteins called mucins together with inorganic salts form mucus (Allen, 1982; Thornton et al, 2008). More specifically it is the mucin component that is responsible for the gel-like, viscoelastic properties which enable mucociliary transport (Thornton et al, 1990). When exposed to a pressure such as cough, stretch in the mucin fibres in addition to the synchronous beating of airway cilia allows for successful expulsion of foreign particles and pathogens trapped within the mucus (Cone et al, 2009).

In addition to mucociliary clearance, there are several antimicrobial peptides produced by airway epithelial cells, such as β-defensins and cathelicidin (LL-37), which encourage pathogen destruction (Bals and Hiemstra, 2004). Together, with mucociliary clearance and antimicrobial host defences, respiratory infection can generally be avoided in the healthy lung. However, in CF, antimicrobial peptides have reduced activity. Recent studies have shown that this reduced activity was attributed to an abnormal ASL pH when investigated in a porcine model (Pezzulo et al, 2012). Others have demonstrated that LL-37 was made inactive via binding to components such as actin and DNA found within sputum (Bucki et al, 2007). Hence, due to defective mucociliary clearance and a reduction in antimicrobial activity, pathogenic bacteria can survive and thrive in the CF lung. But, how they survive, and specifically what they use as a nutrient source to thrive within the airways remains to be determined. Mucins could provide a nutrient source for the pseudomonads.

1.4.2.3. Mucins

Mucins (MUCs) are one of the main components making up mucus. They are an extensive family of large glycoproteins (0.1-50 MDa), which, despite sharing common features vary in size and structure (Harvey et al, 2011). Notably, it is because of their large size that makes them so difficult to study.

They can be categorised into two distinct families: those that are attached to the apical surface of epithelial membranes and are normally monomeric and those that are

secreted, line the epithelium, and are usually polymeric (Adler and Li, 2001). Importantly, it is the secreted polymeric mucins which are responsible for the gel-like properties necessary for the mucociliary escalator (Thornton et al, 2008).

Structurally, mucins are composed of a linearly arranged polypeptide backbone. The central region of this backbone consists of multiple tandem repeats (TRs). A tandem repeat is composed of numerous proline, serine, and threonine residues to form a single repeat section. The sequence of these amino acids can then be repeated to form the TRs. These TRs therefore vary in length depending on the number of amino acids they contain in addition to how many repeats of amino acids there are within the protein backbone. As shown by the orange regions in figure 1.5, they are separated by cysteine-rich domains which break up the TRs. Therefore, it is due to this variability in size and sequence that MUC proteins, of which 20 have been identified, can be characterised since it is the MUC genes which encode the polypeptide backbone. Examples of these polypeptide backbones for the respiratory mucins MUC2, MUC5B, and MUC5AC are shown in figure 1.5. (Rose and Voynow, 2006).

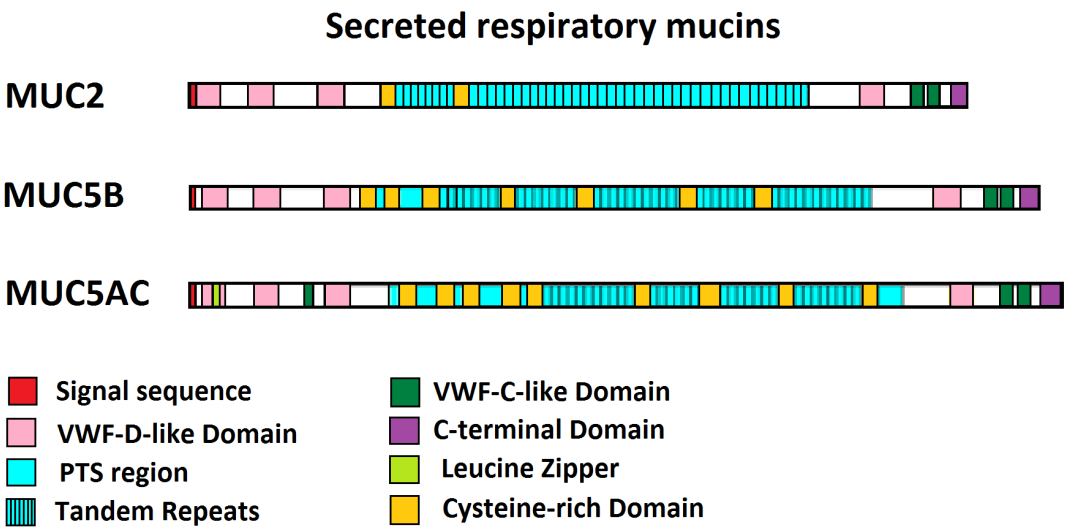

Figure 1.5.

Figure 1.5: Schematic of the polypeptide backbone structure of respiratory mucins MUC2, MUC5B, and MUC5AC. The key shows different coloured boxes representing different regions within the MUC protein backbone. PTS regions represent areas in the protein backbone which are rich in Proline, Threonine, and Serine. (Adapted from Rose and Voynow, 2006; Dekker et al, 2002).

Attached to serine or threonine residues within the TRs of the polypeptide core is extensive O-glycosylation (Lamblin et al, 2001). The first sugar attached to the residues is always N-acetylgalactosamine as shown in figure 1.6, followed by other sugars such as N-acetylglucosamine, fucose, galactose, sulphate and sialic acid, of which the latter two are responsible for the overall negative charge of mucins (Thornton et al, 2008; Rose, 1992). These sugars are added by numerous glycosyltransferases to form O-glycosidic linkages. Therefore, the number of TR domains influences the carbohydrate content. For this reason, up to 85% of the dry weight of mucins is composed of carbohydrate and accounts for their high molecular weight (Wiggins et al. 2001). It is the presence of these glycans that not only contributes to a mucin's enormous size by increasing the length and diameter of the mucin, but because of this, the presence of glycans also increases the rigidity. Therefore, if there are fewer cysteine residues interrupting the TR regions, then these mucins, such as MUC2, will be more rigid than mucins such as MUC5AC and MUC5B which predominate the airway (Wickstrom et al, 1998; Kirkham et al, 2002; Kirkham et al, 2008).

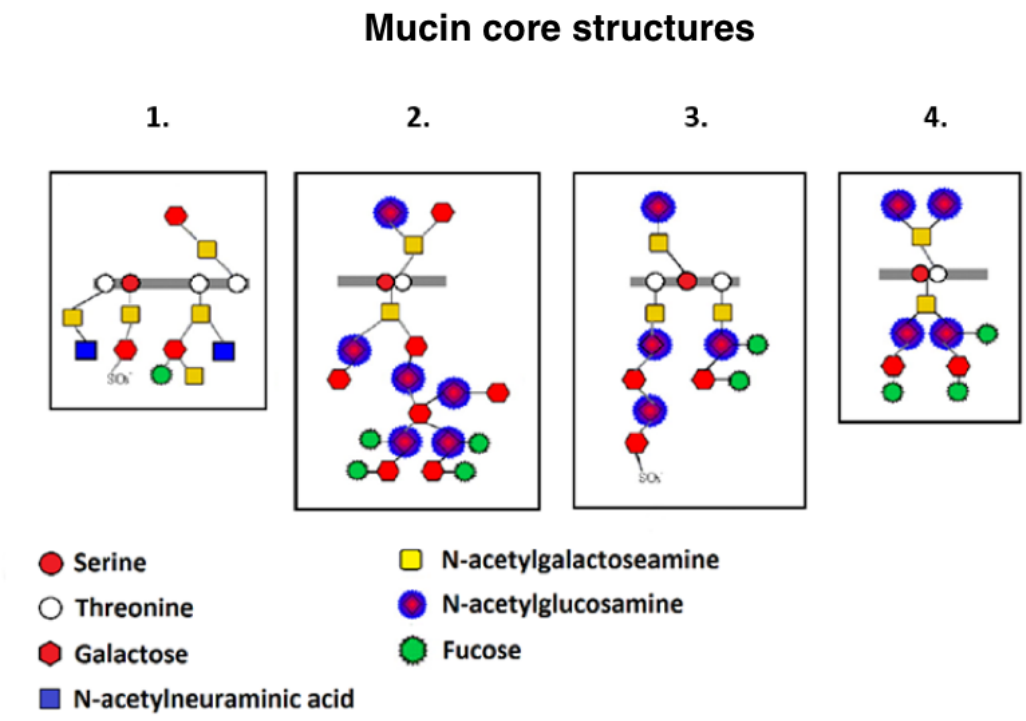

Figure 1.6.

Figure 1.6: Glycosylation mucin core structures. Image demonstrates the four types of mucin core structures in the upper portion of the diagram for each core structure. It shows the variation in structure of the oligosaccharides attached to serine and threonine residues within the TR domains in different mucins. The key shows different coloured shapes which are assigned to different sugars/amino acids. Structure 1 illustrates how N-acetylgalactosamine is attached to serine/threonine residues. Attached to N-acetylgalactosamine is Galactose. Addition of N-acetylglucosamine to N-acetylgalactosamine with galactose forms a branch effect (core structure 2). Alternatively, core structure 3 can be formed if N-acetylglucosamine binds N-acetylgalactosamine attached to serine/threonine with no galactose attached. Finally structure 4 is formed if two N-acetylglucosamine sugars bind to N-acetylgalactosamine forming a branch effect. Subsequent sugars can then be attached to these sugars as shown in the lower portion of the diagrams for each core structure. (Adapted from Rose and Voynow, 2006).

The presence of cysteine residues determines not only the rigidity of the mucin, but also the ability to form polymers. Therefore, depending on the MUC protein, mucins can be cysteine rich or poor. For example, non-secreted mucins, such as MUC1 and MUC4, are tethered to the apical surface membrane and do not require gel-like properties for their function. Thus, they are cysteine poor (Rose and Voynow, 2006). In contrast, the respiratory mucins MUC5B and MUC5AC, possess many cysteine rich regions

allowing the formation of polymers which helps form mucin gels. Therefore, it is the presence of these cysteine residues which allows for mucin polymerisation due to the formation of disulphide bonds as shown in a diagram in figure 1.7. (Strous and Dekker, 1992). Together with how hydrated mucins are, these features contribute to the gel-like properties which makes mucus suitable for its protective and physical role of lubrication at many epithelial surfaces (Yakubov et al, 2007; Harvey et al, 2011; Thornton et al, 2008).

Mucins become hydrated upon their release from secretory granules in which they are stored and condensed following addition of glycan sugars in the Golgi (Verdugo, 1990). These granules are present within goblet cells in the airway surface epithelium, or in mucous cells within the submucosal glands which release MUC5AC and MUC5B, respectively (Groneberg et al, 2002). However due to CFTR dysfunction and the ion imbalance which ensues, mucins in the CF airway are often poorly hydrated and have a reduced ability to expand upon release. This results in increased mucus viscosity and hyper-concentration of the mucins (Henderson et al, 2014). This increased viscosity was previously hypothesised to be due to defective secretion of bicarbonate ions which normally have a role in forming complexes with cations. Thus, removal of these cations enables mucin expansion upon release (Quinton, 2008). For this reason, a defect in bicarbonate secretion was thought to be responsible for the reduced mucin hydration. However, further work in this area has recently clarified what causes these defects. Following granular release of MUC5B, instead of unfolding into a linear form, mucin was shown to remain aggregated in studies. These problems in the maturation of the mucin post-secretion were therefore found to be due to dehydration of the ASL and not due to reduced bicarbonate (Abdullah et al, 2017).

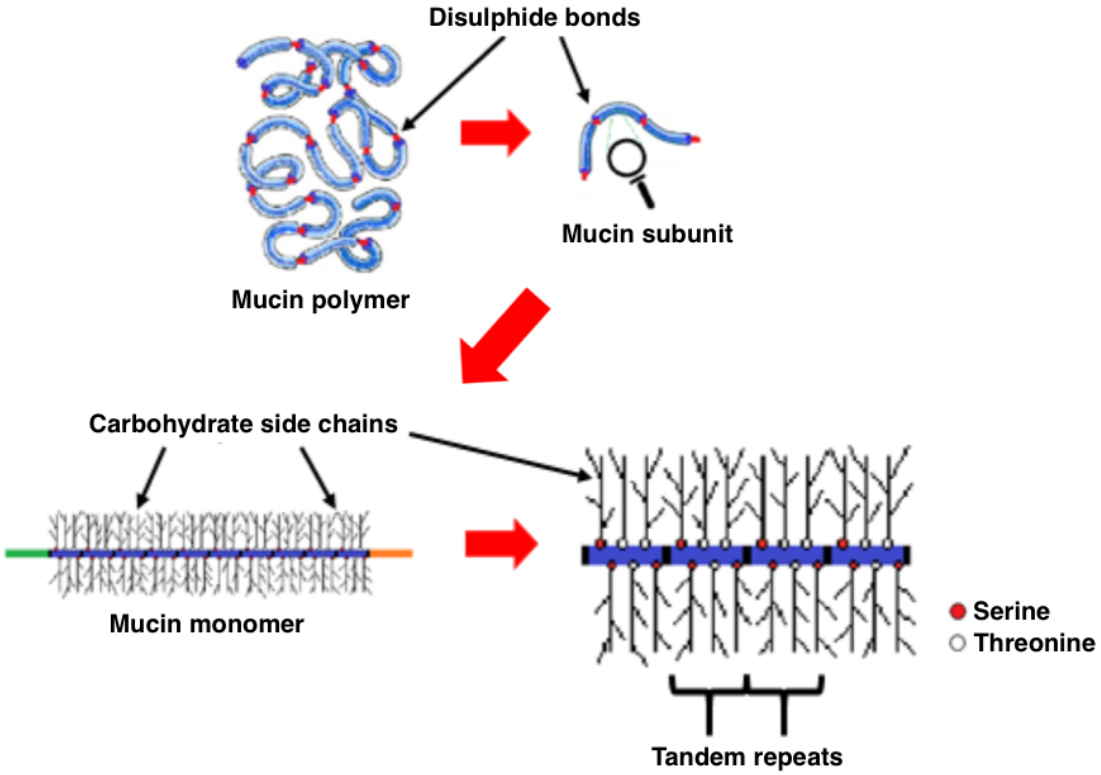

Figure 1.7.

Figure 1.7: Schematic of mucin polymer structure. Diagram shows a mucin polymer with disulphide bonds forming between cysteine residues. Arrows indicate further magnification on regions highlighting how a mucin subunit is formed from mucin monomers. Mucin monomers are illustrated to consist of a linear protein backbone with the N- and C- termini at either end (green and orange respectively) with the central region (blue) consisting of TR domains through which numerous O-glycans or carbohydrate side chains are attached at serine (red circle) and threonine (white circles) residues (Adapted from Ichikawa and Ichihara, 2011).

In the airways, 11 of the 20 identified MUCs are expressed: MUC 1, 2, 3, 4, 5AC, 5B, 6, 7, 8, 13 and 19 (Rose and Voynow, 2006). However as previously discussed, the secreted mucins, MUC5AC and MUC5B, are the main mucins expressed at protein level. This has been illustrated in various studies where they have been frequently detected, such as by analysing the secretions from human epithelial cells using MUC-specific antibodies. Notably, there are two glycoforms of MUC5B. One that has a high charge and another with low charge due to variations in glycosylation during synthesis (Thornton et al, 1997; Thornton et al, 2008; Holmen et al, 2004). MUC 2 is also detected, but when compared to quantities of MUC5AC and MUC5B, there is

significantly less (Martinez-Anton et al, 2006). This is perhaps because MUC2, being more rigid, is less suited to the required role of mucus in the airway.

Interestingly, the two predominating mucins, MUC5AC and MUC5B, have been shown to be upregulated during a CF exacerbation. This has been attributed to the presence of various bacteria, such as *S. pneumoniae* which activate cell surface receptors and specifically up-regulates transcription of the MUC5AC gene during periods of inflammation (Henke et al, 2007; Thai et al, 2008; Li et al, 1997; Ha et al, 2007). Whilst mucins appear to be upregulated during exacerbation, other studies have measured little MUC5AC and MUC5B in the airway secretions of CF patients using immunological techniques (Henke et al, 2004). A more recent study by Henderson and colleagues (2014) demonstrated why levels of mucins appear to be decreased in sputum samples and has attributed this observation to proteolytic degradation of mucin at sites to which antibodies in immunological techniques normally target. In fact, this study revealed that by quantitating mucins using biophysical techniques rather than immunological techniques, the mucin in CF sputum samples were found to be approximately three times more concentrated than in healthy sputum, although more degraded in CF (Henderson et al, 2014). Overall, this successfully demonstrates how mucin concentration increases in CF disease compared to healthy sputum and why mucus and mucin behave differently in CF to an extent that its structure and therefore, its function, is compromised.

1.4.3. Interactions of *Pseudomonas* with mucin in the airway

For chronic infection to be established, pathogens such as *P. aeruginosa* must theoretically, first adhere to a surface to transition from a free-swimming, planktonic, non-mucoid organism to form a community of aggregated microcolonies in a biofilm (Gibson et al, 2003). Although it has been shown that strictly a surface is not required, instead it should be viewed as a platform to which pathogens can colonise. Mucus, being stagnant within the airway can provide this structural platform and a potential source of nutrients for pathogens to thrive. Other components within airway secretions, such as DNA, amino acids, and low iron have all been demonstrated to promote colonisation (Sriramulu et al, 2005; Scharfman et al, 1996). Due to the favourable

environment within the stagnant mucus, pathogens are therefore thought to initially adhere to mucin glycoproteins, present within airway mucus secretions.

Many studies have demonstrated the binding ability of *P. aeruginosa* to regions with glycosylation. For example, early studies initially revealed that *P. aeruginosa* could bind to AsialoGM1 residues within glycolipids present on the CF airway surface epithelium. In this study by Saiman and Prince (1993), epithelial cells from healthy and CF patients were assessed by flow cytometry for AsialoGM1 residues. This study revealed increased amounts of these residues in CF patient epithelial cells vs. 'healthy' cells which were then shown to be receptors for *P. aeruginosa* pili. Consequently, these pili have been shown to be involved in mediating attachment and the establishment of pseudomonad biofilms (Chiang and Burrows, 2003; Woods et al, 1980). In support of *P. aeruginosa* binding to glycosylated regions in the airway epithelium, Bucior and colleagues (2012) revealed that Type IV pili adhesins and flagella were vital in the binding to epithelial cells via N-glycans and heparin sulfate proteoglycans, respectively. However, the literature implies that binding to mucin may be more important than binding to the epithelium. During autopsy of explanted lungs from CF patients, *P. aeruginosa* biofilms were localised deep in the stagnant mucus layer suggesting that binding to mucin is more significant for colonisation than binding to epithelial cells (Lau et al, 2005).

Whilst binding to the epithelial cells was shown to be via the binding of pili and flagella to glycosylated regions, this also appears to be the case with mucins (Landry et al, 2006; Arora et al, 1998), This is because mucins contain many carbohydrate side chain chains which can serve as potential adhesion sites for invading pathogens (Scharfman et al, 1999). Early studies demonstrated binding of non-pilus protein adhesins in *P. aeruginosa* to nasopharyngeal mucins, and more recently, binding has been identified to be to distinct regions within the mucins, such as N-acetylglucosamine and sialic acid receptors (Reddy, 1996; Ramphal and Arora, 2001).

Moreover, the mucin glycoconjugates present within the sputum of CF patients are known to be rich with sialyl-Lewis x (sLe^x), a tetrasaccharide, which attaches to O-glycans. Numerous studies have implicated the involvement of sLe^x in promoting

adherence of *P. aeruginosa* (Scharfman et al, 1999; Scharfman et al, 2001; Lo-Guidice et al, 1994). Supportive of its role is evidence demonstrating that the amount of sLe^x found within mucin directly correlates with CF infection (Davril et al, 1999). The siderophore, pyocyanin, produced by *P. aeruginosa* in iron-limiting conditions, has also been implicated in inducing sLe^x glycosyltransferases. Studies have shown that this leads to increased glycosylation with sLe^x on MUC5AC in a concentration-dependent manner, and finally, increased binding to the epithelium (Jeffries et al, 2016). In addition to numerous virulence factors involved in colonisation, *P. aeruginosa* has interestingly shown to reduce the expression of its virulence factors which is thought to be so that clearance by the host immune response can be avoided (Lau et al, 2005). Additionally, phosphatidylcholine, an important lung surfactant lipid, is degraded by *P. aeruginosa* to aid colonisation by causing further damage to the lungs (Son et al, 2007).

Whilst pili facilitate initial attachment, flagella are responsible with determining whether the attachment is permanent (Chiang and Burrows, 2003). *P. aeruginosa* thus undergoes a series of morphological changes as it establishes a biofilm. First, it makes reversible attachments via flagella as a 'sampling' method to determine whether the area is suitable for biofilm formation (Hinsa et al, 2003; Caiazza and O'Toole, 2004; Sauer et al, 2002). An increase in flagella motility together with twitching of the type IV pili then stimulates aggregation of microcolonies (O'Toole and Kolter, 1998; Yeung et al, 2012). As the biofilm develops, the increased secretions of quorum sensing (QS) molecules serves to regulate the biofilm cell density and coordinate its growth population by regulating gene expression, until it reaches a threshold which signals the production of a molecule to trigger cessation of growth (Cooley et al, 2008; Singh et al, 2000).

The QS systems: Las, Rhl, and PQS have important roles during bacterial growth and in promoting biofilm formation by regulation of numerous virulence factors such as exopolysaccharides, including alginate which is not only responsible for the mucoid phenotype, but also protects the pathogen from antibiotics and clearance via host cell phagocytosis (Ben Haj Khalifa, 2011). QS in addition to several virulence factors released by pathogen secretion systems therefore enables a continued assault on the

airway leading to chronic colonisation (Yeung et al, 2012; Wagner et al, 2003; Bjarnsholt et al, 2010; Ramsey and Wozniak, 2005).

P. aeruginosa possesses five types of secretion systems out of the six in total which have been described in gram-negative bacteria (Bleves et al, 2010; Economou et al, 2006). These secretion systems not only release a wide array of toxic exoproteins which damage the host, but are also regulated by QS. QS molecules induce release of pyocyanin, as previously discussed, but also proteolytic enzymes such as alkaline proteases and elastases secreted from type I (T1SS) and type II (T2SS) secretion systems, respectively. QS also regulates the type III secretion system (T3SS), in which several exoproteins are released and are transported into host cells. ExoU specifically has been strongly associated with lung injury (Bleves et al, 2005; Le Berre et al, 2011; Hauser et al, 2002). A review by Bleves and colleagues (2010) summarises the exoproteins produced by *P. aeruginosa* PAO1 by the five different secretion systems (table 1.1).

Table 1.1: Exoproteins produced by *Pseudomonas aeruginosa* PAO1 (adapted from Bleves et al, 2010)

Secretion system	Secreted protein(s)	Protein type
T1SS (Apr)	AprA (PA1249)	Alkaline protease
	AprX (PA1245)	Unknown
	HasAp (PA3407)	Heme acquisition protein
T2SS (Xcp)	LasB (PA3724)	Elastase
	LasA (PA1871)	Staphylolytic and elastolytic
	PlcH (PA0844)	Haemolytic phospholipase C
	PlcN (PA3319)	Non-haemolytic phospholipase C
	PlcB (PA0026)	Phospholipase C (phosphatidyl-ethanolamine)
	CbpD (PA0852)	Chitin-binding protein
	ToxA (PA1948)	AB toxin, ADP-ribosyl transferase
	PmpA (PA0572)	Putative metalloprotease
	PrpL (PA4175)	Lysine specific endopeptidase
	LipA (PA2862)	Triacyl glycerol acyl hydrolase
	LipC (PA4813)	Lipase
	PhoA (PA3296)	Alkaline phosphatase
	PaAP (PA2939)	Aminopeptidase
T2SS (Hxc)	LapA (PA0688)	Alkaline phosphatase
T3SS	ExoS (PA3841)	ADP-ribosyl transferase and Rho GTPase-activating protein (GAP)
	ExoT (PA0044)	ADP-ribosyl transferase and Rho GTPase-activating protein (GAP)
	ExoY (PA2191)	Adenylate cyclase
T5aSS	EstA (PA5112)	Esterase
T5bSS	LepA (PA4540)	Exoprotease
	CupB5 (PA4082)	Hemagglutinin-like
T6SS	Hcp1 (PA0085)	Nanotubes formation

As previously mentioned, the secretion systems in *P. aeruginosa* are capable of producing proteins which aid and promote its survival. In T1SS, the alkaline protease AprA, is released and interferes with host complement activation (Guzzo et al, 1991; Laarman et al, 2012). AprA produced also activates ENaCs by further reducing the ASL volume and hindering mucus clearance (Butterworth et al, 2012). Also, part of T1SS is the release of the haemophore protein, HasAp, which aids survival of *P. aeruginosa* in the early stages of infection by sequestering haem from haemoglobin when levels of iron may be low (Wandersman and Delepelaire, 2004). However, in the absence of iron, *P. aeruginosa* is also able to produce the siderophores, pyochelin and pyoverdine, to capture iron from the host environment to survive (Hoegy et al, 2014; Gi et al, 2015). Elastase (LasB) released by T2SS has been linked to severity of lung injury by reducing lung elasticity (Braun et al, 1998; Le Berre et al, 2011). It not only cleaves elastin and affects the structural integrity of the lung, but also promotes survival. The collectins; surfactant proteins A and D, are involved in the binding and aggregation of *P. aeruginosa* in preparation for phagocytosis by neutrophils. However, these surfactants can be degraded by elastase produced by *Pseudomonas* (Alcorn and Wright, 2004). The production of elastase therefore prevents pathogen clearance and illustrates one example by how *P. aeruginosa* colonises the airway.

Therefore, despite neutrophils accumulating in the lung, it appears to a certain degree their roles of inducing phagocytosis, degranulation, and ROS generation are rendered redundant. This is because *Pseudomonas* can avoid clearance via biofilm formation and the production of a vast array of virulence factors (Mayadas et al, 2014). However, when near to pathogens, neutrophils have been shown to undergo programmed cell death resulting in the release of NETs, as previously mentioned. This is termed 'NETosis' and is part of the innate immune response. The release of NETS, composed of neutrophil elastase and myeloperoxidase, in addition to other antimicrobial molecules and chromatin helps to destroy these virulence factors and prevent spread of further infections (Lethem et al, 1990; Brinkmann et al, 2004; Dubois et al, 2012). Their lysis therefore causes the production of proteins and DNA which associate with the *P. aeruginosa* biofilm, making it increasingly impermeable to immune cell and antimicrobial attack (Walker et al, 2005; Parks et al, 2009). Thus, neutrophils themselves inadvertently contribute to CF disease.

This illustrates that whilst the initial involvement of *P. aeruginosa* damages the airways by attaching to airway mucin and allowing formation of biofilms, the host immune response further fuels damage via further binding of *Pseudomonas* (Lam et al, 1980; Sriamulu et al, 2005). It does so by the production of inflammatory cytokines such as IL-6 and IL-8 which have been shown to be stimulated by pyocyanin production by *P. aeruginosa* (Denning et al, 1998). In response to respiratory distress due to abnormal chloride secretion, IL-8 secretion is also induced by the submucosal glands (Tager et al, 1998). Consequently, the presence of IL-8 and IL-6 have more recently been shown to increase the amount of sLe^x in mucins, which further increases binding (Ishibashi et al, 2005; Colomb et al, 2012, Colomb et al, 2014). TNF which is also released during inflammation has been shown to stimulate increased binding of *P. aeruginosa* to mucins containing sLe^x moieties. When this is coupled with increased expression of sLe^x glycosyltransferases this explains why sLe^x has been found to correlate with CF severity of infection. Furthermore, it demonstrates how CF disease escalates with progression to chronic colonisation of the pseudomonads (Colomb et al, 2014; Delmotte et al, 2002; Davril et al, 1999).

1.4.4. Mucins as a nutrient source

Because *P. aeruginosa* is closely associated with glycans during initial attachment, it may be possible that pathogens utilise airway mucins as a nutrient source for growth. Supportive of this are earlier studies which have demonstrated that PGM can be used as a nutrient source. In a study by Roberton and Stanley (1981), *Bacteroides fragilis* was shown to be able to use PGM as a nutrient source and more recently experiments conducted by King and colleagues (2006) revealed that *S. pneumoniae* could specifically express glycosidases such as NanA, BgA, and StrH which enable the breakdown of host sugars (King, 2010). This finding is supported by other *in vitro* studies that *S. pneumoniae* can utilise PGM via the EMP pathway of carbohydrate metabolism to yield ATP to establish and maintain survival. In this study colonies were shown to be larger when grown in the presence of mucin vs. when grown in the absence of mucin (Yesilkaya et al, 2008).

Furthermore, it has been established that bacteria present within the gut and oral cavity can also utilise intact mucins as their main nutrient source for survival. In one study, oral biofilms demonstrated an ability to degrade and utilise salivary MUC5B (Wickstrom and Svensater, 2008; Wickstrom et al, 2009). Additionally, in a study by Nelson and colleagues (2013), *P. aeruginosa* PAO1 virulence in response to monosaccharides present in human milk was compared to monosaccharides found in formula milk. It was found that there were significant differences in growth after 24 hours. When grown in xylose and galactose, a significant increase in virulence gene expression was observed. Whilst this study demonstrated a promising indication that *P. aeruginosa* can utilise galactose, which is present within mucin, as a nutrient source, a more recent study has argued that *P. aeruginosa* has a limited ability to utilise intact PGM mucin. Instead *P. aeruginosa* was found to utilise short chain fatty acids (Flynn et al, 2016). Additionally, it was demonstrated by Mirkovic and colleagues (2015) that anaerobic bacteria produced short chain fatty acids in the CF airway and in studies by Ghorbani and colleagues (2015) that they could affect bacterial growth. Therefore, it remains to be determined whether pathogens such as *S. pneumoniae* and *P. aeruginosa* are able to utilise mucins within patient sputum as a nutrient source for growth.

1.5. Hypothesis

I hypothesise that the prevalent respiratory pathogen, *Pseudomonas aeruginosa,* which colonises the CF lung survives in the CF airway by liberating carbohydrate found in airway mucins within secretions from CF patients as a nutrient source for growth.

1.6. Aims

1) To identify any differences in growth of *Streptococcus pneumoniae* D39, *Pseudomonas aeruginosa* PAO1, *Pseudomonas aeruginosa* PA14, and *Pseudomonas aeruginosa* clinical isolates in sputum from cystic fibrosis patients and sputum from healthy control patients.

2) To identify whether mucin in CF sputum is a nutrient for *Pseudomonas aeruginosa*

3) To identify other nutrients within airway sputum.

4) To identify *P. aeruginosa* genes involved in nutrient metabolism which are upregulated or downregulated when exposed to CF sputum.

Chapter 2. Materials and methods

This section will describe the general methods that were used throughout this study.

This chapter will include details on:

- Materials and suppliers
- Buffer recipes
- Bacterial species (including preparation of bacterial stocks, media recipes, growth conditions, and methods of identification)
- General method for assessing growth
- General techniques
- Statistical analyses

2.1. Materials

The materials used for this project are described in table 2.1.

Table 2.2: Materials and suppliers

Company/Supplier	Materials
Ambion®, Inc.	RNase *AWAY*™ Decontamination Reagent, TRIzol® Reagent, Ultrapure DEPC-treated Water
Axygen® Scientific, Inc.	1.5 ml clear microtubes, 1-20 µl maximum recovery (racked, sterile tips), 1-200 µl maximum recovery (racked, sterile tips)
Bioline Reagents	SensiMix™ SYBR Hi-ROX, Agarose
Eurofins Genomics	PCR primers listed in chapter 7.
G-Biosciences/Geno Technology, Inc.	CB-X™ Protein Assay
GE Healthcare Life Sciences	Amersham™ Protran™ 0.45 µm Nitrocellulose Blotting Membrane
Greiner BioOne North American, Inc.	Cell culture microplate, 96 well, polystyrene, u-bottom, clear, cellstar® tc, with lid, sterile, single packed
Invitrogen by Thermo Fisher Scientific	PureLink™ Genomic DNA Mini Kit, Random Primers, Superscript® III Reverse Transcriptase, TURBO DNA-free™ kit
LICOR® Biosciences	IRDye® 800CW Goat Anti-Rabbit
Merck Millipore, Ltd.	Amicon ® Ultra – 0.5 ml Centrifugal filters 10K, 30K, and 50K
Melford Biolaboratories Ltd.	Caesium chloride, Agarose
Molecular Probes® by Life Technologies™	Alexa Fluor® 680 goat anti-mouse IgG (H+L)
New England Biolabs®, Inc.	100 bp DNA ladder, 1 kbp DNA ladder, Gel Loading Dye (6X concentrated), no SDS
Oxoid Ltd.	Blood agar base, Brain heart infusion, Horse blood defibrinated, Phosphate buffer saline tablets
Pall Life Sciences	Acrodisc®25 mm syringe filter with 0.2 μm Supor® Membrane, Acrodisc®25 mm syringe filter with 0.45 μm Supor ®Membrane

Pierce Biotechnology	Snakeskin™ dialysis tubing (10 kDa MWCO)
Qiagen	HotStarTaq® *Plus* Master Mix Kit, Acetic acid, DTT, EDTA, EGTA, Glycerol, Iodoacetamide, Luria agar, Luria Broth, Phosphate buffered saline, Periodic acid, Schiff's reagent, Sodium Chloride, Sodium hydroxide, Sodium metabisulfite, TBST, Tris-HCl
Sarstedt	Tips 200 µl, yellow (1920 x Stack Pack), Petri dish 92 x 16 mm, PS, with ventilation cams, sterile, 20 pcs. /tube bag (stacked)
Sigma-Aldrich Ltd.	Agar, Bovine serum albumin (BSA), Corning® Costar® Stripette® serological pipettes, individually paper/plastic wrapped: 5 ml, 10 ml, and 25 ml, Corning® 15 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap, bulk packed, sterile, natural, 500/cs, Corning® 50 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap, bulk packed, sterile, natural, 500/cs, Galactose, Glucose, Glucose (GO) assay kit, Guanidine hydrochloride, Parafilm® 'M' film, Porcine gastric mucin (type III), Potassium phosphate dibasic, Potassium phosphate monobasic, Salmon sperm DNA, Sicard's defined medium components (see Table 2.4), Sodium azide, Sodium benzoate, Thymine, Tryptone, Tween®-20, Water, Molecular Biology Reagent, Yeast Extract
Spectra/Por®, Spectrum labs, U.K.	Spectra/Por® 7 Standard RC, Pre-treated Dialysis Tubing (1 kDa MWCO)
Star Lab	TipOne 10/20 µl XL Graduated Filter Tip, Max. Vol 20µl (Sterile), TipOne 200 µl Graduated Filter Tip (Sterile), TipOne 1000 µl Graduated Filter Tip (Sterile), 0.1 ml 4-strip Rotor-Gene® Style Tubes and Caps
Thermo Fisher Scientific	20 ml EPA vial and PTFE cap, Sterilin™ 7 ml Polystyrene bijou containers, Sterilin™ 30 ml Polystyrene bijou containers.
VWR International	PCR tube with attached flat cap

2.2. Buffer recipes

10X GuHCl reduction buffer: To prepare the GuHCl reduction buffer, 24.228 g of Tris base (1 M, pH 8), in addition to 29.224 g EDTA (0.5 M) were added to 187.5 ml of 8 M GuHCl. This was then adjusted to 200 ml via the addition of 12.5 ml dH_2O. Next, 0.077125 g of DTT was measured and added to 5 ml of 10X GuHCl reduction buffer which was adjusted to 50 ml using 4 M GuHCl. DTT was therefore added to a final concentration of 10 mM in 50 ml of 1X GuHCl reduction buffer (0.1 M Tris, pH 8, 0.05 M EDTA).

10X Non-reducing loading buffer: contains 1% SDS, 40-50% glycerol bromophenol blue in 10% TAE.

PBS: Potassium buffered saline was prepared by dissolving 2.35 g Na_2HPO_4, 7.35 g NaCl, and 1.3 g $NaH_2PO_4.2H_2O$ in 1 L of dH_2O. It was then adjusted to pH 7.1 using HCl to decrease the pH and NaOH to increase the pH. Alternatively, ten phosphate buffer saline tablets were dissolved in 1 L dH_2O. The PBS was then autoclaved at 121^{o}C at 15 pounds per square inch (psi) for 20 minutes.

1 M potassium phosphate buffer: 1 M potassium phosphate buffer was prepared by firstly dissolving 17.418 g of K_2HPO_4 in 100 ml dH_2O. Then 100 ml of 1 M KH_2PO_4 was prepared in the same way by weighing 13.609 g and dissolving in dH_2O. To make 1 M of buffer, 61.5 ml of 1 M K_2HPO_4 was mixed with 38.5 ml of 1 M KH_2PO_4.

10X reduction loading buffer: contains 1% SDS, 40-50% glycerol bromophenol blue in 10% urea reduction buffer (URB) where URB is composed of 2 M Tris (pH 8), 100 mM EDTA, 8 M urea.

0.1% SDS in TAE buffer: To prepare 10% SDS solution, 100 g of SDS was dissolved in 80 ml and the volume adjusted to 1 L using dH_2O. Then 10 ml was taken from the SDS stock and 1X TAE was used to adjust the volume to 1 L.

4X SSC: Initially 20X saline sodium citrate buffer was prepared. This was done by dissolving 175.3 g of NaCl and 88.2 g of $Na_3C_6H_5O_7$ in 800 ml dH_2O. This was then

adjusted to pH 7.2. The final volume was then adjusted to 1 L. To get 4X SSC, 20 ml of 20X SSC was added to 80 ml dH_2O.

1X TAE buffer: Initially 50X TAE was prepared by dissolving 242 g Tris base, 57.1 ml glacial acetic acid, and 37.2 g $Na_2EDTA.2H_2O$ in 1 L dH_2O. The pH was then adjusted to 8.5. This was diluted to 1X TAE by taking 100 ml of 50X TAE and the volume adjusted to 5 L using dH_2O.

1X TBST buffer: Initially 20X TBST was prepared by dissolving 60.57 g Tris base in 200 ml dH_2O. This was then adjusted to pH 8 and the volume adjusted to 250 ml. To this, 438.3 g NaCl was added with 25 ml of Tween®-20. The final volume was then adjusted to 2.5 L (0.2 M Tris-HCl at pH 8, 3 M NaCl, and 1% Tween®-20). Single strength TBST was prepared when needed by taking 300 ml of 20X TBST and adjusting the final volume to 6 L using dH_2O (10 mM Tris-HCl at pH 8, 150 mM NaCl, 0.05% (v/v) Tween®-20).

2.3. Bacterial species

Streptococcus pneumoniae D39 (serotype 2), *Pseudomonas aeruginosa* PAO1, and *Pseudomonas aeruginosa* PA14 were selected as the reference strains for growth studies. The pneumococcal strain, D39, was taken from the bead stock collection belonging to Professor Peter W. Andrew, Department of Infection, Immunity, and Inflammation, University of Leicester, UK. The *Pseudomonas* clinical isolates were obtained from CF patient sputum samples delivered to the Microbiology department at the Leicester Royal Infirmary Hospital and isolated by the senior biomedical scientist, Hemu Patel (table 2.2). *P. aeruginosa* reference strains, PAO1 and PA14, were kindly donated by Dr. Kumar Rajarkumar, Department of Infection, Immunity, and Inflammation, University of Leicester, UK.

The PAO1 reference strain used in this study is a chloramphenicol resistant mutant of the PAO strain which was initially isolated in 1954 from the wound of a patient in Australia (Holloway, 1955). PAO1 is a moderately virulent strain and the most frequently used strain for researching the pathophysiology of this organism, especially since its full genome was sequenced in 2000 (Clarke, 1975; Lee et al, 2006; Stover et al, 2000). The other strain used in this study, PA14, is more virulent than PAO1 and interestingly belongs to the most common global clonal group, which PAO1 in contrast does not belong to (Wiehlmann et al, 2007). This is despite high conservation being observed between their respective genomes (Stover et al, 2000; He et al, 2004).

Table 2.3: *Pseudomonas* clinical isolates obtained from CF patient sputum

Assigned laboratory number	DOB of patient	Organism ID	Sample type from patient
485399X	02/01/14	*P. aeruginosa*	Sputum
485368Z	04/10/99	*P. aeruginosa*	Sputum
485424G	27/06/91	*P. aeruginosa*	Sputum
485411V	03/09/91	*Pseudomonas* spp.	Sputum
485392T	28/08/97	*P. aeruginosa*	Sputum

2.3.2. Media and growth conditions for *Streptococcus pneumoniae*

S. pneumoniae D39 was either grown in liquid culture using Brain Heart Infusion (BHI) broth or on Blood Agar Base (BAB) with 5% (v/v) defibrinated horse blood in Petri dishes. The media compositions are listed in table 2.3.

Table 2.4: Compositions of BAB and BHI

Medium	Composition
BAB	16 g in 400 ml dH_2O
BHI	14.8 g in 400 ml dH_2O
Recipe: BAB and BHI were sterilised via autoclaving at 121^oC, 15 psi for 20 minutes. After autoclaving, the molten BAB was left to cool to approximately 50-55^oC so that upon addition of the blood it did not burn. The defibrinated horse blood was then slowly added to prevent the formation of bubbles. The agar was mixed by gentle swirling of the bottle before being poured into Petri dishes and allowed to set.	

Sicard's defined medium was used when appropriate for investigating the growth of bacterial species in 0.4% (w/v) porcine gastric mucin (PGM) or sputum obtained from CF patients. The components of Sicard's defined medium and its recipe are described in table 2.4 (Sicard, 1964). Sicard's defined medium will be referred to as 'Sicard's' in this thesis.

When grown in liquid medium, *S. pneumoniae* was statically incubated at 37^oC. When plated, the Petri dishes were inverted, placed in a jar with a lit candle to remove available oxygen, and then incubated at 37^oC.

Table 2.5: Composition of Sicard's defined medium

Part A.	
Minerals and buffer	**Quantity (g) in 1 L of dH_2O**
NaCl	5
NH_4Cl	2
KCl	0.4
Na_2HPO_4	0.12
$MgSO_4$	0.024
$CaCl_2$	0.010
$FeSO_4.7H_2O$	0.00055
Tris base	4.84
Part B.	
Amino acids	**Quantity (g) in 1 L of part A**
L-arginine	0.2
L-asparagine	0.01
L-cysteine-HCl	0.1
L-glutamine	0.02
Glycine	0.12
L-histidine	0.15
L-isoleucine	0.00655
L-leucine	0.00655
L-lysine	0.42
L-methionine	0.18
L-threonine	0.175
L-valine	0.00585
Part C.	
Vitamins	**Quantity (g) in 10 ml of dH_2O**
Biotin	0.000015
Choline	0.005
Nicotinamide	0.0006
Pantothenate	0.0024
Pyridoxal HCl	0.0006
Riboflavine	0.0003
Thiamine	0.0006

Part D.	
Other	**Quantity (g)**
Sodium pyruvate	0.8
Uracil	0.001

Recipe: The balanced salt solution (part A) and sodium pyruvate (part D) were prepared initially in 900 ml dH_2O and adjusted to pH 7.55. The volume was adjusted to 1 L and the pH checked to ensure no change in pH. The amino acids (part B) and uracil (part D) were then dissolved in the 1 L solution with L-cysteine-HCl and L-glutamine excluded. This solution was then sterilised by autoclaving at 121^{o}C for 15 minutes at 15 psi. Vitamins (part C), L-cysteine-HCl, and L-glutamine were prepared in 10 ml dH_2O and added prior to use with 20 ml of 4% (w/v) BSA and 16 ml of 25% (w/v) glucose or galactose which were filter-sterilised through a 0.2 μm acrodisc®. The quantities described were doubled and prepared as previously described with 4% (w/v) BSA and 0.4% (w/v) glucose/galactose excluded for growth experiments when investigating growth in PGM or CF sputum.

2.3.2.2. Identification of *Streptococcus pneumoniae*

Gram staining: A loop of *S. pneumoniae* was smeared over a slide and fixed by passing the slide quickly through a flame several times. Excess crystal violet was then added to the slide and left for 1 minute. This was then washed off with dH_2O, followed by the addition of iodine solution which was left for 1 minute. Iodine was then rinsed off with dH_2O. The slide was then washed with 95% acetone for 2-5 seconds to decolourise and quickly rinsed with dH_2O again. Safranin was then added in excess to counter-stain for 2 minutes. Finally, the slide was rinsed with dH_2O before being dried near the Bunsen flame. Because *S. pneumoniae* is gram-positive, the thick peptidoglycan wall of the bacterial cell wall retains the crystal violet stain following decolourisation. This results in a purple appearance of the stained organism when examined under a light microscope. *P. aeruginosa* is an example of a gram-negative bacterium since its cell wall is thin and cannot retain the purple stain. It appears pink under the light microscope (Coico, 2005).

Catalase test: *S. pneumoniae* is catalase negative. A drop of hydrogen peroxide was added to a slide. A loop of *S. pneumoniae* was then added to the hydrogen peroxide. Absence of bubble formation confirmed that the organism was catalase negative. The presence of catalase would mean that the hydrogen peroxide is converted into water and oxygen. Bubble production would therefore be observed. A positive example of this test would be with *Staphylococcus aureus* (Clarke and Cowan, 1952).

Optochin sensitivity test: *S. pneumoniae* was streaked onto a BAB plate. A sterile optochin disk was then placed on the agar. The plate was inverted and placed in a jar sealed with a lit candle to remove available oxygen. This was then incubated overnight at 37^oC. After 24 hours, the plates were inspected. Optochin was used for identifying *S. pneumoniae* since it causes lysis of the bacterium surrounding the disk. Therefore, no colonies would be observed in this region surrounding the disk (Kellogg et al, 2001).

Haemolysis determination: *S. pneumoniae* is an alpha-haemolytic bacterium. Therefore, when grown on blood alpha haemolysis occurs. Hydrogen peroxide produced by the bacterium reduces haemoglobin in the BAB to oxidised methaemoglobin. This results in a green colour change surrounding the colonies (Lorian and Markovits, 1973).

2.3.3. Media and growth conditions for *Pseudomonas aeruginosa*

Pseudomonas was either grown in Sicard's (table 2.4) Luria broth, or the minimal medium, Medium 9 (M9) (LaBauve and Wargo, 2012). The components and recipe of M9 are described in table 2.5.

PAO1 and PA14 reference strains were grown in M9 (2X concentration) added to an equal volume of sterile nH_2O to get single strength M9 (0.1 mM $CaCl_2$, 2 mM $MgSO_4$). For growth in PGM, M9 (2X concentration) was added to an equal volume of 8 mg/ml PGM to get a final concentration of 4 mg/ml PGM supplemented with single strength M9. Finally, for investigating growth in purified sputum, PAO1 was grown in 0.1% (w/v) sputum by adding 2 mg/ml sputum to double strength M9.

During growth assays, *P. aeruginosa* was plated on Luria agar plates at each time point. The plates were inverted and incubated at 37^{o}C in aerobic conditions. For extraction of genomic DNA, *P. aeruginosa* was grown in Luria broth (table 2.6). When grown in liquid medium, *Pseudomonas* was grown at 37^{o}C, 200 rpm.

Table 2.6: Composition of M9

Part A. M9 salt	
5X concentration	**Quantity (g) in 1 L dH_2O (autoclaved)**
Na_2HPO_4	30
KH_2PO_4	15
NH_4Cl	5
NaCl	2.5
Part B.	
1 M	**Quantity (g) in 10 ml dH_2O (f/s)**
$CaCl_2$	1.47
$MgSO_4$	1.20

Recipe: M9 medium salt (5X concentration) was prepared in 1 L dH_2O and sterilised by autoclaving at 121^oC for 15 minutes at 15 psi. Components of part B were prepared separately and filter-sterilised (f/s) through a 0.2 µm acrodisc®. Then, 400 ml of 5X concentration M9 was added to 500 ml of sterilised dH_2O, 200 µl of 1 M $CaCl_2$, and 4000 µl of 1 M $MgSO_4$. The final volume was then adjusted to 1 L with sterile dH_2O so that the final concentration of $MgSO_4$ and $CaCl_2$ in the doubled strength M9 was 4 mM and 0.2 mM, respectively.

Table 2.7: Compositions of Luria agar and Luria broth

Medium	**Composition**
Luria agar	10 g tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar
Luria broth	10 g tryptone, 10 g NaCl, 5 g yeast extract

Recipe: Luria agar was made by dissolving tryptone, NaCl, and yeast extract in 800 ml dH_2O. The pH was then adjusted to 7.5 and the agar was added. The volume was then adjusted to 1 L using dH_2O and microwaved to melt the agar. It was then sterilised via autoclaving. For Luria broth, the same method was followed without the addition of agar.

2.3.3.2. Identification of *Pseudomonas aeruginosa*

Gram stain: As described in section 2.3.2.2. Identified under microscope due to rod shape.

Catalase test: As described in section 2.3.2.2. *P. aeruginosa* is catalase positive.

Pigment: *P. aeruginosa* was also identified via pigment production. Production of pyoverdine results in a yellow appearance whereas pyocyanin results in a blue appearance. The combined production of both results in blue-green colonies on a plate (El-Fouly et al, 2015).

Oxidase test: Following incubation of *P. aeruginosa* on a Luria agar plate, Gordon-McLeod (Oxidase) reagent was added to some colonies. A change in colour after 10 seconds to a deep blue-purple meant that the organism was oxidase positive, as is the case with *P. aeruginosa* (Jurtshuk and McQuitty, 1976).

2.3.4. Preparation of bacterial stocks

2.3.4.1. Stocks in BHI

S. pneumoniae D39 from the laboratory bead stocks were streaked to single colonies on BAB plates with 5% (v/v) defibrinated horse blood, incubated overnight as previously described, and the next day a loop of colonies were suspended in 3 ml PBS until the OD_{600} was approximately 0.18-0.2. From this, 100 µl was added to 10 ml BHI and statically incubated at 37^{o}C. Growth was assessed at each time point via OD_{600} readings using the spectrophotometer. When OD_{600} reached 0.6-0.8, cultures were frozen at -80^{o}C in 10% (v/v) glycerol.

After 48 hours, Colony Forming Unit (CFU) counts were performed (Miles et al, 1938). Serial dilutions (10 fold) were performed in 96-well plates and for each dilution 60 µl was added to a correspondingly sector on a BAB plate with 5% (v/v) defibrinated horse blood. This was then incubated at 37^{o}C overnight in jars with a lit candle. The candle served to remove available oxygen. Colonies on the incubated BAB plates were counted in the sector where 25-250 CFUs could be easily counted.

The below calculation was used to determine CFU/ml:

$$\textbf{CFU/ml} = \frac{\textbf{Number of colonies x Dilution}}{\textbf{(60 µl/1000 µl)}}$$

P. aeruginosa reference strains, PAO1 and PA14, were streaked to single colonies from aliquots onto Luria agar plates and incubated overnight at 37^{o}C in aerobic conditions. The same was done with *Pseudomonas* clinical isolates described in table 2.2. After 24 hours, they were streaked onto fresh Luria agar plates and incubated overnight again. After 24 hours *Pseudomonas* was re-suspended in BHI and frozen at -80^{o}C in 30% (v/v) glycerol (LeBauve and Wargo, 2012).

2.3.4.2. Stocks in Sicard's defined medium

A frozen stock of D39 in BHI was thawed and used to inoculate 10 ml BHI. This was then statically incubated at 37^{o}C overnight. After 8 hours, 1 ml of the overnight culture was taken for measuring the OD. Samples with OD_{600} readings indicating late exponential phase of growth (0.6-0.8 OD_{600}) were used further and 100 µl of this culture was added to 10ml of Sicard's (1:100) containing 0.4% (w/v) glucose. The glucose in this culture served as the sole carbon source. This was then incubated again at the same temperature and cultures were frozen in 10% (w/v) glycerol when OD_{600} readings exceeded 0.2.

An aliquot of D39 previously grown in Sicard's with 0.4% (w/v) glucose and frozen in 10% (v/v) glycerol was thawed. Then, 100 µl was added to 10 ml of Sicard's with glucose and incubated at 37^{o}C. After 10 hours, when the OD_{600} measured was approximately 0.4, 1 ml from this culture was transferred to a sterile 1.5 ml microtubes and centrifuged at 13000 rpm for 2 minutes. The supernatant was then discarded and the pellet re-suspended in 500 µl PBS before centrifuging again and suspending the pellet in 500 µl Sicard's. Then 100 µl was added to 10 ml Sicard's before being incubated. After 10 hours, the culture was frozen in 10% (v/v) glycerol. For unknown reasons, this was the only method by which it was possible to get D39 growing in Sicard's with glucose. D39 would not grow in 0.4% (v/v) glucose in Sicard's via direct sub-culture.

2.4. Growth studies

2.4.1. Viable counting

Growth was assessed over 24 hours by CFU counts (Miles et al, 1938). Serial dilutions were performed in 96-well microtitre plates and for each dilution 60 µl was added to the corresponding sector on an appropriate nutrient plate. For *S. pneumoniae* D39 60 µl of each dilution was added to a BAB plate with 5% v/v defibrinated horse blood, and incubated at 37^{o}C overnight in jars with a lit candle whereas for *P. aeruginosa* 60 µl of each dilution was added to Luria agar plates which were incubated overnight at 37^{o}C in aerobic conditions. After 24 hours, colonies on the incubated plates were counted in the sector where 25-250 CFUs could be easily counted. The CFU/ml was then calculated as previously described in section 2.3.4.1 and the specific growth rate (µ) was calculated at exponential phase of growth using the following equation (Widdel, 2010):

$$\mu = \frac{2.303\ (\log \text{CFU/ml}_2 - \log \text{CFU/ml}_1)}{(t_2 - t_1)}$$

2.5. General techniques

2.5.1. Agarose gel electrophoresis

Samples were run in 1-1.5% (w/v) agarose and 1X TAE buffer. The agarose was heated until the agarose had fully dissolved which was apparent by the production of bubbles and the transition to a clear liquid. The solution was then allowed to cool temporarily to reduce the production of ethidium bromide vapour formation upon its addition to hot agar. Next, ethidium bromide was added to a final concentration of 0.5 µg/ml and gently mixed. The gel was then poured into a tray sealed with autoclave tape and a well forming comb which allowed the formation of distinct wells as the gel cooled and set. Once the gel was set, the comb was carefully removed with care taken to not damage the wells formed. The tape used to seal the tray was also removed before transfer of the gel to the electrophoresis tank.

The gel was then submerged in 1X TAE buffer and wells were loaded with DNA marker solution prepared from a ratio of 1 volume DNA ladder: 1 volume 6X loading

dye: 4 volumes RNase/DNase-free dH_2O. DNA ladder (100 bp or 1 kbp) was added to the wells to enable assessment of the size of DNA fragments. The gel was then run at 80-120 V for approximately 30-45 minutes and visualised under UV-light. The image was photographed using ImageQuant 100 (GE healthcare) with IQuant Capture 100 software.

2.6. Statistical analyses

Graph Pad prism 7.0 was used to analyse all data. Statistical analysis was performed on a minimum of three replicates per experiment. When data was normally distributed and more than two data sets were being analysed, a one-way ANOVA followed by a Tukey's multiple comparison test was performed. When the Brown-Forsythe test revealed that differences between the variances were significant and therefore not normally distributed, data was transformed to its logarithm to make the data more Gaussian and a one-way ANOVA was performed again. If the Brown-Forsythe test still revealed differences in variance, then a Kruskal-Wallis test followed by a Dunn's multiple comparisons test was performed. For analysis between two groups, statistical differences between the groups were determined for parametric data, via an unpaired or paired t-test, or for non-parametric data, a Mann-Whitney test. For comparison of two independent variables where data was normally distributed, a 2-way ANOVA followed by a Sidak's or Tukey's multiple comparison test was performed. Statistical significance was determined when the p-value was less than 0.05.

Chapter 3. Sputum processing and growth assays

3.1 - Introduction and aims

The first aim of this project was to establish growth conditions for the reference strains to be grown in sputum from cystic fibrosis patients. *S. pneumoniae* was initially investigated due to its unknown involvement in CF pathogenesis since it is difficult to culture from patient sputum (Maeda et al, 2011). Suggestive of its potential involvement in CF disease is its proven ability to utilise PGM as a nutrient source and that it is known to upregulate MUC5AC (Yesilkaya et al, 2008; Lim et al, 2009). The literature suggests an underlying role of *S. pneumoniae* which is so easily overwhelmed by other pathogens in CF, such as *P. aeruginosa*. For this reason, *P. aeruginosa* was the primary focus of this study.

In dealing with the patient sputum, the aim was to remove smaller molecular weight glycoproteins which are already degraded by serine proteases (Henke et al, 2011). This is in addition to sugars such as glucose; argued to be a main factor in the growth of *Pseudomonas* in the airway (Aristoteli and Willcox, 2003; Garnett et al, 2013). DNA was measured because it is known to be at high concentrations in CF sputum (Brandt et al, 1995; Lethem et al, 1990). It was therefore necessary to measure the concentration of glucose, protein, and DNA within the samples. Low molecular weight components needed to be removed prior to growth studies so that it could be determined that the growth of organisms in the sputum was not due to utilisation of previously degraded components. Differences in growth rate in adult and paediatric samples could allude to potential differences between adult and paediatric cystic fibrosis mucin structure.

3.2. Methods

3.2.1. Preparation of porcine gastric mucin (PGM) for growth studies

PGM was initially used to investigate growth of *S. pneumoniae* and *P. aeruginosa* as it has in other studies (Yesilkaya et al, 2008; Aristotelli and Willcox, 2003). It was weighed and 10 g was mixed with 200 ml dH_2O. Once solubilised it was then dialysed at 4^{o}C using 10 kDa Snakeskin™ membrane against a large volume of dH_2O in a beaker

containing a magnetic stirrer. Water was exchanged initially after 2 hours, then again after 4 hours, 6-8 hours, and then once more before being left overnight. This was to remove low molecular weight sugars such as glucose from the samples (Yesilkaya et al, 2008). Glass EPA vials were weighed without their lids. PGM was then lyophilised in these glass vials and covered with perforated Parafilm® 'M' film to prevent loss of sample during the initial stages of freeze-drying. The weight of the glass vial was measured after freeze-drying and the sample weight was calculated from the difference of the two measurements. Once freeze-dried, samples were stored at -20^{o}C until needed for growth studies.

3.2.2. Collection and preparation of sputum for growth studies

Non-induced expectorated sputum samples from CF patients were collected in sterile sputum pots at clinics in Glenfield Hospital and the Leicester Royal Infirmary (LRI). Samples were assigned a number, transported on ice, and frozen at -80^{o}C within 1 hour to prevent degradation of the sputum environment (Horsley et al. 2013).

Healthy patients were recruited as part of study conducted by Prof. C Brightling. Sputum induction was kindly performed by Dr. Kathryn Staley using the following method (Pavord et al, 1997). Expectoration was induced via inhalation of 3% (v/v) saline which was prepared by adding 2.1 ml of 7% (v/v) saline stock to 2.9 ml of sterile dH_2O. Since saline inhalation can cause chest tightness, lung function was assessed by spirometry to check the FEV_1 (forced expiratory volume in the first second) prior to inhalation. Subjects were advised to discontinue inhalation at any point during the procedure if they experienced discomfort. Saline was then inhaled through a nebuliser over a period of 5 minutes (figure 3.1). Patients were encouraged to cough up any sputum into a provided sputum pot during this period if they could. Lung function was assessed between each use of the nebuliser. If FEV_1 remained stable, i.e. a reduction in FEV_1 of less than 10%, then further inhalations could occur. However, the collection was discontinued if FEV_1 values fell greater than 20% following inhalation of the saline. With FEV_1 values falling below 10-20%, the sputum induction could proceed, but only with the same concentration of saline being provided. The total duration of the inhalations was not allowed to exceed 15 minutes in any instance. Finally, the effects of

the saline, such as chest tightness and cough, were reversed via the inhalation of salbutamol through a spacer. All sputum collected was assigned a number, stored on ice, and frozen at -80^{o}C within 1 hour. When required for experiments, sputum was dialysed and lyophilised as described in section 3.2.1.

Figure 3.1.

Figure 3.1: Sputum induction demonstration. Image showing demonstration of subject (printed with permission) during inhalation of the saline through the nebuliser.

Table 3.1: Origin of sputum samples used in the study.

	'Healthy' adult samples	Paediatric CF samples	Adult CF samples
Total number of subjects	7	17	20
Number of samples from male patients	3	6	9
Number of samples from female patients	4	11	11
Number of BAL samples	0	5	0
Number of induced sputum samples	7	0	0
Number of non-induced sputum samples	0	12	20
Mean age of patients at the time of sputum collection	34.9	9.8	29.8

3.2.2.2. PGM and sputum preparation prior to growth studies

When samples were required for growth studies, the lyophilised samples in the glass vials were re-suspended in nH_2O based on their freeze-dried weight to the required concentration. Potassium phosphate buffer (1 M, pH 7) was added to the PGM/sputum to a final concentration of 10 mM. Its presence prevents pH-induced cleavage of mucin monosaccharide residues during autoclaving (Beighton et al, 1988; Aristoteli and Willcox, 2003; Yesilkaya et al, 2008). The role of potassium phosphate buffer was investigated during preliminary experiments (table 3.4). Samples were then sterilised by autoclaving at 121^{o}C, 15 psi for 15 minutes.

For investigation of which concentration to use in growth assays, PGM was suspended in nH_2O to either 2, 4, 6, 8, or 10 mg/ml and sterilised via autoclaving as previously

described. PGM was then added to an equal volume of double strength Sicard's/M9 (described in chapter 2), so the final concentration of PGM would be 1 (0.1%), 2 (0.2%), 3 (0.3%), 4 (0.4%), or 5 (0.5%) mg/ml respectively in single strength medium for growth assays.

Prior to growth experiments, double strength Sicard's or M9 were mixed in equal parts with 8 mg/ml sputum/PGM to obtain a final concentration of 0.4% (w/v) (4 mg/ml). This concentration was decided based on optimisation experiments as described in table 3.4.

3.2.3. Preparation of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* prior to growth in PGM and sputum samples

S. pneumoniae D39, *P. aeruginosa* PA01, PA14 and CF clinical isolates were all grown in 0.4% (w/v) PGM, adult CF sputum, and paediatric CF sputum supplemented with Sicard's. *P. aeruginosa* PA01 and PA14 were also grown in 0.4 (w/v) PGM and CF sputum supplemented with M9.

Aliquots of *S. pneumoniae* D39 stock were thawed and centrifuged at 13000 rpm for 2-3 minutes. The supernatant was discarded and the bacterial pellet re-suspended in 3 ml of PBS. Then, 45 µl of inoculum was added to 3 ml of medium in a 7 ml bijou with a sealed cap and statically incubated at 37^{o}C.

P. aeruginosa aliquots were thawed the day before growth studies and streaked to single colonies onto Luria agar plates to check for contamination. These were then incubated overnight at 37^{o}C in aerobic conditions. After 24-36 hours, *P. aeruginosa* was re-suspended in 3 ml PBS until OD_{600} ~ 0.18. A total of 5 µl of *P. aeruginosa* in PBS was then added to each 3 ml of medium (1:600) before incubation on a shaking platform at 37^{o}C, 200 rpm.

3.2.4. Antibiotic spiking experiments

To investigate whether the presence of antibiotics was a factor in influencing the growth of bacterial species, *S. pneumoniae* was grown in PGM with the addition of autoclaved or filter-sterilised antibiotic. PGM was used as a control sample to avoid wastage of

patient samples. Pen-G, vancomycin, streptomycin, kanamycin, ceftriaxone, erythromycin, and chloramphenicol were all prepared to a stock concentration of 10 mg/ml in nH_2O. Antibiotic stocks were sterilised via passage through a 0.2 µm acrodisc® filter near a Bunsen flame or were autoclaved under conditions previously described. Antibiotics were then prepared to final concentrations of 2, 20 or 200 µg/ml in sterile 0.8% (w/v) PGM. PGM with antibiotic was then added to an equal volume of double strength Sicard's to get a final concentration of antibiotic at 1, 10, or 100 µg/ml in 0.4% (w/v) PGM supplemented with Sicard's. Growth studies were performed as described in section 2.4.1 (Miles et al, 1938).

3.2.5. Assessment of DNA in sputum samples

The quantity of DNA was assessed by Nanodrop One spectrophotometer using the corrected values generated when contaminants such as protein and sugars are present. DNA was measured because it is at high concentrations in CF sputum (Brandt et al, 1995; Lethem et al, 1990).

3.2.6. Assessment of glucose in sputum samples

The glucose (GO) assay kit (Sigma) was used to determine the amount of glucose in unprocessed and processed samples. Processed samples were those which were dialysed before being lyophilised whilst unprocessed samples were not dialysed. Glucose measurements were required to determine levels of glucose in unprocessed samples and to be aware of glucose concentrations in processed samples. This is because glucose is known to affect bacterial growth (Garnett et al, 2013). Therefore, to eliminate glucose being a nutrient source in the culture it had to be confirmed that it had either been removed or that the concentration measured in the samples would not promote growth.

A capsule from the kit containing 500 units of glucose oxidase and 100 purpurogallin units of peroxidase with buffer salts was dissolved in 39.2 ml of nH_2O to form the Glucose oxidase/peroxidase reagent. This was then added to 0.8 ml of the o-dianosidine reagent and mixed by inverting several times. The bottle was covered in foil to minimise exposure to the light. A 300 µl volume of the sputum samples at a concentration of 8 mg/ml were then diluted to 4 mg/ml with the addition of an equal

volume of nH_2O before being passed through a 10 kDa centrifugal filter. Samples were centrifuged at 14000 x g for 10 minutes until all 600 µl of the samples had passed through the filter. If glucose was present in the samples then a pink solution was produced as explained by the calculations in table 3.3. To ensure that each assay was successful, a series of glucose standards were prepared as indicated in table 3.2.

Table 3.2: Volume of water and glucose standard needed to make the standards for the glucose assay

Tube	Water (µl)	Sample (µl)	Glucose Standard (µl)
Reagent Blank	500	-	-
Standard 1	490	-	10
Standard 2	480	-	20
Standard 3	470	-	30
Standard 4	460	-	40
Test sample	-	500	-

To start the assay 1 ml of the assay reagent was added to the first tube (reagent blank), and mixed. A time interval of 30-60 seconds was allowed between additions of assay reagent to the subsequent sample tubes being tested: standard 1, 2, 3, 4, and test sputum samples. Hydrogen peroxide produced in this reaction then reacts with o-dianisidine and is converted to a brown solution (reaction 2, table 3.3). After 30 minutes at 37^oC, to stop the reaction 1 ml of 12 N H_2SO_4 (reaction 3, table 3.3) was then added to each tube followed by the same 30-60 second intervals between the tubes. The intensity of the pink solution formed was proportional to the original glucose concentration in the sample. Absorption was measured at 540 nm against the reagent blank. Absorbance at 540 nm of the standards was then plotted against the known glucose concentrations in the standards. The results of the test sputum samples were determined from the standard curve or calculated from the calculation in table 3.3.

Table 3.3: Glucose assay reactions and calculation

Reaction 1. Glucose oxidase		
D-glucose + H_2O + O_2	→	D-gluconic acid + H_2O_2
Reaction 2. Peroxidase		
H_2O_2 + Reduced o-dianisidine (colourless)	→	Oxidized o-dianisidine (brown)
Reaction 3. H_2SO_4		
Oxidized o-dianisidine (brown)	→	Oxidised o-dianisidine (pink)
Glucose assay calculation: $\text{mg Glucose} = \frac{(\Delta A540 \text{ of Test})(\text{mg Glucose in Standard})}{\Delta A540 \text{ of Standard}}$		

3.2.7. Assessment of protein in sputum samples

Protein was measured in samples to determine whether growth differences correlated with availability of protein present in the sputum. *P. aeruginosa* has previously been shown to grow in cultures with high abundance of amino acids (Barth and Pitt, 1996; Palmer et al, 2005; Palmer et al, 2007). Since amino acids make up the protein backbone of mucins, and mucins are known to be degraded, it was important to measure protein content, since a decrease in protein measurement before and after growth could indicate that protein is being used as a nutrient source for growth (Henderson et al, 2014).

To measure the protein content in unprocessed and processed 'healthy' adult, paediatric CF, and adult CF sputum, the CB-X protein assay from G-Biosciences was used.

Before use, the CB-X reagent was chilled at - 20°C. Provided BSA standard was diluted to the following concentrations; 0, 0.2, 0.4, 0.6, 0.8, 1.0 µg/µl, and 50 µl of each was transferred to separate 1.5 ml microtubes before the addition of CB-X reagent. For all concentrations, this was performed in duplicate. The tubes were then vortexed briefly, followed by centrifugation for 5 minutes at 16000 x g. The supernatant containing any interfering agents was removed with care taken to not disturb the protein pellet. Following this, 50 µl of both CB-X solubilisation buffer I and II were added to the tube and vortexed for up to 10 minutes. The assay dye was then gently inverted several times before adding 1 ml to each tube and mixed again by vortexing. The tubes were then incubated at room temperature for 5 minutes before being transferred to cuvettes with a 1 cm light path. Absorbance was then read on a spectrophotometer at 595 nm against nH_2O. From the readings collected, the line equation was used to generate a CB-X table to allow all future assays to be performed without repeated protein standards. When this experiment was performed with patient sputum samples, sample volumes ranging between 5-100 µl were used. The initial sample volume was used to estimate the protein concentration (µg/µl) by dividing the amount of protein (µg), as read from the CB-X table generated based on the absorption at 595 nm, by the sample volume (µl).

3.3. Results

3.3.1. Preliminary experiments

Several preliminary experiments were needed for this thesis as described in table 3.4.

Aim	Why was this needed?	How was this achieved?	What was found?
Determine method of sterilisation for sputum samples	Previous studies have stated that mucin which is sterilised by autoclaving does not affect the "biological activity of its constituents" (Beighton et al, 1988; Aristoteli and Willcox, 2003). Others have stated that the addition of 10 mM potassium phosphate buffer protects the mucin from pH-induced cleavage of the monosaccharides which occurs during autoclaving (Yesilkaya et al, 2008; Terra et al, 2010; Roberton and Stanley, 1982). Use of potassium phosphate buffer is therefore desirable since it is expected that pathogens will favour growth on degraded mucin *in vivo* vs. intact mucins which would be assumed to be more difficult to degrade. An alternative method of sterilisation is via filter sterilisation through a through a 0.45 µm acrodisc® (Beighton et al, 1988). Since studies have used different methods to sterilise sputum/PGM prior to growth studies, this work was necessary to determine how processing of the samples affects bacterial growth.	*Pseudomonas aeruginosa* PAO1 was grown in 0.4% (w/v) PGM supplemented with M9. The PGM was then either sterilised via autoclaving in the presence of potassium phosphate buffer, autoclaved in the absence of potassium phosphate buffer, or filter-sterilised through a 0.45 µm acrodisc®. The growth was assessed via CFU counts (Miles et al, 1938). Samples of each were reserved for freeze-drying. with the initial weight of the glass vial being recorded. The sample was frozen at -80°C for 10 minutes so that the sample was frozen prior to freeze-drying to prevent loss of liquid during the initial part of the freeze-drying process. This ensures weight of lyophilised sample is representative of PGM available to PAO1 during the growth assay. Measurement of the weight of the glass vial after freeze-drying meant that the weight of PGM in the sample could be recorded.	There was an increased growth rate of PAO1 when grown in PGM which had been autoclaved in the absence of buffer compared to when buffer was present or the PGM was filter-sterilised. It was observed that the filter-sterilised samples were more transparent than the autoclaved samples. This was thought to be due to a loss of mucin within the filter. This was confirmed following measurements of the glass vials after freeze-drying. Filter-sterilisation resulted in a significant reduction in PGM in the sample.
Determine medium to supplement sputum in growth studies	To determine differences in growth rate of *P. aeruginosa* when grown in samples supplemented with Sicard's or M9 medium. This is because Sicard's was selected for growth studies of *S. pneumoniae* D39 based on previous studies (Yesilkaya et al, 2008; Terra et al, 2010).	*P. aeruginosa* PAO1 was grown in 0.4% (w/v) PGM supplemented with M9 or Sicard's. The growth was assessed via CFU counts (Miles et al, 1938).	No difference in growth rate of *P. aeruginosa* PAO1 when grown in PGM supplemented with Sicard's vs. M9

Determine concentration of mucin/sputum to use in growth studies	The nature of patient samples is that they can vary in their volume and weight. For this reason, conserved usage of samples was favourable so that enough sample would be available for gene expression experiments at the end of this study. It was decided to select a concentration where favourable growth was observed. A higher concentration would only be selected if there was a clear benefit as indicated by the growth studies.	*P. aeruginosa* PAO1 and PA14 were grown in 0.1, 0.2, 0.3, 0.4 or 0.5 % (w/v) PGM supplemented with M9 or Sicard's. The growth was assessed via CFU counts (Miles et al, 1938).	No difference in growth rate of PAO1 or PA14 when grown in different concentrations of PGM when supplemented with M9 or Sicard's.
Determine whether autoclaving antibiotics reduces ability to kill bacteria	Because autoclaving was selected as the method to sterilise samples, it was necessary to determine its effect on antibiotics present in the sample. It was questioned whether differences in growth could be due to residual contaminating antibiotics present within sputum samples at the time of spontaneous expectoration. Most patients are on some form of antibiotic treatment necessary to treat reoccurring infections. It was therefore necessary to identify whether samples which were autoclaved and were known to contain antibiotics were affected via the sterilisation process. It was necessary to determine whether the antibiotic effect remained following autoclaving.	*S. pneumoniae* was grown in PGM with Sicard's with the addition of autoclaved or filter-sterilised antibiotic. PGM was used as a control sample to avoid wastage of patient samples. PGM samples were prepared as previously described in section 3.2.1. The following antibiotics were prepared to 10 mg/ml: pen-G, vancomycin, streptomycin, kanamycin, ceftriaxone, erythromycin, and chloramphenicol, in nH_2O and sterilised via passing through a 0.2 µm acrodisc® filter, or were autoclaved before adding to a final concentration of 2, 20 or 200 µg/ml in sterile 0.8% (w/v) PGM. PGM with antibiotic was then added to an equal volume of double strength Sicard's to get a final concentration of antibiotic of 1, 10, or 100 µg/ml in 0.4% (w/v) PGM supplemented with Sicard's. The growth was assessed via CFU counts (Miles et al, 1938).	The data revealed variability in how the autoclaving procedure affects the efficacy of antibiotics.

Identify differences in growth of *Pseudomonas aeruginosa* PAO1, PA14, and clinical isolates in Sicard's, PGM, and CF sputum	Growth differences could be indicative of differences in sputum composition.	*P. aeruginosa* reference strains, PAO1 and PA14, were grown in 0.4% (w/v) PGM, adult CF sputum, or paediatric CF sputum supplemented with Sicard's. Five *Pseudomonas* isolates which were isolated from CF patient sputum were also grown in Sicard's alone, PGM, and CF sputum. The growth was assessed via CFU counts (Miles et al, 1938).	Data revealed increased growth rates of PAO1 and PA14 when grown in adult vs. paediatric CF sputum. Both reference strains could also grow in Sicard's alone. There were many differences in the growth rates and ‹yields after 24 hours when the clinical isolates were grown in Sicard's alone, PGM, and CF sputum. Suggests different nutritional requirements of the isolates.

Based on the preliminary experiment results, it was decided that:

1) Autoclaving in the presence of 10 mM potassium phosphate buffer was the best method for sterilising sputum.
2) The concentration of 0.4% (w/v) would be used for growth studies since no difference was observed between growth of *P. aeruginosa* PAO1 and PA14 when grown at different concentrations. It was also the same concentration as used in other studies (Yesilkaya et al, 2008; Terra et al, 2010).
3) The study would proceed using sputum regardless of the presence of antibiotics since bacterial growth had been observed in the CF sputum samples.
4) It was decided that Sicard's was not an appropriate medium for investigating the growth of *P. aeruginosa* even though a statistically significant increase in growth rate had been observed in adult vs. paediatric CF sputum. (See appendix 1). This was because considerable growth was observed when grown in Sicard's alone.
5) M9 would be used as the medium for growth of *Pseudomonas aeruginosa*. Sicard's would be used for the growth of *Streptococcus pneumoniae* D39.

3.3.2. Growth assays

3.3.2.1. Growth of *Streptococcus pneumoniae* in PGM and CF sputum supplemented with Sicard's

Following identification of a suitable method to process samples and identification of a concentration to use in growth studies, the next aim of this project was to grow bacterial species in 0.4% (w/v) PGM/CF sputum and assess growth via CFU counts (Miles et al, 1938).

Figure 3.2a. shows the log CFU/ml growth of *S. pneumoniae* D39 in PGM, adult CF, and paediatric CF sputum. Results of this growth assay revealed that the longest lag phase was observed when grown in Sicard's without any mucin present. The next longest lag phase of D39 was observed when grown in adult CF sputum followed by paediatric CF sputum and PGM. The growth profile reveals that the log CFU/ml growth yield after 15 hours appears to be greater when grown in paediatric CF sputum in

comparison to adult CF sputum. A one-way ANOVA on the log growth yield data (figure 3.2c.) revealed that this increase in growth yield in paediatric sputum was significant compared to the yield in adult sputum (*$p < 0.05$). Interestingly, whilst it was found that the growth yield of D39 in paediatric CF sputum and PGM was significant compared to the yield in Sicard's alone, this was not the case with adult CF sputum. Statistical analysis revealed that there was no significant difference in growth yield of D39 when grown in adult CF sputum compared to Sicard's alone ($p > 0.05$). Notably the error bars were larger when D39 was grown in adult CF sputum and therefore the larger error is likely to be affecting statistical analysis. Furthermore, when a one-way ANOVA was performed on the raw CFU/ml data, the growth yield of D39 was only statistically significant in the paediatric samples compared to Sicard's alone (****$p < 0.0001$, figure 3.2d.). However, analysis of the raw data revealed that the difference in growth yield of D39 after 15 hours in adult and paediatric CF sputum was even more statistically significant (****$p < 0.0001$), compared to when performed on the log growth yield data (*$p < 0.05$).

Seemingly, the growth yield data suggests that pneumococcus cannot grow in adult CF sputum. However, when observing the growth profile in figure 3.2a., visually the growth rate does appear to be increased in the adult samples compared to Sicard's alone as evidenced by the steeper gradient of the pink line representing D39 growth. Figure 3.2b. shows the calculated specific growth rate of D39 when grown in the samples. It was observed that the greatest growth rate was observed in PGM followed by adult and then paediatric CF sputum. The difference in growth rate of D39 in adult vs. paediatric CF sputum was shown to be not statistically significant ($p > 0.05$). When a one-way ANOVA followed by a Tukey's multiple comparison test was conducted on all data sets analysis revealed that the difference in growth rate of D39 in PGM (****$p < 0.0001$), adult CF sputum (****$p < 0.0001$), and paediatric CF sputum (***$p < 0.001$), was significant compared to the growth rate of D39 when grown in Sicard's alone. Whilst this ANOVA revealed that there was no significant difference in growth rate of D39 when grown in adult vs. paediatric CF sputum, when an unpaired two-tailed t-test was carried out on the two data sets, the difference in growth rate was found to be significant (*$p < 0.05$).

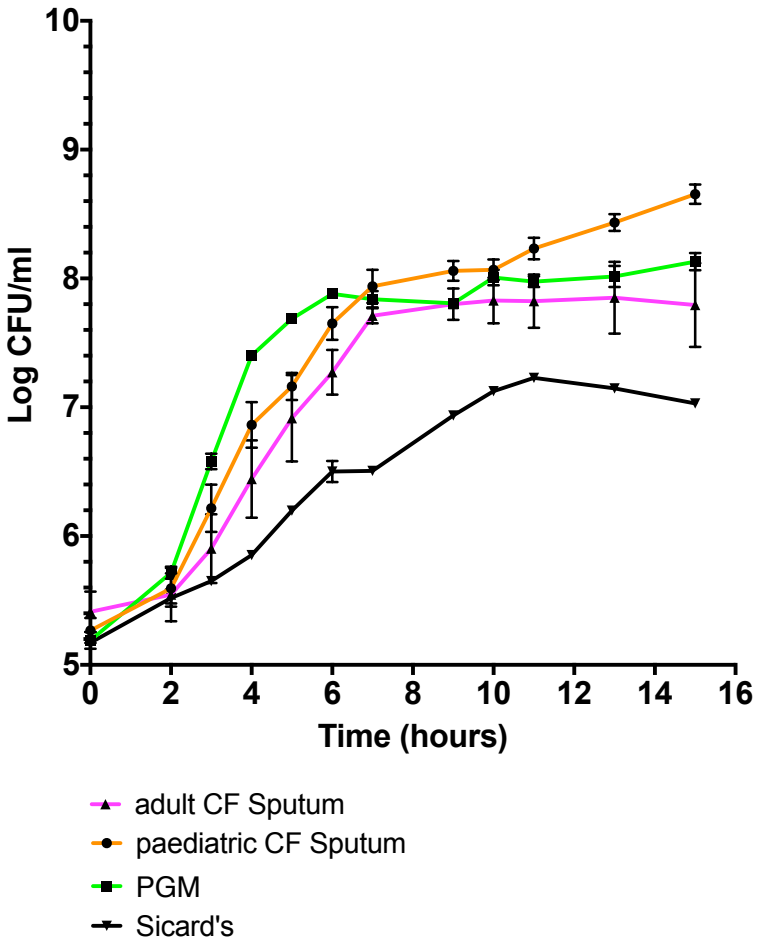

Figure 3.2a.

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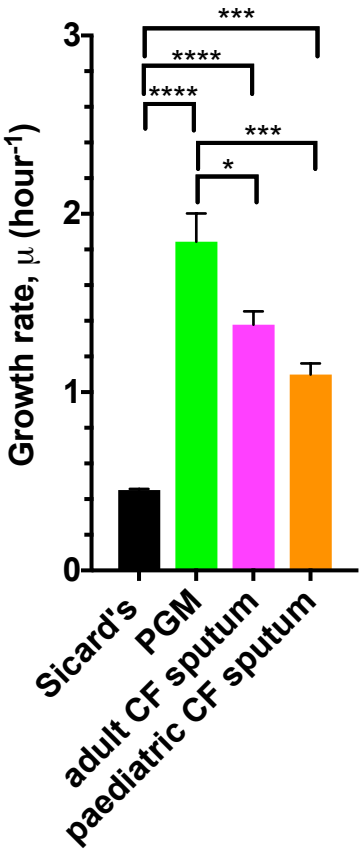

Figure 3.2b.

See legend on the next page

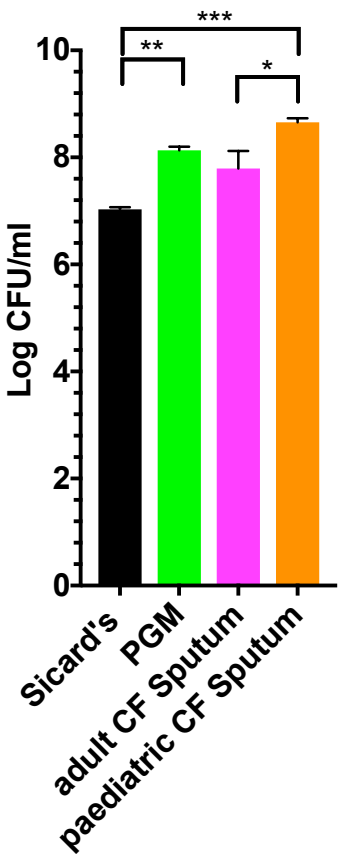

Figure 3.2c.

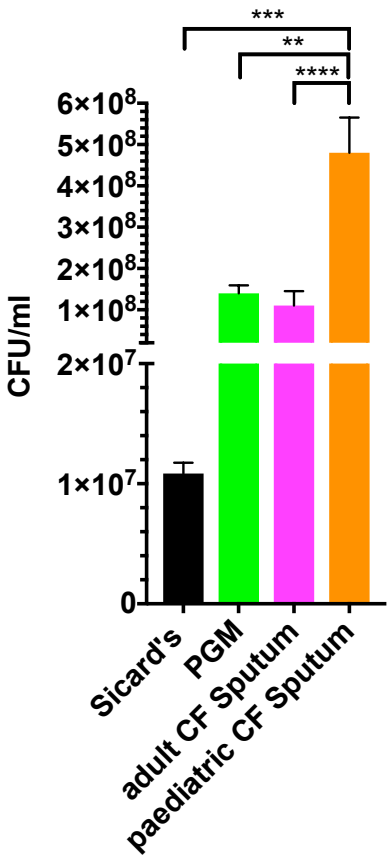

Figure 3.2d.

Figure 3.2: a) Growth of *Streptococcus pneumoniae* D39 (log CFU/ml) over 15 hours in 0.4% (w/v) PGM/sputum supplemented with Sicard's. The pink (adult CF sputum) and orange (paediatric CF sputum) lines indicate the mean log CFU/ml from 5 biological replicates at each time point whereas the green (PGM) line indicates the mean log CFU/ml from 4 biological replicates. b) Mean specific growth rate (μ, hour^{-1}) of *S. pneumoniae* D39 calculated from each replicate. c) Mean log growth yield (log CFU/ml) of D39 after 15 hours calculated from each replicate. d) Mean growth yield (CFU/ml) of D39 after 15 hours. Error bars show standard error of the mean (SEM) of replicates. P values on the graphs (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

3.3.2.2. Growth of *Pseudomonas aeruginosa* in M9

Since differences in growth rates of the reference strains, PAO1 and PA14, had been observed in adult vs. paediatric CF sputum when supplemented with Sicard's (see table 3.4), it was decided at this point in the study to change the medium used to supplement the sputum samples.

Although Sicard's was suitable for investigating the growth of *S. pneumoniae*, analysis of the growth of the isolates in sputum samples supplemented with Sicard's revealed the difficulty in comparing between the isolates. Although it sufficed to reveal differences in bacterial growth rates between adult and paediatric CF sputum, because it was demonstrated that there was a clear ability of *Pseudomonas* reference strains and clinical isolates to grow very well in Sicard's alone, it could not be deemed an appropriate negative control to compare growth against (see appendix 1). Therefore, Sicard's was changed to M9, a minimal medium, for growth of *P. aeruginosa* (LaBauve and Wargo, 2012).

3.3.2.3. Growth of *Pseudomonas aeruginosa* reference strains PAO1 and PA14 in processed cystic fibrosis sputum supplemented with M9

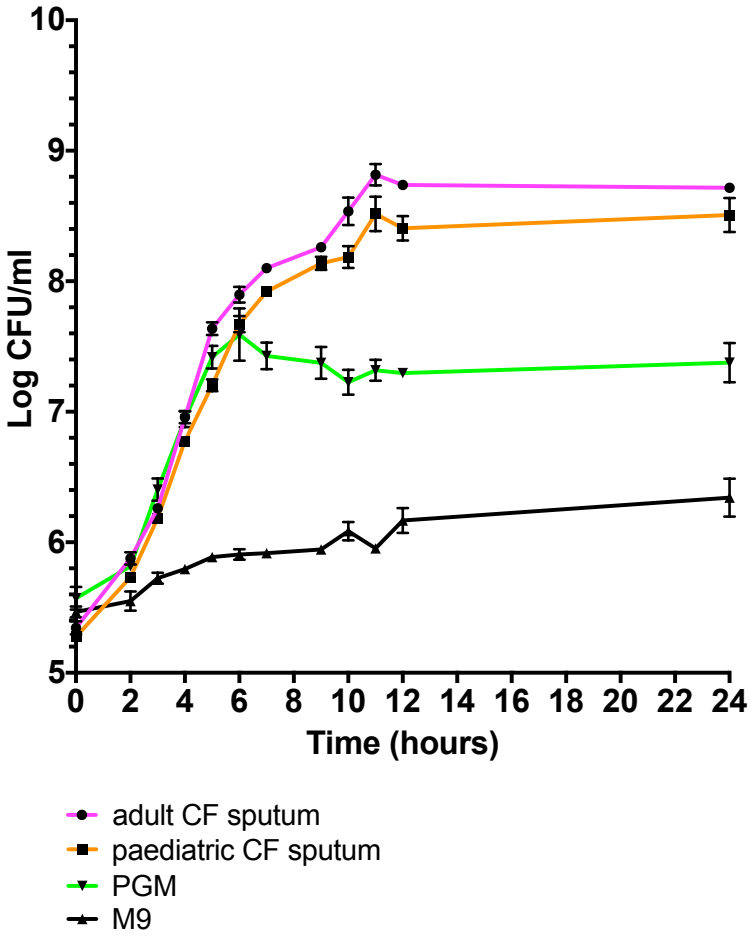

Figure 3.3a.

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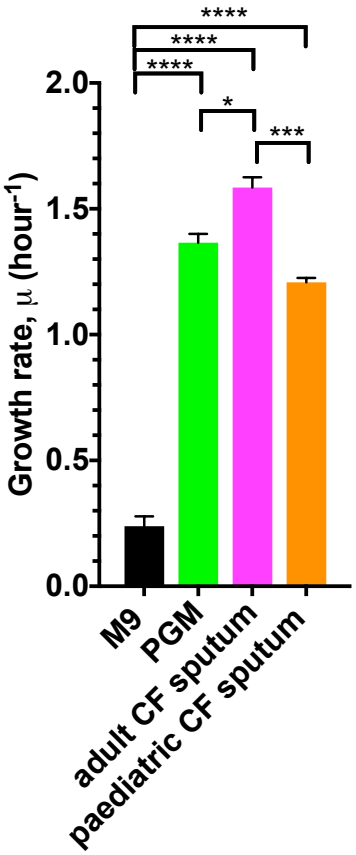

Figure 3.3b.

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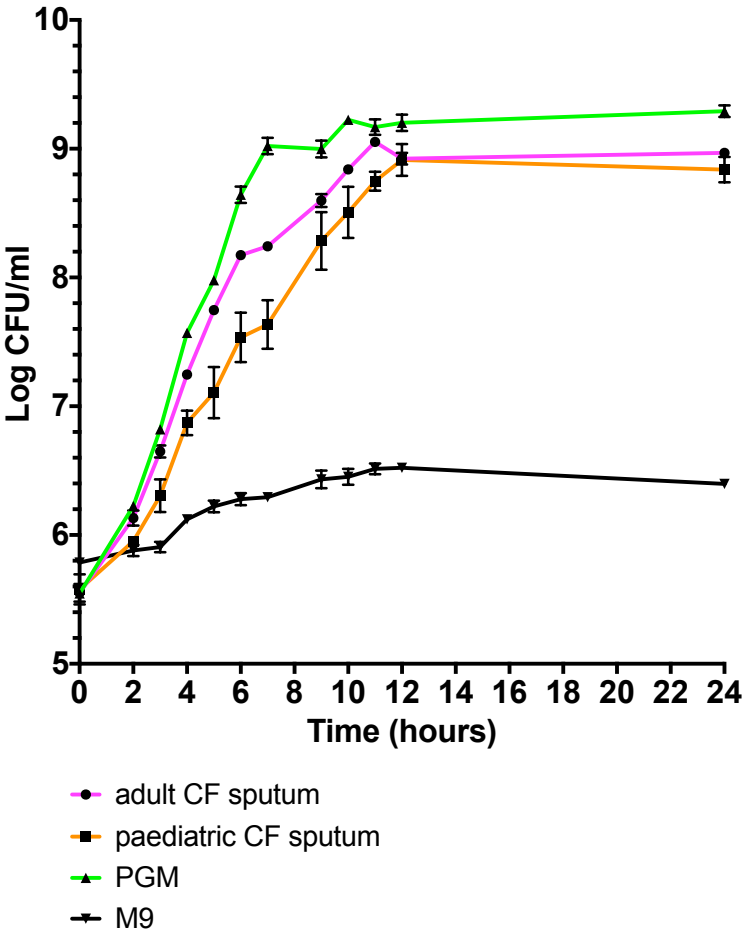

Figure 3.3c.

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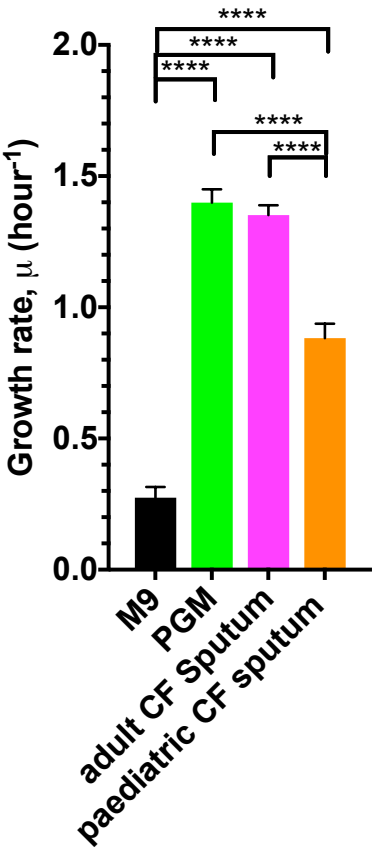

Figure 3.3d.

Figure 3.3: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.4% (w/v) PGM/CF sputum supplemented with M9. The pink (adult CF sputum) and orange (paediatric CF sputum) lines indicate the mean log CFU/ml from 4 biological replicates respectively at each time point whereas the green (PGM) line indicates the mean log CFU/ml from 6 biological replicates. All samples were supplemented with M9 (black line, 10 replicates). b) Mean specific growth rate (μ, hour^{-1}) of PAO1 calculated from graph a). c) Growth of *Pseudomonas aeruginosa* PA14 over 24 hours. The pink (adult CF sputum) and orange (paediatric CF sputum) lines indicate the mean log CFU/ml from 4 biological replicates at each time point whereas the green (PGM) line indicates the mean log CFU/ml from 5 biological replicates. d) Mean specific growth rate (μ, hour^{-1}) calculated from graph c). All samples were supplemented with M9 (black line, 5 replicates). Error bars show standard error of the mean (SEM) of all replicates. P values on the graphs (**p < 0.0001, ***p < 0.001, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

When PAO1 and PA14 were grown in PGM, adult, and paediatric CF sputum supplemented with M9, in all conditions there was an increased growth rate which was significant compared to the growth rates of PAO1 and PA14 in M9 alone (****p < 0.0001, figures 3.3b. and 3.3d.). This demonstrates that M9 was more suitable as a

medium for comparing growth because the growth rates of PAO1 and PA14 in M9 were considerably lower than when grown in Sicard's. For example, PAO1 and PA14 had growth rates of 0.24 μ ($hour^{-1}$) $\pm$ 0.04 SEM and 0.27 μ ($hour^{-1}$) $\pm$ 0.04 SEM in M9, respectively. The growth profiles of PAO1 and PA14 in figures 3.3a. and 3.3c. also demonstrate minimal growth in M9 over 24 hours.

These data in figures 3.3b. and 3.3d. reveal that there were differences between the growth rates of PAO1 and PA14 in the different mucin/sputum conditions. Similarly, to growth studies using Sicard's, increased growth rates of both PAO1 (***$p < 0.001$) and PA14 (****$p < 0.0001$) were observed in adult vs. paediatric CF sputum. However, when grown in the presence of PGM, adult CF sputum, or paediatric CF sputum, there was not only an increase in growth rate which was significant, but there also appeared to be an increase in growth yield compared to the M9 control. When observing the growth profile in figure 3.3a., PAO1 appears to grow 'better' as determined by the growth yield after 24 hours in adult and paediatric CF sputum compared to PGM. In contrast, PA14 appears to grow 'better' as determined by growth yield after 24 hours in PGM, followed by adult CF sputum, and paediatric CF sputum (figure 3.3c.). Analysis via a one-way ANOVA on the raw growth yield data (CFU/ml) revealed that the variances were different in both the PAO1 and PA14 data following application of the Brown-Forsythe test (****$p < 0.0001$). Therefore, to equalise these differences in the variances, the data was converted to its logarithm to make it more Gaussian. When the ANOVA was performed again, there were no significant differences in the variances. For this reason, the log growth yield graphs are shown in figure 3.4.

Figures 3.4a. and 3.4b. represent the log CFU/ml growth yield of PAO1 and PA14 after 24 hours. Following a one-way ANOVA, the differences in growth yield, as observed in the growth profile in figures 3.3a. and 3.3c., were confirmed to be significant. For example, PAO1 had a decreased growth yield after 24 hours in PGM compared to adult CF sputum (***$p < 0.001$) and paediatric CF sputum (**$p < 0.01$). In contrast, it was confirmed that the increased growth yields of PA14 in PGM compared to adult CF sputum (**$p < 0.01$) and paediatric CF sputum (***$p < 0.001$), were also significant. Notably, whilst differences in growth rate were observed between adult and paediatric CF sputum when PAO1 and PA14 were grown in these samples, no significant

difference was found between adult and paediatric CF sputum in terms of the log growth yield after 24 hours ($p > 0.05$). Finally, with both reference strains, it must also be noted that the increased growth yields after 24 hours in PGM, adult, and paediatric CF sputum were highly significant compared to the M9 control. The p-values for all conditions were less than 0.0001, except for PAO1 in PGM vs. M9 (***$p < 0.001$). This thereby illustrates the suitability of M9 as a negative control for growth in the sputum samples.

Following the observation that there was an increased growth rate of PAO1 and PA14 in adult vs. paediatric CF sputum when supplemented with both Sicard's and M9, it was decided to continue the study in the direction of assessing growth in adult CF sputum compared to sputum obtained from healthy patients. It was therefore decided at this point to not proceed with the *S. pneumoniae* work, although presented in this thesis, because greater statistical differences were observed in terms of growth rate and yield in the results obtained for *P. aeruginosa*.

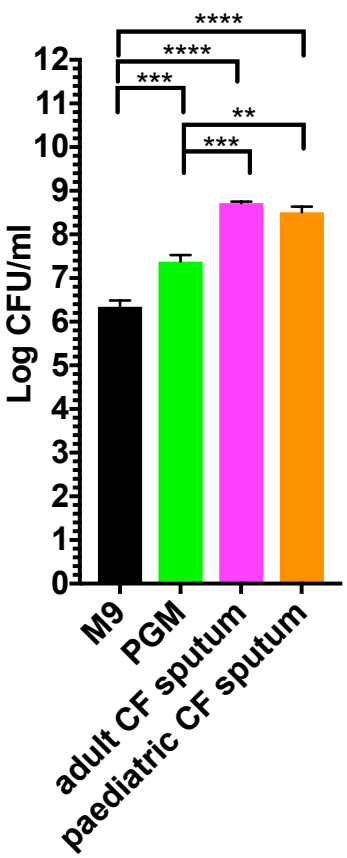

Figure 3.4a.

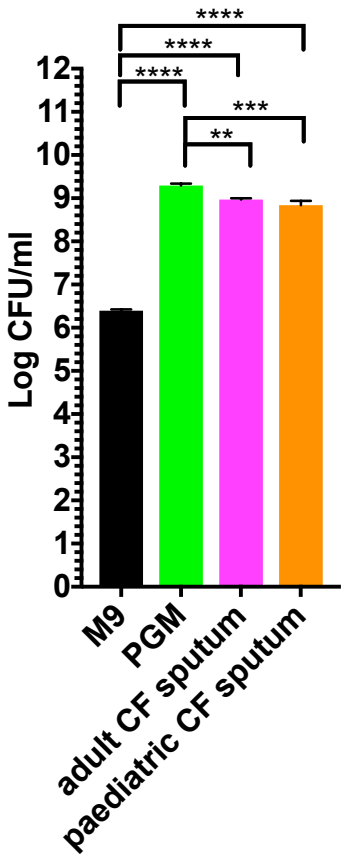

Figure 3.4b.

Figure 3.4: a) Growth yield (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 after 24 hours of growth in 0.4% (w/v) PGM/CF sputum supplemented with M9. The pink (adult CF sputum) and orange (paediatric CF sputum) bars indicate the mean log CFU/ml from 4 biological replicates whereas the green (PGM) bar indicates the mean log CFU/ml from 6 biological replicates. All samples were supplemented with M9 (black bar, 10 replicates). b) Growth yield (log CFU/ml) of *Pseudomonas aeruginosa* PA14 after 24 hours of growth in 0.4% (w/v) PGM/CF sputum supplemented with M9. The pink (adult CF sputum) and orange (paediatric CF sputum) bars indicate the mean log CFU/ml from 4 biological replicates whereas the green (PGM) bar indicates the mean log CFU/ml from 5 biological replicates. All samples were supplemented with M9 (black bar, 5 replicates). Error bars show standard error of the mean (SEM) of all replicates. P values on the graphs (**p < 0.0001, ***p < 0.001, **p < 0.01) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

3.3.2.4. Growth of *Pseudomonas aeruginosa* PAO1 in 'healthy' adult sputum compared to adult CF sputum supplemented with M9.

Since a difference in growth rate of *P. aeruginosa* had been observed in adult vs. paediatric CF sputum, it was questioned whether there would be a difference in growth rate and yield compared to sputum from healthy patients. Unfortunately, it was not possible to obtain sputum from healthy children due to the difficulty in recruiting enough healthy children to provide sputum for a study. Instead sputum was obtained from healthy adults who all gave informed consent. For the rest of this thesis, the sputum from the healthy patients will be referred to as 'healthy' adult sputum, acknowledging that the sputum itself is not healthy, and rather it is the patient from which the sputum was collected from. The sputum collected from the healthy patients was handled in the same manner as the CF samples, as previously described in section 3.2.2.

Growth in 'healthy' adult sputum vs. adult CF sputum, as shown by the data in figure 3.5c., revealed an increased growth rate of PAO1 in the adult CF sputum compared to 'healthy' adult sputum which was found to be significant when analysed by a one-way ANOVA (*$p < 0.05$). For example, the growth rate in 'healthy' adult sputum was 1.20 μ ($hour^{-1}$) $\pm$ 0.16 SEM compared to 1.59 μ ($hour^{-1}$) $\pm$ 0.04 SEM in adult CF sputum. Interestingly, figures 3.5a. and 3.5b. suggest there was no difference in growth yield of PAO1 in adult vs. paediatric CF sputum. However, a highly significant difference in growth yield was found between adult CF sputum and 'healthy' adult sputum when a one-way ANOVA was performed on the raw CFU/ml data as presented in figure 3.5d. (****$p < 0.0001$).

An interesting point is that regardless of the sputum origin, *P. aeruginosa* PAO1 was found to grow in all samples, despite originating from individuals who were healthy. Growth was also comparable to growth observed in CF sputum, although there were significant differences in terms of the growth rate (*$p < 0.05$) and the growth yield (****$p < 0.0001$). Therefore, these data suggest that *P. aeruginosa* would have the capability to grow on healthy sputum in circumstances where mucus may not be cleared efficiently as it is in healthy individuals. This is because the data in this study has found

that PAO1 is able to grow very well in both 'healthy' adult and adult CF sputum. The statistical differences, however, suggest that nutritional content in adult CF sputum differs to an extent that it allows 'better' growth in CF sputum vs. 'healthy' sputum. For this reason, at this point in the study it was necessary to attempt to identify nutrients which differ in the sample types. Since differences had been observed in processed samples, i.e. samples which were dialysed prior to growth studies, it was decided to investigate growth in unprocessed sputum samples.

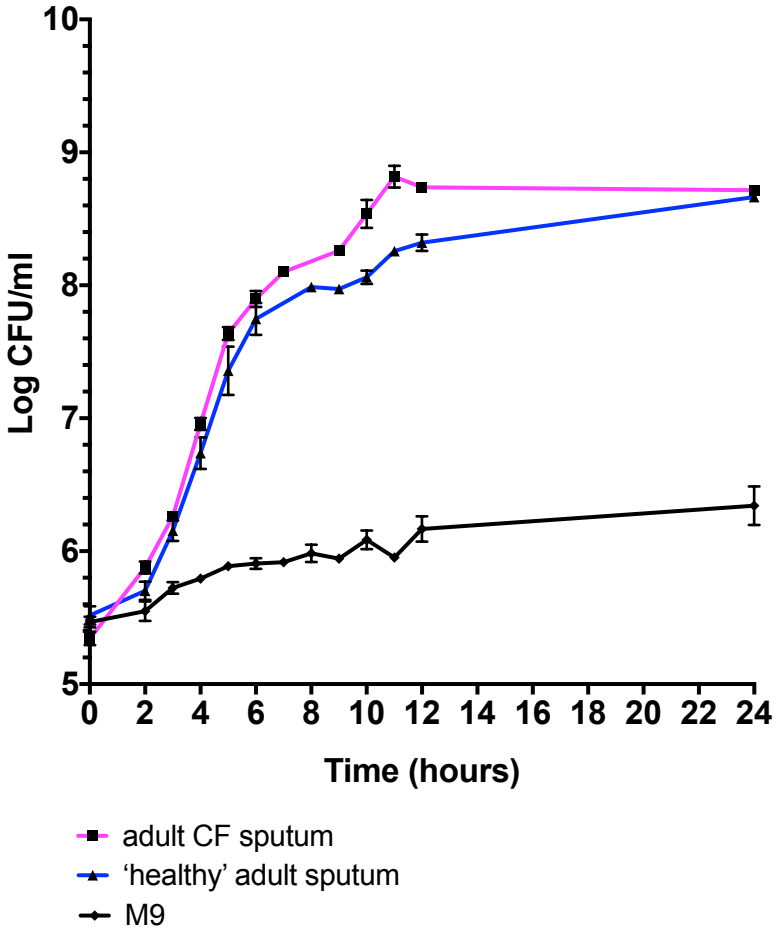

Figure 3.5a.

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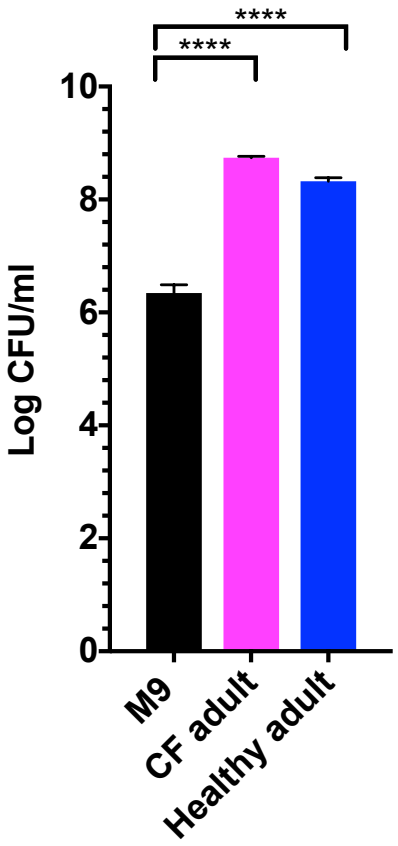

Figure 3.5b.

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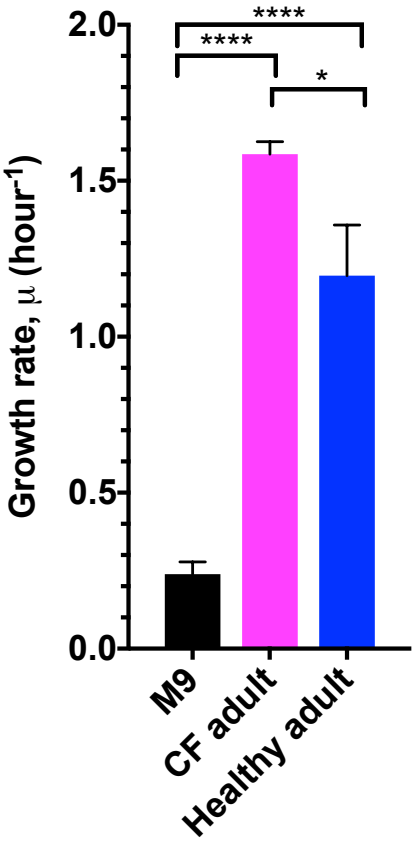

Figure 3.5c.

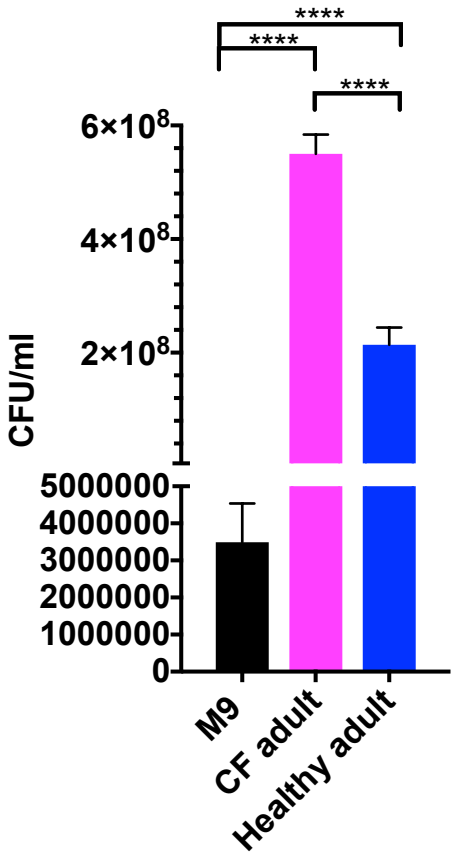

Figure 3.5d.

Figure 3.5: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.4% (w/v) processed 'healthy' adult and adult CF sputum. The pink (adult CF sputum) and blue ('healthy' adult sputum) lines indicate the mean log CFU/ml from 4 and 3 biological replicates, respectively. Samples were supplemented with M9 (black line, 10 replicates). b) Mean growth yield (log CFU/ml) after 24 hours. c) Mean specific growth rate, μ (hour^{-1}) of PAO1. d) Mean growth yield (CFU/ml) after 24 hours. Error bars show standard error of the mean (SEM) of all replicates. P values on the graphs (**p < 0.0001, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

3.3.2.5. Growth of *Pseudomonas aeruginosa* PAO1 in unprocessed 'healthy' adult sputum and cystic fibrosis sputum supplemented with M9

Until this point in the study, all growth assays had been conducted in samples which were 'processed' to remove pre-existing low molecular weight sugars. Growth had not been considered in samples which were not dialysed previously. For this next section, rather than dialysing using a 10 kDa membrane, residual samples from the same samples used in the 'processed' sputum growth assays, were thawed and freeze-dried. They were then re-suspended to the same concentration and autoclaved in the presence of potassium phosphate buffer.

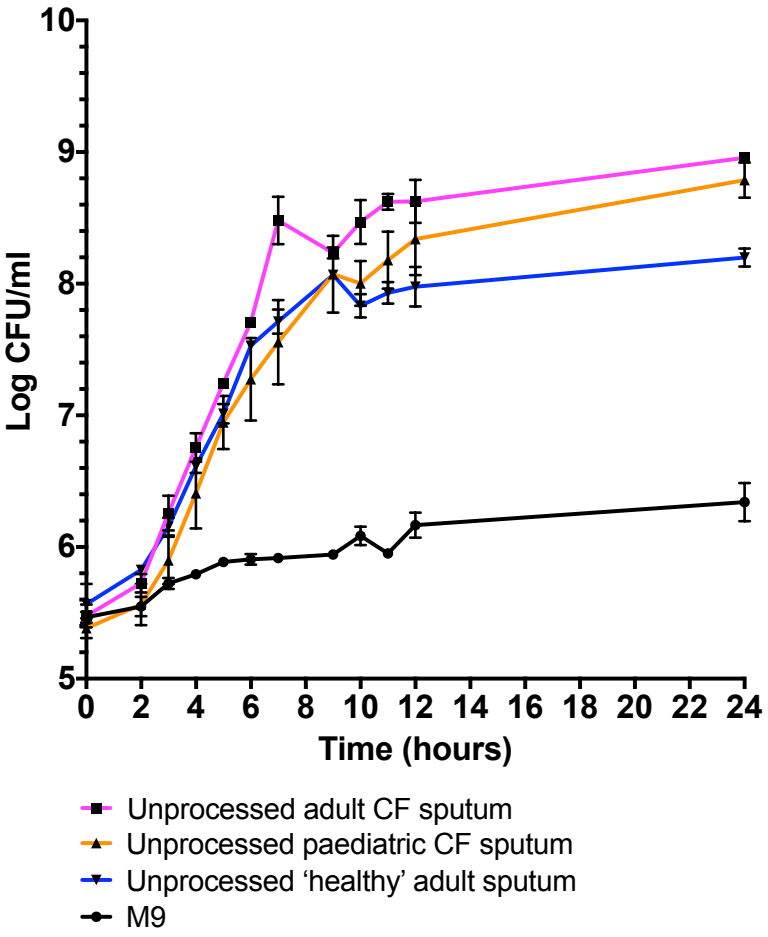

Figure 3.6a.

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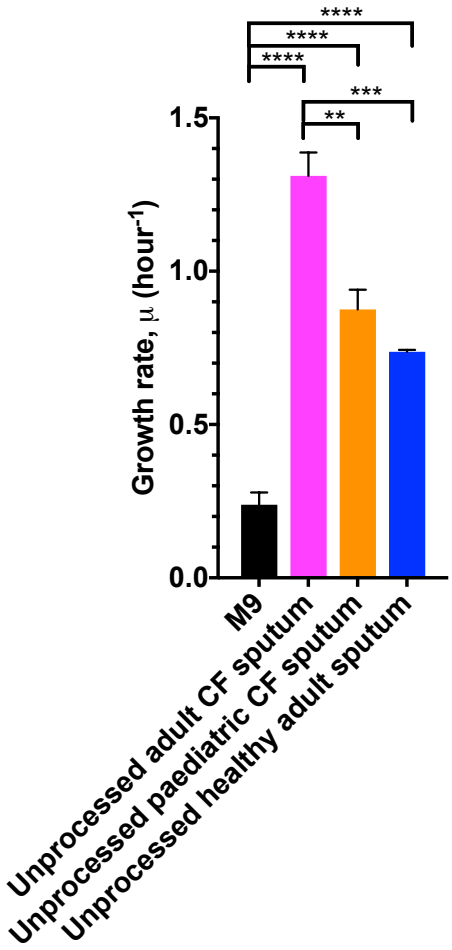

Figure 3.6b.

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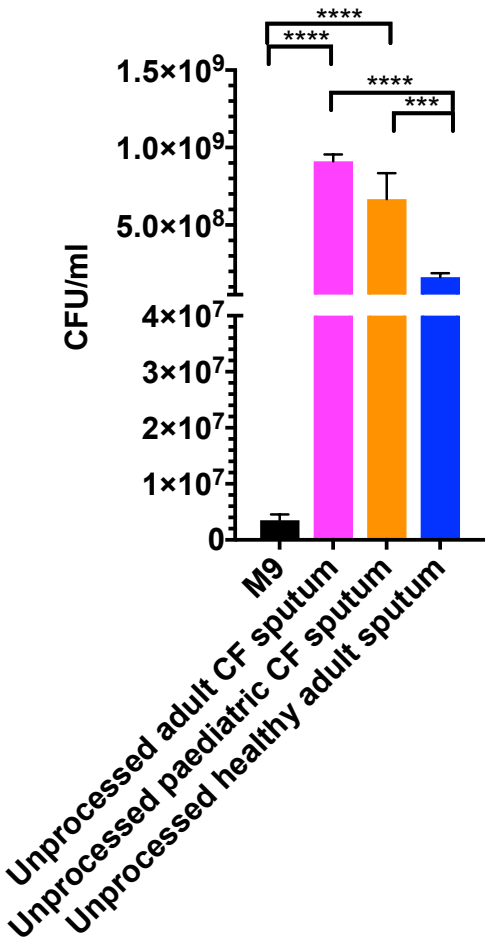

Figure 3.6c.

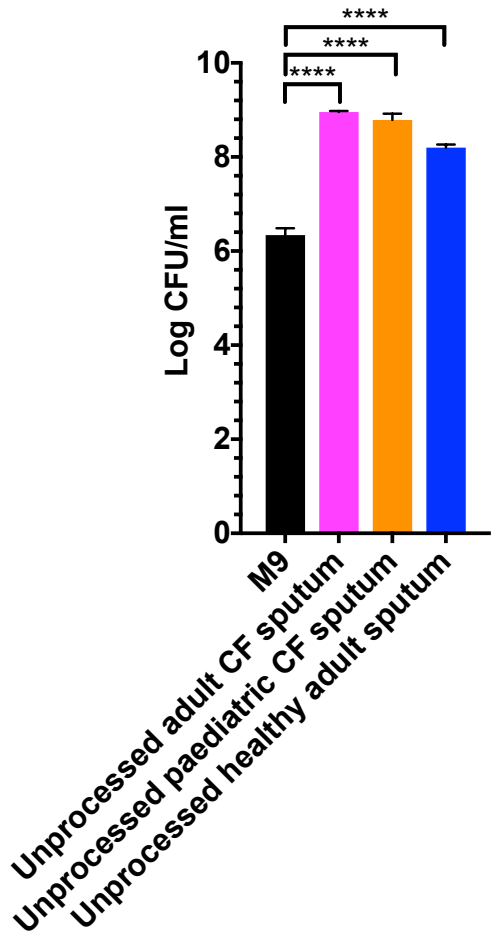

Figure 3.6d.

Figure 3.6: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.4% (w/v) unprocessed sputum. The pink (adult CF sputum), orange (paediatric CF sputum) and blue ('healthy' adult sputum) lines indicate the mean log CFU/ml growth from 3 biological replicates each. Samples were supplemented with M9 (black line, 10 replicates). b) Mean specific growth rate, μ (hour^{-1}) of PAO1. c) Mean growth yield (CFU/ml) after 24 hours. d) Mean growth yield (log CFU/ml) after 24 hours. Error bars show standard error of the mean (SEM) of all replicates. P values on the graphs (** p < 0.0001, ** p < 0.01) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

Firstly, the data revealed increased growth rates of PAO1 when grown in all sputum types which were unprocessed compared to the growth rate in M9 alone (****p < 0.0001, figure 3.6b.). Secondly, there was both an increased growth rate in adult vs. paediatric CF sputum, and in adult CF sputum vs. the 'healthy' adult sputum. In both cases these differences were significant. These findings were consistent with previous results in processed samples as shown in figures 3.3b. and 3.5c., respectively. The

growth rate in adult CF sputum remained high in the unprocessed sample with a growth rate of 1.31 μ (hour^{-1}) ± 0.08 SEM compared to 0.88 μ (hour^{-1}) ± 0.06 SEM in unprocessed paediatric CF sputum (**$p < 0.01$), and 0.74 μ (hour^{-1}) ± 0.01 SEM in unprocessed 'healthy' sputum (***$p < 0.001$). These data suggest that the composition of the sputum samples is different to an extent that *P. aeruginosa* can thrive 'better' as determined by the increased growth rate in CF sputum compared to 'healthy' sputum. The data also suggests that the composition between unprocessed adult and paediatric CF sputum is different because PAO1 was found to have different growth rates which were statistically significant in the two sputum types (**$p < 0.01$).

Interestingly, despite the appearance of the growth profile (figure 3.6a.) which reveals an apparent difference in log growth yield after 24 hours between growth of PAO1 in unprocessed adult CF and 'healthy' adult sputum, when a one-way ANOVA was performed on the log growth yield data as presented in figure 3.6d., this difference was found to be not statistically significant ($p > 0.05$) similarly to data shown in figure 3.5b. However, when this ANOVA was performed on the raw CFU/ml data after 24 hours of growth (figure 3.6c.), differences in yield of PAO1 in unprocessed adult CF sputum vs. unprocessed 'healthy' adult sputum were found to be significant (****$p < 0.0001$), similarly to growth data in processed samples in figure 3.5d. Importantly there was no difference in growth yield of PAO1 when grown in unprocessed adult CF sputum compared to paediatric CF sputum. This observation was consistent with the growth yield found after 24 hours when PAO1 and PA14 were grown in processed sputum (figure 3.4).

Finally, if the growth yields in unprocessed and processed samples are compared, the data in figure 3.7. reveals that the process of dialysing affects the maximum growth yield after 24 hours in adult CF sputum, but not in 'healthy' adult sputum. The data in figure 3.7 demonstrates that the process of dialysing drastically reduces the maximum growth yield of PAO1 after 24 hours. For example, the growth yield in unprocessed adult CF sputum was 9.11 x 10^8 CFU/ml whereas in processed adult CF sputum it was 5.50 x 10^8 CFU/ml. Following a one-way ANOVA, this difference was found to be statistically significant (***$p < 0.001$). The data therefore implies that there is a nutrient within unprocessed sputum which further increases growth yield, which is absent in

healthy sputum since there was no significant difference between the growth yield in unprocessed and processed 'healthy' adult sputum (p = 0.7556). However, the data demonstrates that following removal of this unknown nutrient that PAO1 is still able to grow to a high growth yield in processed sputum which is significant against growth in healthy sputum. This suggests that CF sputum composition is different to the sputum from healthy patients.

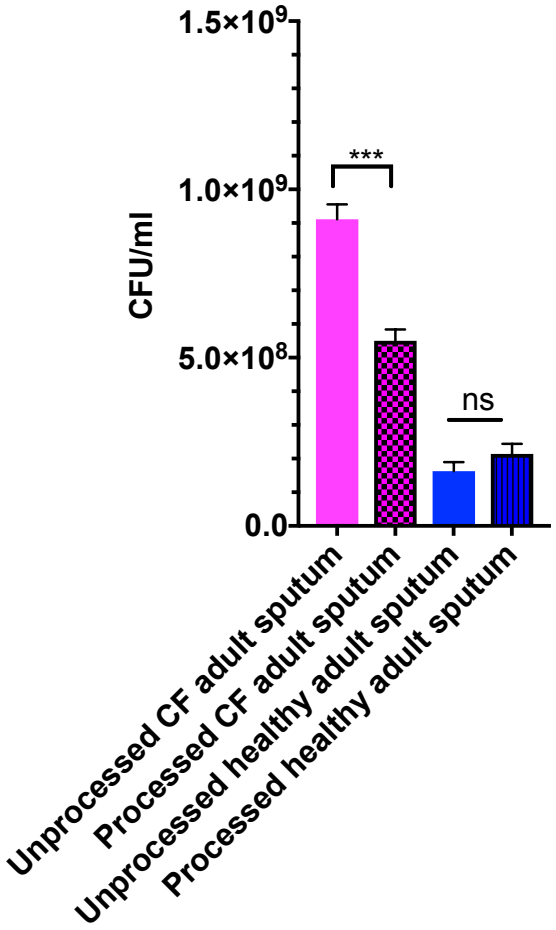

Figure 3.7.

Figure 3.7: Growth yield of *Pseudomonas aeruginosa* PAO1 in 0.4% (w/v) unprocessed and processed adult CF and 'healthy' adult sputum supplemented with M9. Data represent the mean CFU/ml after 24 hours from 3 biological replicates. Error bars show standard error of the mean (SEM) of all replicates. P value on the graph (*p < 0.001) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

3.3.3. Assessment of sputum samples

3.3.3.1. Assessment of glucose in unprocessed and processed sputum samples

Following growth assays of *P. aeruginosa* in unprocessed and processed sputum it was necessary to further investigate the composition of the samples being used in this study.

Initially it seemed logical to measure the amount of glucose in samples since glucose is known to be elevated in the CF airway and is thought to be a main factor in driving growth of respiratory pathogens as investigated by Garnett and colleagues (2013). Glucose was measured using a glucose assay kit as described in section 3.2.6.

3.3.3.2. Glucose is increased in unprocessed adult CF sputum compared to 'healthy' adult sputum but is removed efficiently via dialysis

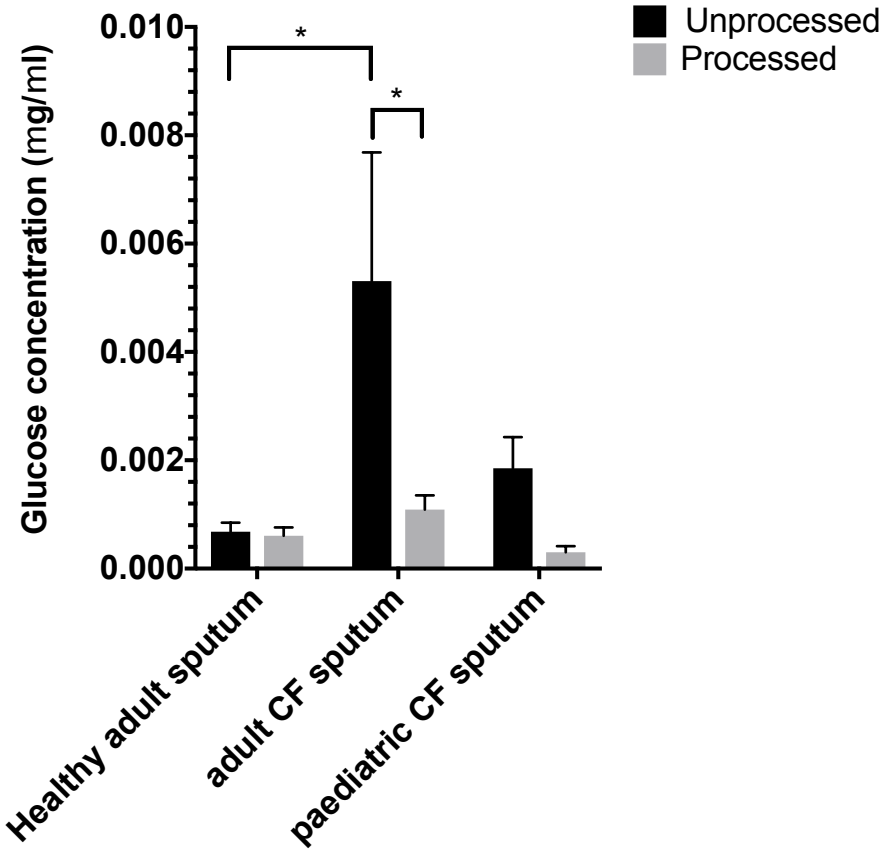

Figure 3.8.

Figure 3.8: Glucose concentration (mg/ml) measured in 0.4% (w/v) sputum. Unprocessed sputum is presented as the black bars and the processed sputum is presented as the grey bars. Data represent the mean concentration of glucose from 3 biological replicates per sputum type. Error bars show standard error of the mean (SEM). P values (* $p < 0.05$) on the graph were obtained via a two-way ANOVA followed by Sidak's multiple comparison test between unprocessed and processed samples and a Tukey's multiple comparisons test between the sputum types.

Glucose was measured in samples which were unprocessed and processed. These samples originated from the same samples which were prepared for growth assays if enough residual volume was available for the glucose assay. For this reason, there were only 3 biological replicates per sputum type.

Statistical analysis using a two-way ANOVA followed by a Sidak's multiple comparisons test between unprocessed and processed sputum revealed that there was no

significant difference in the mean glucose concentration measured at 0.00068 mg/ml in unprocessed 'healthy' adult sputum and 0.00061 mg/ml measured in processed 'healthy' adult sputum prior to growth studies. This demonstrates that not only was there initially very little glucose in the unprocessed sputum samples, but following dialysis there was no further decrease in concentration.

The unprocessed adult CF sputum samples, however, were shown to have considerably more glucose prior to processing, compared to 'healthy' adult sputum. When a two-way ANOVA followed by a Tukey's multiple comparisons test was performed to compare the glucose concentration between the sputum types, the increase in glucose measured in unprocessed adult CF sputum compared to unprocessed 'healthy' adult sputum was found to be statistically significant ($*p < 0.05$). This successfully demonstrates that glucose is increased in adult CF sputum compared to sputum from healthy subjects. This test also revealed that there was no significant difference between the glucose concentration measured in the processed samples.

The larger standard error bar of the mean in the unprocessed adult CF sputum reveals that there was more variation in these samples. This was possibly due to the small sample size in this experiment or because of patient-to-patient variability. However, it was found that processing of these adult CF sputum samples via dialysis significantly reduced the mean glucose concentration from 0.0053 mg/ml to 0.0011 mg/ml. This represents a ~ 4.9-fold decrease, which was found to be statistically significant ($*p < 0.05$) when a two-way ANOVA was performed followed by a Sidak's multiple comparisons test.

The data also suggests a decrease in the concentration of glucose measured in the unprocessed and processed paediatric CF sputum. However, despite a 6-fold decrease, this reduction was found to be not statistically significant ($p > 0.05$). These data show a trend in the reduction of glucose concentration following dialysis, highlighting how it is an important part of the processing procedure, especially for the removal of glucose in adult CF sputum.

3.3.3.3. Growth of *Pseudomonas aeruginosa* PAO1 in glucose concentrations detected in adult and paediatric CF sputum reveal no growth compared to growth in M9

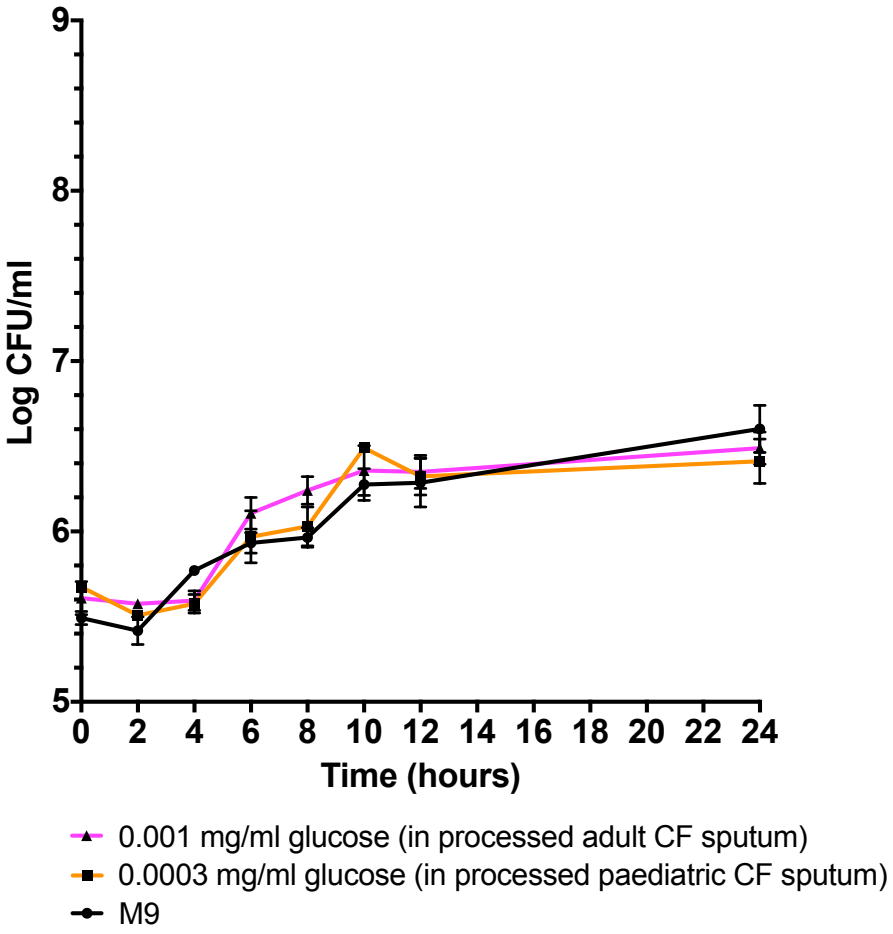

Figure 3.9a.

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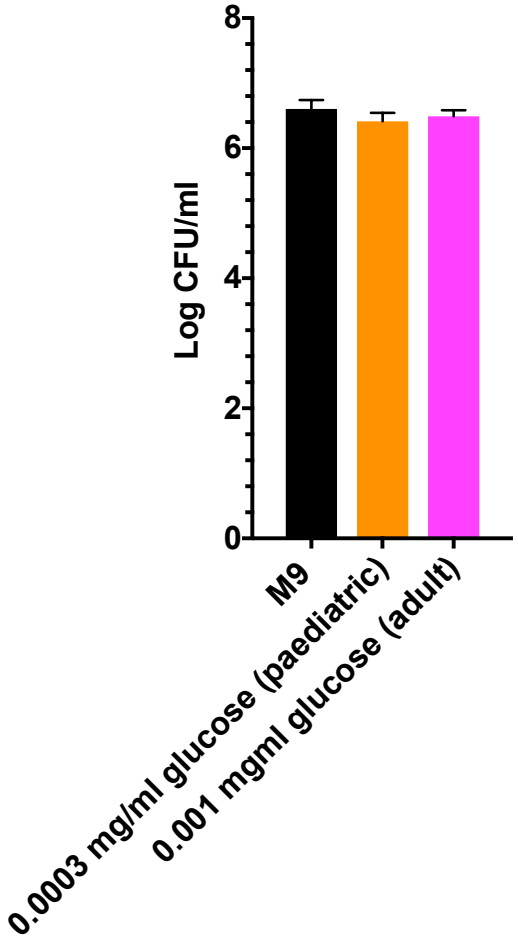

Figure 3.9b.

Figure 3.9: a) Growth of *Pseudomonas aeruginosa* PAO1 in glucose over 24 hours. PAO1 was grown in glucose concentrations found in 0.4% (w/v) processed adult and paediatric CF sputum which was supplemented with M9 (black line/bar). The concentration of glucose at 0.0003 mg/ml represents the glucose concentration measured in paediatric CF sputum (orange line/bar) and the concentration of glucose at 0.001 mg/ml represents the glucose concentration measured in adult CF samples (pink line/bar). Data represent the mean log CFU/ml from 3 biological replicates. Error bars show standard error of the mean (SEM). b) Growth yield (Log CFU/ml) after 24 hours. Data were analysed using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

Based on the glucose concentrations measured in the processed CF sputum, it was necessary to next determine whether growth of *P. aeruginosa* was due to the presence of glucose in these samples. PAO1 was therefore grown in the mean glucose concentrations measured in processed adult and paediatric CF sputum at 0.001 mg/ml and 0.0003 mg/ml, respectively. This was to determine whether glucose present at these

concentrations could stimulate growth of PAO1 to an extent that it affects the growth yield after 24 hours.

Figure 3.9a. shows the growth profile of PAO1 in 0.0003 mg/ml (orange line, 'paediatric') and 0.001 mg/ml (pink line, 'adult') glucose supplemented with M9. The growth profile reveals that there was no difference in growth compared to the M9 control, especially after 24 hours of growth ($p > 0.05$). This was confirmed following statistical analysis using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test which was performed on both the raw and log CFU/ml growth yield data after 24 hours of growth. Initially a one-way ANOVA was performed on the raw CFU/ml data but the Brown-Forsythe test revealed there were significant differences ($*p < 0.05$) between the variances. The data was then log transformed to make the data more Gaussian and the ANOVA performed again. This also revealed there were differences in the variances. Therefore, a Kruskal-Wallis test was performed instead.

3.3.3.4. Protein concentration is increased in unprocessed CF sputum compared to unprocessed 'healthy' sputum

Another component within sputum is protein, in the form of mucin glycoproteins. Therefore, it was necessary to assess the protein content in samples to establish whether its presence can support growth in CF sputum. Protein was measured in unprocessed and unprocessed sputum using the CB-X protein assay.

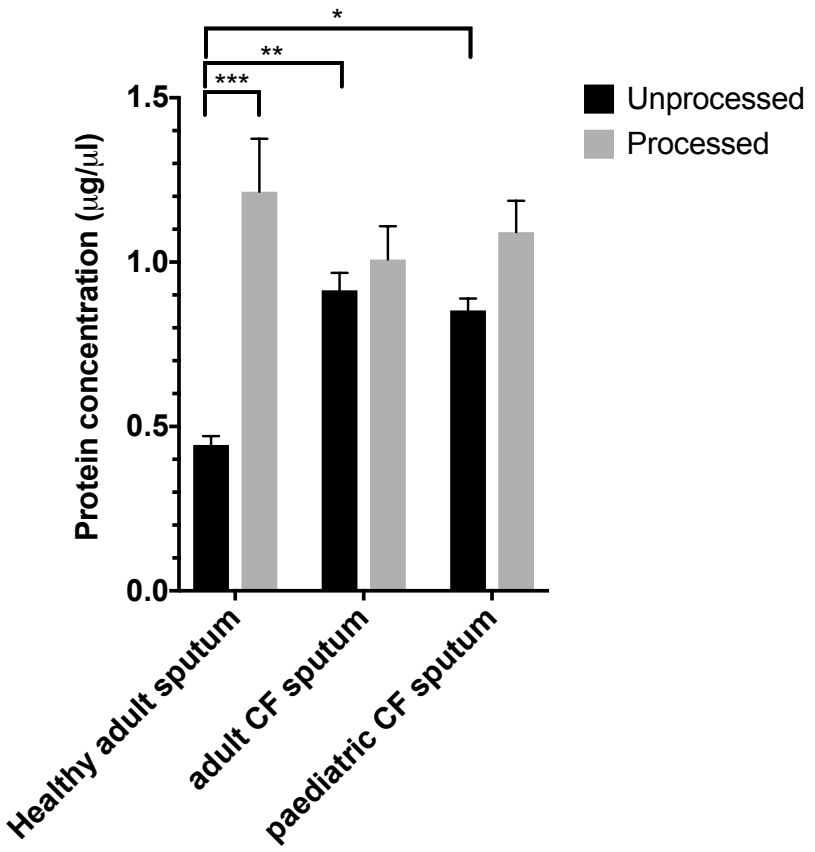

Figure 3.10.

Figure 3.10: Concentration of protein in 0.4% (w/v) unprocessed (black bars) and processed (grey bars) 'healthy' adult, adult CF, and paediatric CF sputum prior to growth studies. Data represent the mean protein concentration (µg/µl) from 3 biological replicates per sputum group. Error bars show standard error of the mean (SEM). P values on the graph (*p < 0.05, **p < 0.01, ***p < 0.001) were obtained by a two-way ANOVA. A Tukey's multiple comparisons test was used to compare between the sputum types. A Sidak's multiple comparisons test was used to compare between unprocessed and processed samples.

Figure 3.10 shows the concentration of protein in unprocessed and processed 'healthy' adult, adult CF, and paediatric CF sputum. Analysis revealed that the concentration of protein in unprocessed 'healthy' adult sputum was 0.44 µg/µl ± 0.03 SEM compared to 0.91 µg/µl ± 0.05 SEM in unprocessed adult CF sputum. This raised protein concentration in unprocessed adult CF sputum represents a 2-fold increase which was found to be statistically significant (**p < 0.01).

A two-way ANOVA followed by a Tukey's multiple comparisons test also revealed a significant 1.9-fold increase in protein concentration in unprocessed paediatric CF sputum vs. 'healthy' adult sputum (*$p < 0.05$).

Interestingly, no difference in protein concentration was observed between the unprocessed and processed CF sputum types ($p > 0.05$). Rather than observing an expected decrease in protein concentration following dialysis using a 10 kDa membrane, the data instead revealed an increased protein concentration following processing in 'healthy' adult sputum (***$p < 0.001$). Notably the increase was not statistically significant in the CF sputum samples when a Sidak's multiple comparisons test was performed between unprocessed and processed samples in each sputum type.

The graph in figure 3.10 also reveals that following processing, the concentration of protein amongst all sputum types was comparable with no significant difference reported between the processed sputum types ($p > 0.05$). It therefore appears that differences in growth in processed samples are not due to the protein concentration measured in these samples and it must be another factor affecting the growth rate and yield. However, this does not exclude protein being a viable nutrient source in unprocessed sputum since protein is measured at higher concentrations in CF sputum compared to healthy.

3.3.3.5. Protein concentration decreases after 24 hours when PAO1 is grown in unprocessed adult and paediatric CF sputum supplemented with M9

P. aeruginosa had previously been grown in unprocessed sputum as presented in figure 3.6. Before inoculation with PAO1, the protein content was measured in these samples used for the growth study. Protein content was also measured after 24 hours of growth. The data in figure 3.11 reveals that protein concentration is decreased in the same six CF sputum samples after 24 hours of growth of *P. aeruginosa* PAO1. This reduction in protein concentration measured after 24 hours was found to be highly significant in the adult and paediatric CF sputum compared to the concentration measured in the same samples at 0 hours. A two-way ANOVA followed by a Sidak's multiple comparisons

test was performed to compare the protein concentration at 0 and 24 hours in the samples (****$p < 0.0001$).

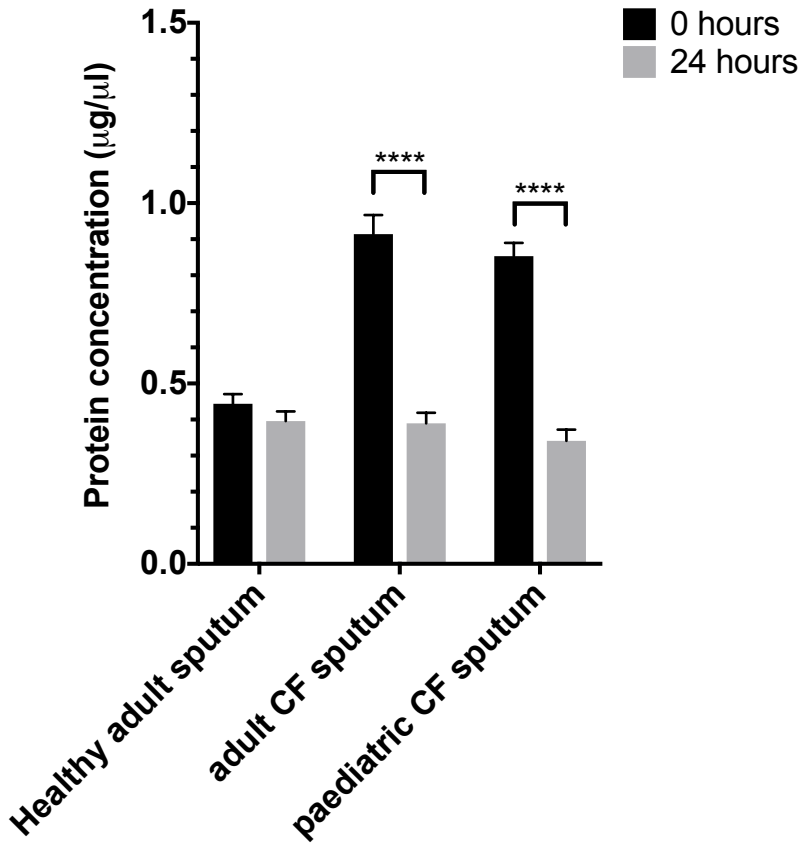

Figure 3.11.

Figure 3.11: Protein concentration (μg/μl) in unprocessed 0.4% (w/v) sputum before growth and after 24 hours of growth of PAO1. Black bar represents concentration at 0 hours and the grey bar represents concentration after 24 hours. Data represent the mean concentration of protein from 3 biological replicates per sputum type. Error bars show standard error of the mean (SEM). P values on the graph (**$p < 0.0001$) were obtained by a two-way ANOVA followed by a Sidak's multiple comparison test.**

However, in 'healthy' adult sputum the data reveals that PAO1 was unable to use the protein as a nutrient source, since there was no decrease in protein concentration after 24 hours compared to 0 hours ($p > 0.05$). Interestingly after 24 hours of growth, the concentration of protein measured in the adult and paediatric CF samples was found to be comparable to baseline concentrations measured in 'healthy' adult sputum. When a Tukey's multiple comparisons test was performed to compare differences between the

sputum types after 24 hours of PAO1 growth, no difference in protein measurement was reported ($p > 0.05$).

3.3.3.6. Assessment of DNA concentration in unprocessed and processed sputum

DNA is known to accumulate in the CF lung due to lysing of host cells such as neutrophils which releases DNA as neutrophil extracellular traps (NETs) within the CF airway (Lethem et al, 1990; Dubois et al, 2012; Walker et al 2005). However, it is also known that *Pseudomonas* itself can release DNA via lysis of a small population of bacterial cells to aid biofilm formation (Allesen-Holm et al, 2006). It is suggested that DNA protects *Pseudomonas* in the CF airway because the DNA secreted from neutrophils binds to proteases which also originate from the neutrophils, thus rendering them inactive (Dubois et al, 2012). DNA is also known to have electrostatic interactions with aminoglycosides and positively charged antimicrobial peptides highlighting its role in causing resistance to aminoglycoside treatment (Landry et al, 2006). In a study by Chiang and colleagues (2013), biofilms formed in a flow chamber with DNA release-deficient *P. aeruginosa* quorum-sensing mutants were susceptible to aminoglycoside treatment. However, the *Pseudomonas* was found to become tolerant to the antibiotic when exogenous DNA was added to the flow chamber. This emphasises the role of DNA in pathogen protection.

For this reason, many patients are given a DNase drug called dornase alfa to degrade the cross-linking caused by DNA. This aids clearance and expectoration of the sputum, and minimises the protective effects which DNA has on the biofilm (Whitchurch et al, 2002; Griese et al, 1997).

The reason why DNA is a potential nutrient of interest in this study is because one of its components is the sugar deoxyribose and deoxyribose may serve as a potential carbon source for *P. aeruginosa*. In previous studies DNA has been extensively measured, with a wide range of concentrations being reported, but a review by Palmer and Whiteley (2014), reported that in most studies the DNA concentration ranged between 400 µg/ml and 700 µg/ml. If *P. aeruginosa* can utilise mucin sugars, then it must be assessed whether it can also use deoxyribose in addition to the nucleobases. For this reason, it

was necessary to be aware of DNA concentration in the sputum samples used in this study.

3.3.3.7. Measurement of DNA in unprocessed and processed sputum reveals that DNA concentration is increased in unprocessed CF sputum compared to healthy sputum and that processing does not reduce DNA concentration

Analysis of the mean DNA concentration in the samples revealed that there were significant differences between the variances according to a Brown-Forsythe test. The data was therefore log transformed and a two-way ANOVA performed on all data sets. Figure 3.12 demonstrates the log DNA concentration measured in unprocessed and processed sputum samples. In the unprocessed samples, DNA was found to be significantly increased in samples originating from CF patients compared to healthy patients (****$p < 0.0001$), but there was no difference between concentrations measured in unprocessed adult and paediatric CF sputum ($p > 0.05$). The Tukey's multiple comparisons test which was used to compare differences between the sputum types also revealed that there were differences in the processed samples also. Once again, statistical analysis revealed that the concentration of DNA was increased in both adult (****$p < 0.0001$) and paediatric (**$p < 0.01$) CF sputum samples compared to 'healthy' adult sputum. However, it was found that following processing there were significant differences in the concentration of DNA measured in processed adult vs. paediatric CF sputum (****$p < 0.0001$).

When a Sidak's multiple comparison test was used to assess differences in DNA concentration between unprocessed and processed samples, the only difference was found in the adult CF sputum (****$p < 0.0001$). Interestingly, despite dialysis using a 10 kDa membrane, it appears that processing was unable to reduce the concentration measured in samples.

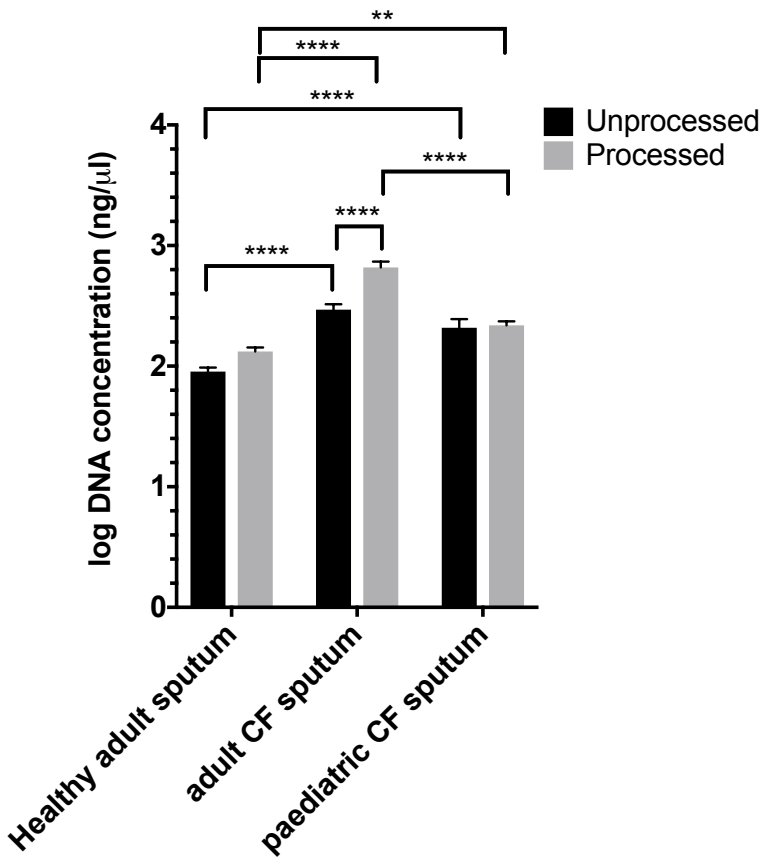

Figure 3.12.

Figure 3.12: Log DNA concentration (ng/µl) in 0.4% (w/v) unprocessed (black bar) and processed (grey bar) 'healthy' adult, adult CF, and paediatric CF sputum. Data represent the mean log DNA concentration from 5, 4, and 4 biological replicates for 'healthy' adult, adult CF, and paediatric CF sputum, respectively. Error bars show standard error of the mean (SEM). P values on the graph (p < 0.01, ****p < 0.0001) were obtained by a two-way ANOVA. A Tukey's multiple comparisons test was used to compare between the sputum types. A Sidak's multiple comparisons test was used to compare between unprocessed and processed samples**

3.4. Discussion - Growth assays and measurements of protein, glucose, and DNA in sputum

To address whether mucin provided a source of nutrients to pathogens, it was first investigated whether there were any differences in growth of the organisms when grown in adult and paediatric CF sputum samples that may allude to differences in composition. Furthermore, since samples were processed for growth studies, the glucose, protein, and nucleic acid content were measured in all samples before and after processing to characterise how this may affect the growth profiles of the organisms.

3.4.1. *Streptococcus pneumoniae* D39 grows in CF sputum

3.4.1.1. Degradation of mucin by *Streptococcus pneumoniae*

It is widely known that pneumococcus can grow in PGM supplemented with Sicard's; the medium used also in this study, by using mucin glycoconjugates as a carbon source for survival (Sicard, 1964; Yesilkaya et al, 2008; Terra et al, 2010; Paixão et al, 2015). Sicard's was therefore selected based on its use in prior studies and because following cessation of growth it sufficiently supports *S. pneumoniae* (Sicard, 1964). A study by Yesilkaya and collaborators (2008) revealed that growth in PGM stimulated upregulation of the *nanA* gene encoding a neuraminidase which cleaves terminal sialic acid in mucin. Interestingly, *nanA* gene transcription was found to be 24 times greater when grown in PGM compared to when grown in glucose. Growth in PGM also demonstrated larger colonies compared to when grown in the absence of mucin. This work illustrated a mechanism by which pneumococcus could begin to sequentially degrade mucin for exploitation of its rich sugar supply.

Other studies have demonstrated that mucin is then further degraded by the expression of glycosidases that destroy the N- or O- linkage of the oligosaccharide side chain which links the glycan to the protein backbone of the mucin. Progressive breakdown of the mucin eventually follows using enzymes including neuraminidase which cleaves terminal sialic acid, followed by galactosidase which cleaves galactose linkages, and N-acetylglucosaminidase which cleaves N-acetylglucosamine, where the latter sugar is attached by an O-glycosidic linkage to serine or threonine in the polypeptide backbone

(King et al, 2006; King, 2010). The production of these enzymes in *S. pneumoniae* was shown to be imperative for successful organism growth (Burnaugh et al, 2008; Rose and Voynow, 2006). The literature strongly suggests that this is the mechanism by which *S. pneumoniae* grows on CF sputum as it has in this study and suggests that invading pathogens should also possess these enzymes if mucin is indeed degraded in the lung. For example, a study by Aristoteli and Willcox (2003) revealed that some *P. aeruginosa* isolates possess galactosidase and N-acetylglucosaminidase activity. Additionally, N-acetylgalactosaminidase activity was exhibited when grown in PGM. Furthermore, the virus, Influenza A has been shown to possess a neuraminidase which helps permeate the mucus layer illustrating how mucin degradation may to be integral for pathogens colonising the lung (Berry et al, 1996; Aristoteli and Willcox, 2003; Cohen et al, 2013).

3.4.1.2. Are growth differences due to mucin glycosylation structure?

Since no difference in growth rate of pneumococcus was observed in this study between adult and paediatric sputum samples, the data could imply that the mucin itself in patient sputum does not differ in structure since pneumococcus grows similarly as determined by growth rate, and instead it's the ability of the organism to exploit the source of carbon provided in its existing structure within mucin. However, this does not explain why there is an increased growth yield after 15 hours in paediatric vs. adult CF sputum. Indeed, it could be that the structure of the mucins is similar that it allows the growth rates to be comparable to a certain extent, but as the mucin is sequentially degraded the sugar content and structure could differ to a degree that paediatric sputum is more favourable for pneumococcal growth than adult sputum. Such observations could be explained by differences in glycosylation of mucins in adult and paediatric sputum, where it is known that respiratory mucin has four core mucin structures as described in the introduction of this thesis.

It is very possible that mucin glycosylation changes through disease progression to an extent that adult mucins differ from paediatric mucins. In support of this, inflammation has been linked to enacting an increase in Siayl-lewis antigens in various cancers by the upregulation of glycotransferases which add sugars to the protein backbone in the Golgi apparatus (Ugorski and Laskowska, 2002; Wu et al, 2009; Linden et al, 2008; Thornton

et al, 2008). Indeed, these differences in Sialyl-Lewis content has also been documented in CF vs. non-CF mucins, as has an increase in sulphation and fucose content (Carnoy et al, 1993; Wesley et al, 1983; Cheng et al, 1989; Davril et al, 1999; Rhim et al, 2000; Xia et al, 2005). In contrast, a study conducted by Schulz and colleagues (2007) revealed that fucose and sulphation was reduced in CF patients compared to controls. Other studies have reported no difference in glycosylation or contrasting findings (Schulz et al, 2005; Holmen et al, 2004). For example, independent studies carried out by Reid (1999), Leir (2005), and their colleagues demonstrated that CFTR expression does not affect glycosylation or sulphation of mucins and it is likely that any differences observed in glycosylation by Schulz and colleagues (2005) were likely to be due to inflammation or infection (Holmen et al, 2004). The literature is therefore extensively unresolved on differences in glycosylation in CF.

3.4.1.3. Are growth differences due to sputum composition?

Furthermore, it is possible that there is a difference in growth rate of pneumococcus when grown in CF sputum which is at present unnoticeable due to the larger error of growth in the adult CF patient samples. Patient-to-patient variability could therefore explain this more varied growth where this could be due to mucin glycosylation differences as previously discussed. It must also be appreciated that the sputum samples themselves are not pure mucin as is the case with the PGM. Instead sputum is a mixed blend of other components such as DNA, iron, proteins, apoptotic inflammatory cells, and antibiotics which are absent in healthy airways, and such components can be influenced by the exacerbation state of the CF patient (reviewed by Palmer and Whiteley, 2014). For example, levels of inflammatory cells will vary depending on infection, whilst MUC5AC and MUC5B has been shown to increase during exacerbations (Henke et al, 2007; Chmiel et al, 2014).

3.4.1.4. Are growth differences due to antibiotics?

It is well documented that different pathogens predominate at distinct periods during CF disease, which could in turn affect the sputum composition and the surrounding environment based on the antibiotics they are treated with (Ciofu et al, 2013; Lyczak et al, 2002; Gibson et al, 2003). To minimise the variability in growth of organisms in the

sputum, this was one of the reasons why sputum samples were separated into the sub groups, adult and paediatric. Ideally, sputum samples would be preferred to come from patients not on antibiotic treatment, but this was not possible due to the nature of the disease and the need for infections to be treated quickly. Therefore, this was a limitation of the study. Whilst the presence of antibiotics may be a cause for affecting the assay, other studies have shown that antibiotics such as collistin can bind to porcine mucin and interestingly reduce the antimicrobial potential (Huang et al, 2015). In our laboratory, when PGM was spiked with a cocktail of antibiotics using concentrations found in the sputum, it was shown that dialysis did not reduce the bacterial killing effect (data not shown). Therefore, the process of dialysis is not sufficient for removing antibiotics. The samples were, however, sterilised via autoclaving. This study investigated as part of preliminary experiments how heat denaturing can reduce antimicrobial effect of some antibiotics (data not shown). Other studies have reported varied effects on antibiotic stability with increases in temperature (Hsieh et al, 2011). Therefore, because growth in these sputum samples was observed, it could be argued that perhaps the presence of antibiotics is less of a contributing factor than first assumed, since the aim of this study was to assess what nutrients promote growth, and not factors that hinder it.

Further growth assays may help to clarify differences in growth yet this study at this point in the project was limited with the further use of samples which were reserved for future growth assays involving *P. aeruginosa*. It would therefore be interesting to further assess the specific role of pneumococcus in promoting growth of other respiratory pathogens, and whether in the complicated airway mixture, whether it degrades mucins for use of carbon for itself, or for other organisms which are better adapted to exploit the CF environment. This could to a certain extent explain why pneumococcus is so infrequently detected in sputum cultures compared to other pathogens (Maeda et al, 2011). This study, however, for the first time demonstrates the ability of *S. pneumoniae* to grow in airway sputum samples from CF patients, using Sicard's defined medium to support growth whereby the medium contains no other carbon source for pneumococci other than what is provided in the sputum.

3.4.2. Increased growth rate of *Pseudomonas aeruginosa* in adult CF sputum

Interestingly, whilst no difference in growth rate was observed when *S. pneumoniae* was grown in both CF sputum types, in contrast, an increased growth rate was observed in adult vs. paediatric CF sputum when *P. aeruginosa* reference strains PAO1 and PA14 were grown in these samples using both Sicard's and M9 to supplement the sputum. An increased growth rate and yield was also observed when PAO1 was grown in sputum which was not processed, i.e. samples which were not dialysed to remove low molecular weight components prior to lyophilisation, where the growth yield after 24 hours in the unprocessed adult CF sputum was significantly greater than the processed sputum. The data alludes to the presence of components within CF sputum which are smaller than 10 kDa which greatly promote growth, because when removed, the growth yield was reduced after 24 hours in processed sputum compared to unprocessed sputum. Smaller components could include proteins and sugars such as glucose (Garnett et al, 2013). No difference in growth yield after 24 hours was found in unprocessed vs. processed sputum from healthy subjects. This finding was expected since it is known many components present within sputum are absent in healthy sputum (reviewed by Palmer and Whiteley, 2014).

3.4.2.1. Growth of *Pseudomonas* in sputum with Sicard's

Sicard's defined medium, used for the growth of *S. pneumoniae*, was modified so that the only sugar provided was supplied by those present in PGM, adult, or paediatric sputum. However, amino acids present in Sicard's are known to support growth (Moreno et al, 2009). In one study, the high levels of amino acids in CF sputum compared to non-CF sputum was demonstrated to support rapid growth of *P. aeruginosa* (Palmer et al, 2007; Barth and Pitt, 1996). Previous work conducted by Palmer and colleagues (2005) has already shown that CF sputum supports growth of *P. aeruginosa* to high densities – 10^8-10^{10} CFU/ml, an observation which was also observed in this project when using M9 as a minimal medium instead of Sicard's. In their study, it was suggested that amino acids within sputum were the likely source of carbon for *Pseudomonas* as evidenced by transcriptome analysis that revealed growth in CF sputum affected its gene expression for uptake and metabolism of amino acids.

However, it must be noted that in contrast to the method of this project, sputum samples were not dialysed in the study by Palmer (2005) to remove low molecular weight sugars or smaller molecules already present, such as amino acids, readily available due to prior mucin degradation which has been shown to occur via the expression of glycosidases in clinical isolates (Aristoteli and Willcox, 2003). Therefore, the presence of free amino acids in these samples, which have been documented to be raised, could attribute as to why gene expression of *P. aeruginosa* was increased in the study by Palmer. Furthermore, other experiments have assessed the gene expression of *Pseudomonas* clinical isolates in sputum from CF patients and revealed that the presence of amino acids and degradation of lung surfactant molecules was required for high density growth. It was also revealed that many metabolic pathways were active, such as the pentose phosphate pathway, the TCA cycle, and oxidative phosphorylation (Son et al, 2007).

Collectively, the literature indicates that amino acids are important nutrients in the CF airway. Therefore, this suggests why growth of PAO1 and PA14 in Sicard's alone during preliminary experiments was so efficient, since Sicard's contains numerous amino acids that *Pseudomonas* can utilise. For this reason, upon reflection, Sicard's may not have been the best medium for studying growth in patient sputum, but does shed light on its ability to readily grow in the presence of amino acids as other studies have also demonstrated.

3.4.2.2. Growth of *Pseudomonas* in sputum with M9

Growth experiments were then repeated using the same patient samples supplemented with M9; a minimal medium used for the growth of pseudomonads, and in contrast to Sicard's possesses no amino acids (LeBauve and Wargo, 2012). It was once again observed that there was an increased growth rate of PAO1 and PA14 when grown in adult CF sputum compared to paediatric CF sputum. Increased growth was also observed in terms of yield with differences being highly significant compared to 'growth' in M9. Although statistically significant increases in growth yield in the adult and paediatric sputum were observed compared to M9, no differences in yield of PAO1 or PA14 were observed between adult or paediatric sputum. This suggests that any

differences in sputum composition between adult and paediatric CF sputum solely affects the growth rate, and not the yield. However, when growth in adult CF sputum was compared to growth in 'healthy' adult sputum, there were differences between the two sputum types in terms of both growth rate and yield. Therefore, it could mean that the sputum composition does indeed differ.

3.4.2.3. What could *Pseudomonas* be using as a nutrient source in sputum?

It is known pneumococci utilise sugar readily. *Pseudomonas*, on the other hand, if offered sugars in a nutritional mixture will favour substrates more easily metabolised including amino acids and organic acids, such as succinate, so that it can survive (Palmer et al, 2007; Siegel et al, 1977). The ability of pseudomonads to thrive in otherwise challenging environments is due to its extensive genome which allows for flexible regulation of metabolic pathways depending on the nutritional milieu available which can best support the most efficient and rapid growth. This is regulated via carbon catabolite control (CCC). This regulation permits both the inhibition of uptake and catabolism of less valuable nutrients, in what is known as carbon catabolite repression, and the activation of genes allowing for metabolism of more preferred nutrients via catabolite control activation. Such preferential use of carbon sources was initially described in *E. coli* by Monod in 1942. In Monod's study, glucose-lactose diauxie with preference for glucose over lactose via CCC was described for the first time (Legout, 2010). This metabolic phenomenon has also been exhibited in various other bacterial species, such as *Listeria monocytogenes*, *Pseudomonas putida*, and *Bacillus subtilis*, as reviewed by Görke and Stülke (2008).

In *Pseudomonas,* glucose is not readily used like it is by *E. coli* due to the repression from the presence of organic acids, although certainly more preferred than other hydrocarbons (Rojo, 2010). For example, the expression of various enzymes involved in glucose metabolism is repressed until levels of succinate are depleted (Siegel et al, 1977). Other organic acids including acetate, malate, and fumarate have also been shown to evoke suppression of carbohydrate utilisation until organic acids have been utilised (Wolff et al, 1991). Therefore, this partially describes why differences in growth of *P. aeruginosa* may not have initially been observed when grown in sputum

supplemented with Sicard's which contains amino acids such as glutamine, histidine, arginine, lysine, and asparagine, which are all preferred amino acids over others such as glycine and valine as determined in in *P. putida* (Moreno et al, 2009). These growth findings in *S. pneumoniae* and *P. aeruginosa* alludes to potential differences in composition between adult and paediatric CF sputum. Differences observed in the growth rate in CF sputum obtained from adult and paediatric subjects have not previously been reported. It is necessary to elucidate whether these differences in growth rate and yield are due to specific mucin utilisation, utilisation of other components within sputum, or the different abilities of pathogens to utilise specific nutrients in the airway. It could indeed be a combination of all factors.

Growth differences could be due to nutritional preferences for specific mucins within 'healthy' adult sputum, adult CF, and paediatric CF sputum. The acidic form of MUC5B is known to predominate in CF airway secretions vs. MUC5AC in healthy (Henke et al, 2004; Kirkham et al, 2002; Davies et al, 1999). If the growth yield and rate is reduced in 'healthy' adult sputum, this could be because MUC5AC is less preferred as a nutrient source. *P. aeruginosa* could therefore be utilising MUC5B which is increased within CF secretions. On the other hand, because MUC5B predominates in CF secretions, conversely this could be indicative that MUC5B is not preferentially used as a nutrient source. A study by Henke and colleagues (2007) assessed MUC5AC and MUC5B protein levels during CF exacerbation and compared it to protein expression during stable disease. Their study demonstrated that MUC5AC protein increased by 908% whereas MUC5B increased by only 59%. This lower percentage increase of MUC5B relative to MUC5AC could suggest that the amount of MUC5B is lower because it is degraded by bacteria and utilised as a nutrient source.

Studies have shown that the presence of invading pathogens can induce mucin expression (Li et al, 1997; Ha et al, 2007). However, it is unknown whether mucin gene expression is induced so that it benefits pathogen survival so that the upregulated mucin can be used as a nutrient source, or because it is part of a host protective mechanism as a counter response to the damage caused by the pathogen. For example, one study has shown that MUC5AC mucin transcription is upregulated by *S. pneumoniae* (Ha et al, 2007). This is interesting because PGM has been shown to support pneumococcal

growth so it is not clear whether MUC5AC is upregulated for use as a nutrient source by *S. pneumoniae* or is upregulated for airway protection. This is because a more recent study has highlighted the protective role of MUC5AC in the CF airway. A study by Ehre and colleagues (2012) found that when MUC5AC secretion was increased in mice it could prevent influenza infection. Therefore, this could also explain why in the previous study that MUC5AC was increased by 908% during CF exacerbation (Henke et al, 2007).

Furthermore, whilst increased levels of MUC5AC have been implicated with the protection of the lung during exacerbation, other studies have suggested that MUC5B also has a protective role. In a study by Roy and colleagues (2014), it was found that in MUC5B-deficient mice there was altered mucociliary clearance and accumulation of apoptotic macrophages. This could also explain why bacterial clearance is so poor in CF, especially since its normal mucin function is impaired if degraded (Henderson et al, 2014). Previous studies have disputed that mucin within CF secretions is reduced but this has been more recently addressed using non-immunological techniques to quantify mucin content which reveal that mucins are present but are proteolytically degraded at sites which affect antibody binding (Henke et al, 2004; Henderson et al, 2014). If degraded, naturally it would be presumed that sugars within mucins would be more easily liberated for use as a nutrient. To address the aims of this study, further purification of the samples was necessary to determine if growth is due to utilisation of mucin as a nutrient source.

3.4.2.4. *Pseudomonas* clinical isolates grow differently in CF sputum, PGM, and Sicard's defined medium

Preliminary experiments revealed varied differences in growth of clinical isolates in Sicard's alone, PGM, and CF sputum which sheds light onto the fact that isolates have varied nutritional requirements possibly because of adaptation to an ever-changing lung environment during colonisation. A recent study conducted by Perez and colleagues (2011) revealed that the amino acids: methionine, arginine, and asparagine, were necessary for growth. Notably, these amino acids were also present in Sicard's. However, my data reveals that there were differences in the growth of the *Pseudomonas*

clinical isolates when grown in Sicard's alone. It could be that different isolates require different amounts of amino acids or types of amino acids for efficient growth as described in a study by Moreno and colleagues (2009) which demonstrated a hierarchical nature of assimilation and utilisation of amino acids.

Since *Pseudomonas* clinical isolates were shown to grow very well in Sicard's, this itself reveals that there were metabolic differences between the isolates in terms of nutritional requirements which is unsurprising when considering the pseudomonads in general. Most *Pseudomonas* species lack the phosphofructokinase enzyme involved in the Embden-Meyerhoff-Parnas pathway, whilst *P. stutzeri*, in contrast expresses this enzyme (Fuhrer et al, 2005; Lalucat et al, 2006). These clinical isolate growth data do not reveal any real differences in growth in adult and paediatric sputum in which conclusions can be drawn. Therefore, it would be necessary to repeat these growth assays using M9 to supplement the culture in future experiments.

3.4.3. Glucose detected in sputum did not promote growth

It was previously acknowledged that *P. aeruginosa* and *S. pneumoniae* could grow very well in cultures with glucose (Garnett et al, 2013; Yesilkaya et al, 2008). Glucose is often elevated within CF patient sputum samples to levels that are approximately 5-fold greater than in healthy airways (Baker et al, 2007; Brennan et al, 2007). For this reason, samples in this project were dialysed to remove glucose and other low molecular weight sugars as has been conducted in other studies (Yesilkaya et al, 2008). This was because it was essential to ensure that any growth that occurred in sputum was not due to the presence of glucose which has been argued to be a main nutritional factor in promoting growth of pathogens in the CF airway. A study by Garnett and colleagues (2013) demonstrated that a rise in the basolateral glucose concentration elicited an increased growth yield, as determined by CFU counts, at the apical surface of the airway epithelium in both non-CF and CF human bronchial epithelial cells. However, this proposal of glucose being the important nutrient driving bacterial growth is inconsistent with other studies that have instead demonstrated that amino acids within CF sputum are utilised by *Pseudomonas* in the CF airway (Barth and Pitt, 1996; Palmer et al, 2005; Palmer et al, 2007; Moreno et al, 2009). This is interesting considering the presence of

amino acids is known to repress enzymes such as glucose-6-phosphate dehydrogenase involved in glucose metabolism (Moreno et al, 2009; Collier et al, 1996). Therefore, the findings with respect to glucose utilisation in the CF airway need to be interpreted with caution because amino acids may be the preferred nutrient source over glucose.

3.4.4. Increased protein content in unprocessed CF sputum vs. healthy sputum

The data in this study reveals that in unprocessed CF sputum there is significantly more protein compared to sputum from healthy subjects. This was expected since proteins have been confirmed to be in abundance in the CF airway following analysis using multidimensional protein identification technology (Pattison et al, 2017). More specifically it was revealed that many of these proteins originated from NETs. NETs arise in the airway as result of neutrophil recruitment to the infected airways and a special type of programmed cell death which causes the release of proteins (Brinkmann et al, 2004; Downey et al, 2009).

The data also shows that following processing there is no significant difference amongst protein content in adult 'healthy' sputum or CF sputum. This demonstrates that the processing was successful, although interestingly it appears that protein content has increased. This observation is extremely likely to be a processing artefact due to dialysis of the sample. In the process of removing sputum components smaller than 10 kDa, the contribution of original protein content makes up a greater proportion of the original sample. This is because samples are suspended to the same concentration as used to assess the protein concentration in unprocessed sputum, so if other components are removed and a greater amount of sputum is needed to suspend to the same concentration, then this would explain why seemingly there is an increase in protein concentration.

This study also assessed the contribution of protein in unprocessed samples in promoting growth. This was done indirectly by measuring the total protein content in unprocessed samples before and after (24 hours) growth. Whilst there was no further decrease in protein content in healthy sputum, there were significant reductions in protein measurement in the CF samples after 24 hours. This suggests that growth observed in healthy sputum is not due to protein in the samples, but in part, protein

present in CF samples can indeed support growth of *Pseudomonas*, as evidenced by the data in this study where protein measurements were reduced following growth, possibly due to their degradation and the assimilation of amino acids as a nutrient source. In hindsight, it would have been interesting to also measure glucose concentration in the same manner to determine whether the concentration remained unchanged since amino acid presence is known to inhibit glucose metabolism as previously described.

3.4.5. Increased DNA measurements in unprocessed CF sputum compared to sputum from healthy control samples

DNA is known to accumulate in the CF lung due to lysing of host cells such as neutrophils which releases DNA as NETs within the CF airway (Lethem et al, 1990; Dubois et al, 2012; Walker et al 2005). Additionally, it is known that *Pseudomonas* itself can release DNA via lysis of a small population of bacterial cells to aid biofilm formation (Allesen-Holm et al, 2006). For this reason, DNA was measured in sputum samples to see if DNA concentration in CF sputum significantly varied from measurements in 'healthy' sputum. A review by Palmer and Whiteley (2014), reports that most studies measured DNA in a range from 400 μg/ml and 700 μg/ml. This study, in contrast, reveals a lower range of approximately 240 to 360 μg/ml in CF sputum, with significantly less measured in the 'healthy' sputum at approximately 100 μg/ml. Measurements of DNA following processing revealed that DNA was still present in all sputum samples. This was despite sputum samples coming from patients on dornase alfa treatment in which the active ingredient DNase is used to degrade DNA cross-linking the sputum (Whitchurch et al, 2002; Griese et al, 1997).

Interestingly, similarly to data demonstrating increased protein measurements in sputum following processing, the same appears to be the case with the measurements of DNA. It is likely that the process of dialysis removes degraded DNA which is small enough to pass the 10 kDa pore of the dialysis membrane, but larger intact DNA which could not be accessed by dornase alfa in the patient lung because of its complex association with mucin, cannot be removed via this described method. Therefore, the same explanation applies in that following removal of sputum constituents via dialysis, the components which do remain make a greater contribution to the total sample, thus explaining why

when sputum is suspended to the same concentration, there is an increased measurement.

A limitation in measuring the DNA via absorbance using NanoDrop One was that only one method was used. Although it must be stressed that the corrected value for DNA measurements were obtained using NanoDrop One which detects the presence of contaminants and accordingly makes measurement adjustments. It would have been interesting to also measure DNA more specifically, for example, via Qubit fluorometric quantitation.

3.5. Chapter conclusions

The first aim of this project was as follows:

"1) To identify any differences in growth of *Streptococcus pneumoniae* D39, *Pseudomonas aeruginosa* PAO1, *Pseudomonas aeruginosa* PA14, and *Pseudomonas aeruginosa* clinical isolates in sputum from cystic fibrosis patients and sputum from healthy control patients."

Based on the experiments presented in this chapter, *S. pneumoniae* and *P. aeruginosa* have been shown to grow in both adult and paediatric CF sputum. Following processing of the samples via dialysis with the aim of removing lower molecular weight sputum components, differences in growth of *P. aeruginosa* in adult and paediatric CF sputum do not appear to be due to the presence of glucose. Whilst *P. aeruginosa* was found to grow in unprocessed samples by the utilisation of protein as evidenced by a reduction in its measurement after 24 hours, it appears likely that *P. aeruginosa* is using protein as a nutrient source in processed CF sputum as it is in unprocessed sputum.

Finally, the processing procedure used in this chapter was found to have no impact in reducing DNA concentration following processing. Therefore, to determine whether mucin is used as a nutrient source, which was the second aim of this project, it was necessary to further purify the sputum samples from CF patients to assess the growth in these purified mucin samples.

Chapter 4. Assessment of potential nutrient sources in promoting growth of *Pseudomonas aeruginosa* in the airways

4.1. Introduction and aims

The next aim of this project was to identify what *P. aeruginosa* was specifically using as a nutrient source when growing in CF patient sputum. The complexity of the composition means that this has previously proven difficult to determine and therefore further purification of the samples was required.

Several previous studies have grown *Pseudomonas* in sputum to investigate which nutrients they metabolise. An early study by Ohman and Chakrabarty (1982) found that *Pseudomonas* mucoid strains were associated with chronic infections. Since then, *P. aeruginosa* has been repeatedly grown in CF sputum and has been shown to support growth to high densities (10^8-10^{10} CFU/ml) (Hoiby, 1998; Palmer et al, 2005). A more recent study used an enhanced similar artificial sputum medium (ASM) previously developed by Sriramulu and colleagues (2005). This study revealed various virulence genes which were upregulated in the presence of sputum (Fung et al, 2010). This therefore highlights how sputum not only provides nutrients for growth, but the nutrients themselves can influence several virulence and metabolic genes which aids its survival. A clear understanding of these nutrients is therefore essential.

Since one of the sputum components is mucin and contains an abundance of carbon, it was logical to assess the role of mucin as a potential nutrient source for *P. aeruginosa* growth. It is already known that mucin plays a role in the colonisation of *P. aeruginosa* since it not only promotes biofilm formation by decreasing the pathogen's motility, but it is also known that the bacterial adhesins; pili and flagella, adhere to the airway epithelium (Landry et al, 2006; Bucior et al, 2012). Numerous other studies have reported pathogen-mucin interactions where *Pseudomonas* is able to bind degraded mucins more readily than intact mucins (Ramphal et al, 1989). It is now known that this binding is via sialic acid receptors (Scharfman et al, 1999). However, binding to mucin is not unique to *P. aeruginosa*. Other respiratory pathogens such as *Staphylococcus aureus* and *Haemophilus influenzae* have also been reported to exhibit this mucin

binding ability (Shuter et al, 1996; Reddy et al, 1996). This therefore illustrates the importance of mucin to the CF microbiome and how mucin could act as an abundant nutrient source for *P. aeruginosa*.

4.2. Methods

4.2.1. Mucin purification

4.2.1.1. Preparation of sample for density gradient ultracentrifugation

Sputum samples were initially thawed before solubilising in 8 M guanidine hydrochloride (GuHCl) at 4^{o}C overnight. GuHCl is a chaotropic agent used for denaturing mucin prior to fractionation. Samples were then diluted to 4 M GuHCl with the addition of an equal volume of ddH_2O to the sample. A refractometer was then used to check the index of refraction of each sample against ddH_2O which has a refractive index (RI) of 1.3325 (figure 4.1a.). Based on the RI obtained for each sample, the molarity of GuHCl was calculated using the following equation,

$$\mathbf{M\ (GuHCl) = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3,}$$

... where ΔN is the difference between the RI of the GuHCl within the sputum sample and that of the buffer. Samples were further diluted by adding more 4 M GuHCl if required. An RI value of ~ 1.4 meant that GuHCl within the sample was at 4 M. The online calculator can be found at: http://sosnick.uchicago.edu/gdmcl.html

The amount of caesium chloride (CsCl) required in the sample was then calculated based on the density of CsCl at 1.4 g/ml being required in the sample. The following calculation was used for calculating the amount of CsCl required for each sample:

$$\mathbf{x = a\ ((1.347\ x\ 1.4) – (0.0318M) – 1.347),}$$

… where 'x' is the amount of CsCl (g) needed, 'a' is the final volume of the solution (ml), and 'M' is the molarity of GuHCl in the sample.

The final weight (FW) of the solution which was needed once CsCl had been added was calculated using the following calculation:

$$\mathbf{FW = 1.4 \times a}$$

The required amount of CsCl was weighed, and then topped up to the final weight using the sputum sample in 4 M Gu-HCl. Once the CsCl had dissolved, the sample was then added to 40 ml or 100 ml centrifuge tubes before heat sealing (figure 4.1c.).

Samples were then balanced using foil in the caps before being centrifuged at 40000 rpm for 60 hours at 15^{o}C (Beckman coulter Ti70 rotor). After 60 hours, the tubes were carefully removed and each sample was separated into 20 fractions using a pump as shown in figure 4.2a. The OD_{280} and the density (g/ml) as shown in figures 4.2b. and 4.2c, respectively were then measured for each fraction. The density was measured by taking 1 ml of each fraction, and weighing the sample. This was to ensure that the CsCl gradient had been established (Thornton et al, 1991; Davies and Carlstedt, 2000; Ridley et al, 2016).

Figure 4.1a.

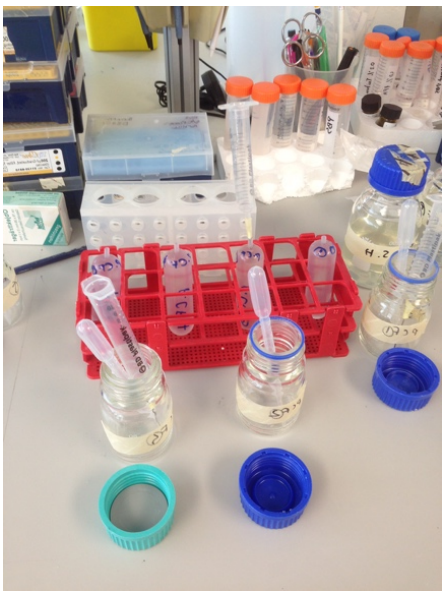

Figure 4.1b.

Figure 4.1c.

Figure 4.1: Images showing a) the refractometer, b) loading of the samples into 40 or 100 ml centrifuge tubes, and c) sealing of centrifuge tube via melting of the neck opening of the tube. Please note the pink liquid within the tube shown is not of sputum within the tube, but that of another sample, but is included for the interest of the reader.

Figure 4.2a.

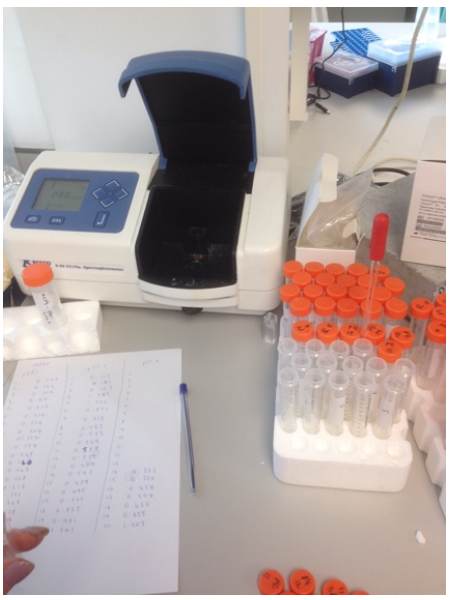

Figure 4.2b.

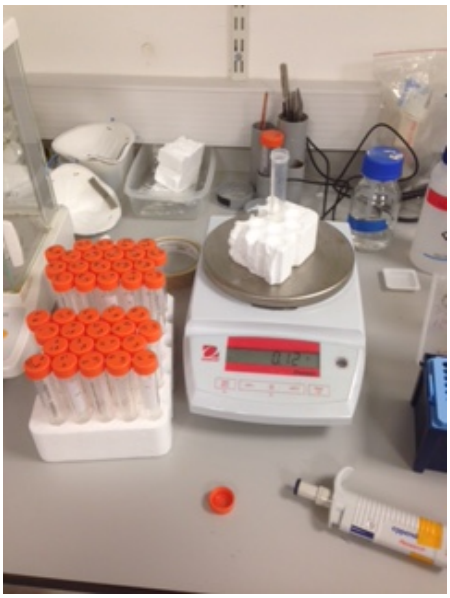

Figure 4.2c.

Figure 4.2: a) unloading of fractions. Tubes are held by a clamp whilst a pump expels the fractions by steady drops into the 15 ml falcon tubes. b) measurement of OD_{280} using a spectrophotometer. c) illustrates the weighing of 1 ml of the fractions.

4.2.1.2. Periodic acid-Schiff (PAS) stain

The method by Thornton and colleagues (1989) was developed to assess carbohydrate content in the mucin fractions via utilisation of the Periodic acid-Schiff reaction (Hotchkiss, 1948). This assay was developed from the use of the PAS stain used for detection of mucins in tissue samples so that it could be used on glycoprotein samples in chaotropic agents which have been immobilised on nitrocellulose membrane via slot blotting (Thornton et al, 1996).

Two pieces of Whatman™ filter paper were soaked in a basin with ddH_2O before placing on the plastic sleeve of the slot blot base apparatus. The nitrocellulose blotting membrane covered with protective film was also soaked in ddH_2O and the protective film carefully peeled off. Care was taken to not touch the centre of the exposed membrane. The membrane was then placed on top of the wet filter paper followed by the top apparatus which was sealed with the white plastic side blocks as shown in figure 4.3a.

This assay was used to detect carbohydrate in each of the twenty fractions per sputum sample. A sample volume of 50 µl from each fraction was ejected in the slots in order of the fraction number down each column for each sample, e.g. fraction 1 in row 1, fraction 2 in row 2, etc. The liquid in the slots was then aspirated onto the nitrocellulose membrane before removing from the apparatus and washing in ddH_2O. Then 50 ml of ddH_2O containing 1% (v/v) periodic acid and 3% (v/v) acetic acid was added and left on a rotating platform. After 30 minutes the solution was discarded and the membrane washed three times in ddH_2O. The membrane was then washed twice in freshly prepared 0.1% (w/v) sodium metabisulfite in 0.01 M HCl. Next, Schiff's reagent was added until it just covered the membrane and discarded when a magenta colour change was observed (figure 4.3b). The membrane was then washed three times in sodium metabisulfite followed by three washes in ddH_2O and quickly air-dried (figures 4.3c. and 4.3d.). Intensity of periodic acid – Schiff was measured using the Bio-Rad Chemidoc MP1.

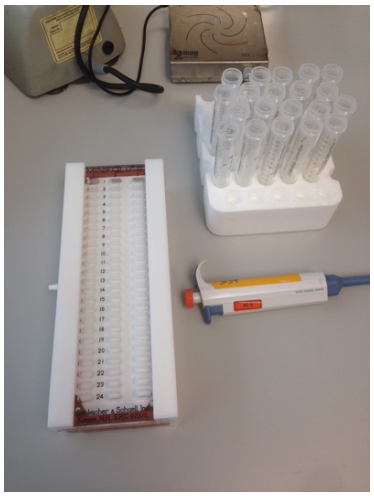

Figure 4.3a.

Figure 4.3b.

Figure 4.3c.

Figure 4.3d.

See legend on the next page

Figure 4.3e.

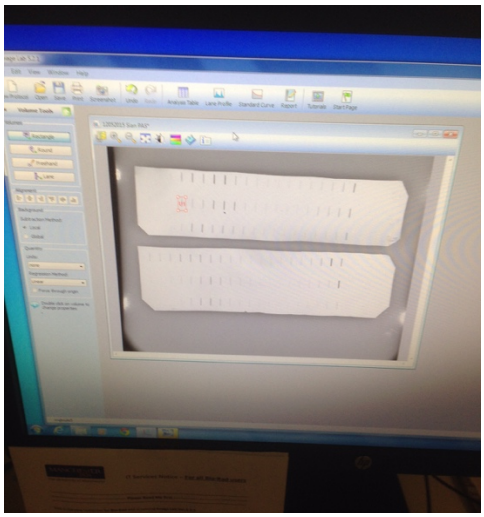

Figure 4.3f.

Figure 4.3: a) set-up of the slot blot apparatus, b) appearance of magenta stain following addition of Schiff's reagent, c)/d) drying of the membrane, and e)/f) use of the BioRad Chemidoc MP1 for measuring intensity of the PAS stain on mucin fractions which have been transferred onto a nitrocellulose membrane.

4.2.1.3. Western slot blot

To detect the presence of MUC5B and MUC5AC proteins in sputum fractions, slot blots were set up as described in section 4.2.1.2. A volume of 15 µl of each fraction was added to each slot per fraction sample. The sample was then aspirated onto the nitrocellulose membrane before removing from the apparatus and washing in ddH_2O. Disulphide bonds between cysteine residues in mucin were then reduced with the addition of 25 ml 0.01 M (w/v) DTT in GuHCl reduction buffer containing 0.1 M Tris-HCl (pH 8) 0.05 M EDTA. After 30 minutes on a rotating platform, to prevent disulphide bonds reforming, an alkylation process was performed. A volume of 25 ml iodoacetamide (25 mM) in GuHCl reduction buffer was added and the sample left on the rotating platform. After 30 minutes the iodoacetamide was discarded and replaced with 25 ml of 1X TBST (10 mM Tris-HCl at pH 8, 150 mM NaCl, 0.05% (v/v) Tween®-20) mixed with 5% (w/v) milk powder and left on the rotating platform for 30 minutes. The membrane was then washed twice in 1X TBST before adding 25 ml of 1X TBST. Following this, 12.5 µl (1:2000) of polyclonal (rabbit) antibody MAN5ACI sequence of the immunisation peptide: RNQDQQGPFKMC (Hovenberg et

al, 1996; Kirkham et al, 2002) and 25 µl (1:1000) of monoclonal (mouse) antibody EU-MUC5Bb sequence of the immunisation peptide: RNREQVGKFKMC (Rousseau et al, 2003) was added. This was then left overnight on a rotating platform.

After 24 hours, the membrane was washed four times in 1X TBST over 1 hour, followed by several quick washes in 1X TBST to remove any unbound antibody. After washing off the primary antibodies, 1 µl of each of the secondary antibodies was added in 25 ml of 1X TBST (1:25000). IRDye® 800CW goat anti-rabbit (LI-COR®) polyclonal antibody binds to MUC5AC primary antibody (MAN5ACI), and Alexa Fluor® 680 goat anti-mouse IgG monoclonal antibody (H+L) (Life Technologies) binds to the MUC5B primary antibody (EU-MUC5Bb). The membranes within the container were then quickly covered in foil and put on a rotating platform. After 1 hour, secondary antibodies were removed by washing three times in 1X TBST. After the final wash TBST was discarded and the membrane was put in 10 ml of 1X PBS. The membrane was then scanned by the Odyssey CLx LiCor using Image studio v5 software (Thornton et al, 1991; Thornton et al, 1996).

4.2.2. Preparation for growth studies in purified mucin fractions

4.2.2.1. Processing of sputum fractions

Following analysis of the mucin fractions via PAS staining and western slot blotting, fractions 1-12 (containing mucin) and 13-20 (not containing mucin) were pooled. The pooled fractions were then dialysed using 1 kDa standard grade regenerated cellulose dialysis membrane (Spectra/Por) overnight against nH_2O with several changes to remove GuHCl and CsCl present in the samples which would prevent bacterial growth.

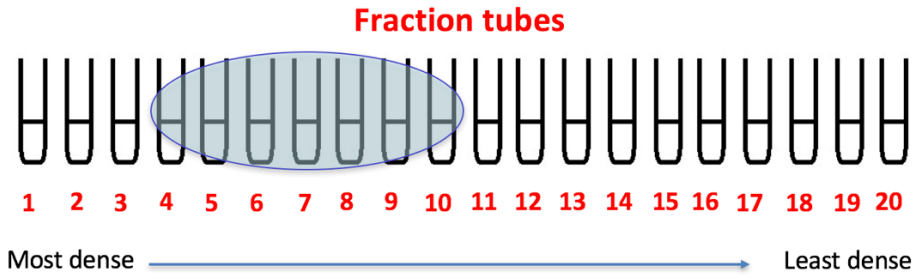

Figure 4.4.

Figure 4.4: Illustration of the 20 fraction tubes collected after density gradient centrifugation. The blue oval region represents the fractions in which the mucins were mostly found as determined by PAS stain and Western slot blot techniques.

The pooled fractions were then lyophilised in 20 ml EPA vials and re-suspended to 2 mg/ml with nH_2O. This was because following processing it was not possible to suspend the sample to 8 mg/ml and have enough volume for growth studies. The sample was then sterilised via autoclaving in the presence of 10 mM potassium phosphate buffer in preparation for growth studies. Samples were assessed using NanoDrop One to determine nucleic acid quantity within the sample.

4.2.2.2. DNase treatment of sputum fractions

To investigate growth in DNase treated mucin-containing fractions, fractions which had been suspended to a concentration of 2 mg/ml were treated with 60 U/ml DNase in volumes of 100 µl so that a total sample volume of 600 µl was treated with DNase.

To each 100 µl reaction volume of the mucin-containing fractions, 10 µl of 10X DNase buffer (0.1X volume) and 1.5 µl of TURBO DNase was added and left for 30 minutes at 37^{o}C. After 30 minutes, another 1.5 µl of TURBO DNase was added and incubated for the same duration of time so a total of 6 U of DNase had been added to each 100 µl sample. Following a total of 1 hour incubation, the DNase within the 100 µl sample was then inactivated with the addition of 20 µl DNase inactivation reagent (0.2X volume) that had been vortexed before use. This was then incubated at room temperature for 5 minutes before centrifuging at 10000 x g for 2 minutes. The supernatant was then carefully removed and transferred to a fresh 1.5 ml microtube where the supernatants from all six 100 µl sample reaction volumes were pooled. Following this, the total volumes were often greater than 600 µl due to the addition of buffer and DNase. They were therefore lyophilised in the same microtubes before being re-suspended to 600 µl with nH_2O.

4.2.2.3. Attempted removal of degraded DNA following DNase treatment

The samples were gently pipette mixed, and transferred to a Pur-A-Lyzer 1 kDa dialysis tube to remove the degraded DNA. Prior to its use, the tube was loaded with 800 µl of nH_2O, placed within the floating rack, and soaked in nH_2O for 30 minutes at room temperature. This was done to remove the residual preservative. The sample was then dialysed overnight in nH_2O at 4^{o}C with a magnetic stirrer with several changes as previously described. Following dialysis, the sample was transferred to a fresh EPA vial before being lyophilised. After 24 hours, the lyophilised samples were weighed, re-suspended to 2 mg/ml with nH_2O, and autoclaved in the presence of 10 mM potassium phosphate buffer in preparation for growth studies.

4.2.2.4. Further processing of mucin containing sputum fractions for growth studies

In further experiments, it was necessary for the mucin-containing fractions (1-12) to be further purified following DNase treatment. This method was initially trialled using PGM and centrifugal filters of different membrane pore sizes: 10 kDa, 30 kDa, and 50 kDa.

As described in section 4.3.7.1, the 30 kDa centrifugal filter was eventually selected. Following centrifugation at 14000 x g for 10 minutes, between each wash with nH_2O, the flow-through was collected and stored at -20^{o}C. The flow-through was reserved for further growth studies to assess whether *P. aeruginosa* can grow on the flow-through sample containing degraded DNA following DNase treatment. Washing of the filter, followed by centrifugation was repeated four times to ensure as much of the flow-through as possible had been collected. After four centrifugation cycles, the column was inverted into a fresh 1.5 ml microtube and the 30 kDa filtered mucin was collected. The volume was then measured and adjusted to 600 µl with nH_2O. These samples are referred to as the 30 kDa filtered PGM/adult or paediatric CF mucin-containing fractions in later growth studies. DNase treated, 30 kDa filtered, or flow-through samples were sterilised in the presence of 10 mM potassium phosphate buffer as previously described (section 3.2.2.2).

4.2.3. Assessment of mucins following processing

Mucin structure was assessed to determine whether processing influences growth. To determine this the processed mucin structure would need to be compared to the structure of the unprocessed mucins. Therefore, unprocessed mucin fractions in GuHCl and CsCl following density gradient centrifugation were pooled and dialysed in 1.5 ml microtubes with 1 kDa Spectro/Por dialysis tubing covering the lid which was secured with an elastic band (figure 4.5a.). These were inverted in a beaker containing 6 M urea and secured with autoclave tape (figure 4.5b.). Alternatively, samples were concentrated via use of a 1 kDa centrifugal filter centrifuged at 14000 x g for 10 minutes, washed in 6 M urea, and repeated for four cycles. GuHCl was exchanged for urea so that mucin structure could be assessed via Western blotting following separation via SDS-agarose gel electrophoresis (Thornton et al, 1995). The concentrated sample volume was then adjusted to one third of the original volume with 6 M urea so that the sample was 3X concentrated compared to the original sample.

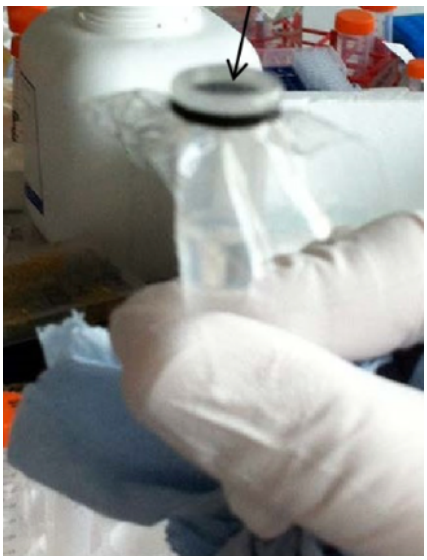

Figure 4.5a.

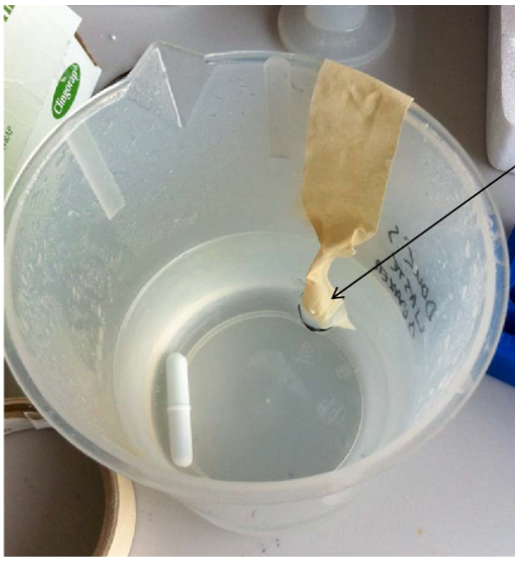

Figure 4.5b.

Figure 4.5: Dialysis of samples in 6 M urea.

Determination of how concentrated the processed sample was vs. the 3X concentrated, unprocessed mucin fractions was calculated from the starting volume of the unprocessed pooled mucin fractions before dialysis compared to the final volume in which the freeze-dried mucin was re-suspended prior to growth experiments. Based on how concentrated this processed sample was compared to the unprocessed sample, the processed sample was either diluted to be 3X concentrated or re-concentrated via centrifugal filters as previously described. This was so that the 3X concentrated mucins could be compared directly. Evaluation of mucin loss during processing was assessed via SDS-agarose gel electrophoresis which enabled separation of these large glycoproteins followed by Western blotting (Thornton et al, 1995; Ridley et al, 2016).

4.2.3.2. SDS-agarose gel electrophoresis

Prior to SDS-agarose gel electrophoresis and following density gradient centrifugation of sputum samples, 60 µl was taken from each of the twelve fractions containing mucin and pooled so that the final volume was 720 µl. Each sample was then dialysed in 6 M urea overnight in a beaker with a magnetic stirrer at 4^{o}C. The next day a 0.7% (w/v) agarose gel was prepared in 1X TAE buffer, 0.1% SDS buffer. The agarose was then

heated until fully dissolved and left to cool for 6 minutes. The agarose was then poured into the tray sealed with tape. The gel was left to solidify for 30 minutes before careful removal of the well-forming comb and transfer to the tank. The gel was then submerged in 1X TAE buffer, 0.1% SDS buffer. The reducing agent, dithiothreitol (DTT) was then prepared to a final concentration of 0.1 M in 10X reduction loading buffer (section 2.2). For samples to be reduced, 10 µl of 10X reduction loading buffer containing 0.1 M DTT was added to 90 µl of the samples so that the final concentration of DTT in the sample became 10 mM in 1X reduction loading buffer. These samples were then heated to 95^{o}C for 10 minutes to reduce the disulphide bonds between cysteine residues in the polymeric mucins (Strous and Dekker, 1992). Whole samples instead had 10 µl of 10X loading buffer added to each of the 90 µl samples without the presence of 10 mM DTT so that the polymeric structure could be assessed. Samples were then loaded into the wells so that the whole samples were loaded in the top wells of the gel, and the reduced samples loaded in the middle wells of the gel. The gel was then run at 60 V for 3 hours (figure 4.6.). This method allows separation of large mucin glycoproteins so that polymeric (whole) and reduced forms can be transferred and immobilised on nitrocellulose membrane prior to Western blotting (Thornton et al, 1995).

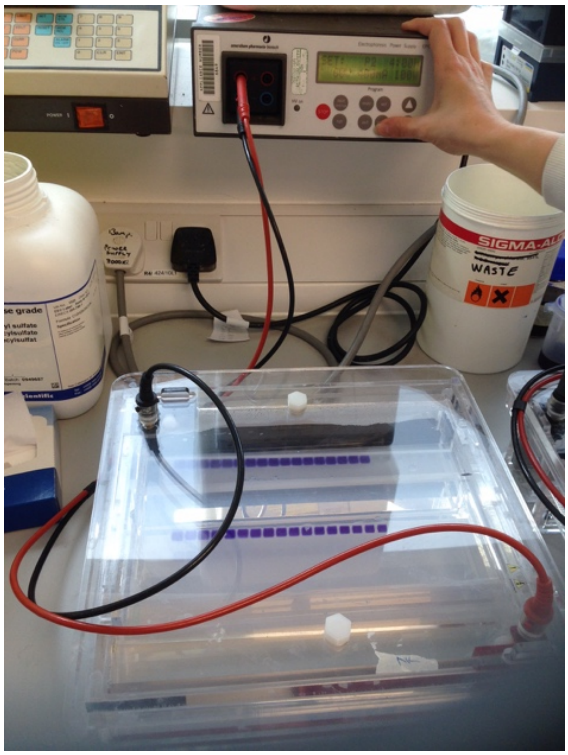

Figure 4.6.

Figure 4.6: Experimental set-up of the SDS-agarose gel electrophoresis for studying whole and reduced mucins.

4.2.3.3. Western blotting following SDS-agarose gel electrophoresis

After 3 hours, the gel was removed from the tank and transferred to a dish containing 4X SSC and 20 mM DTT as shown in figure 4.7a. This was to reduce the whole mucins so they could be transferred more easily onto the membrane. Following incubation at room temperature on a rocker, the gel was gently washed in 4X SSC to remove the DTT. The samples were then transferred over 1.5 hours to the nitrocellulose membrane using transfer apparatus to create a vacuum and the pump set at 50. During the entire transfer duration, it was ensured that the gel remained wet with the addition of 4X SCC when needed as shown in figure 4.7c.

When complete, the vacuum was switched off, before pouring off the liquid and marking the wells with pencil. The membrane was blocked with the addition of 50 ml of 5% (w/v) milk powder in 1X TBST. After 30 minutes the membrane was probed with

50 ml of 1X TBST containing the primary antibodies: 25 µl (1:2000) of polyclonal (rabbit) antibody MAN5ACI sequence of the immunisation peptide: RNQDQQGPFKMC (Kirkham et al, 2002) and 50 µl (1:1000) of monoclonal (mouse) antibody EU-MUC5Bb sequence of the immunisation peptide: RNREQVGKFKMC (Rousseau et al, 2003) and left overnight on a rocker. The following day the membrane was washed four times in 1X TBST over 1 hour and placed on the rocker between washes. After washing off the primary antibodies, 2 µl (1:25000) of the secondary antibodies previously mentioned were added in 50 ml of 1X TBST, left for 1 hour covered in foil, and placed on the rocker as described in section 4.2.1.3 for the slot blots. After 1 hour, the antibodies were removed by washing four times in 1X TBST over 1 hour. After the final wash TBST was discarded and the membrane was put in 10 ml 1X PBS. The membrane was then scanned by the Odyssey CLx LiCor using Image studio v5 software (figure 4.8).

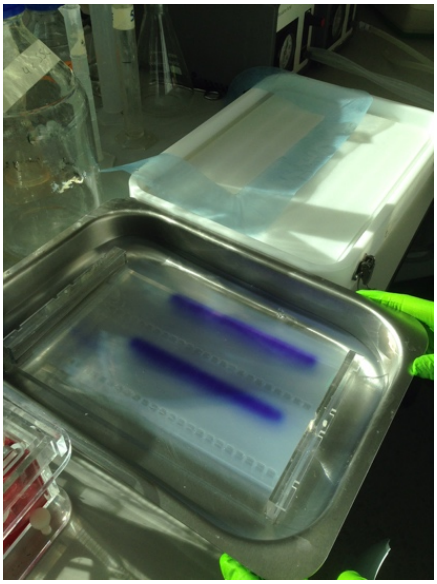

Figure 4.7a.

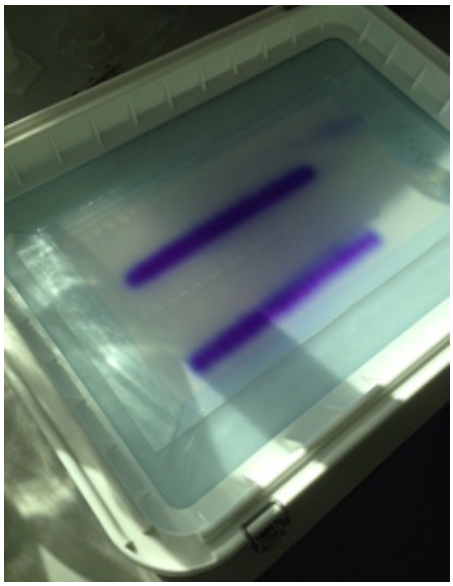

Figure 4.7b.

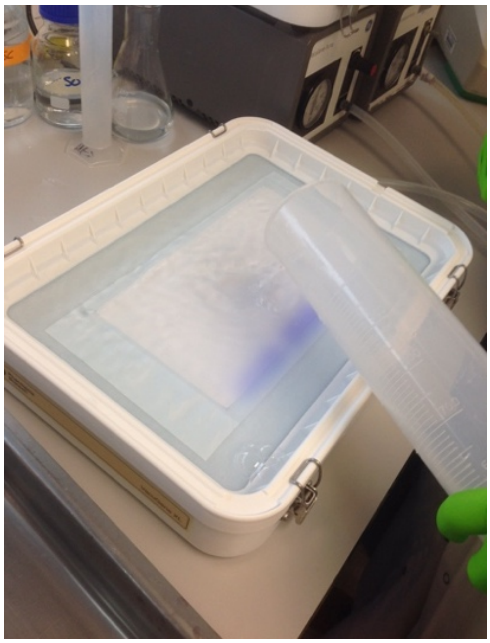

Figure 4.7c.

Figure 4.7: a) reduction of whole mucins within a dish in 4X SSC and 20 mM DTT, b) transfer of the mucins onto the nitrocellulose membrane, and c) showing addition of 4X SSC during transfer.

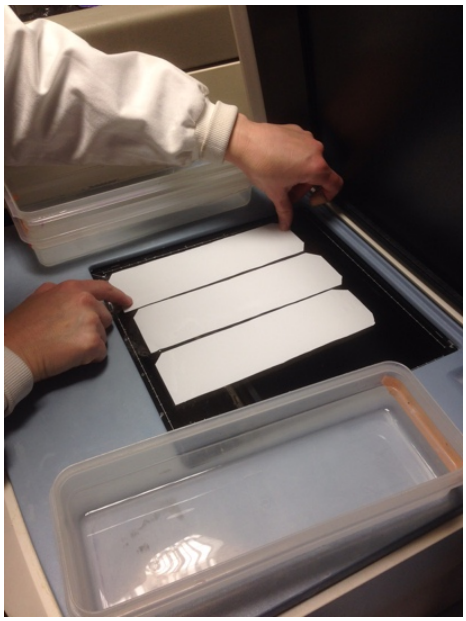

Figure 4.8a.

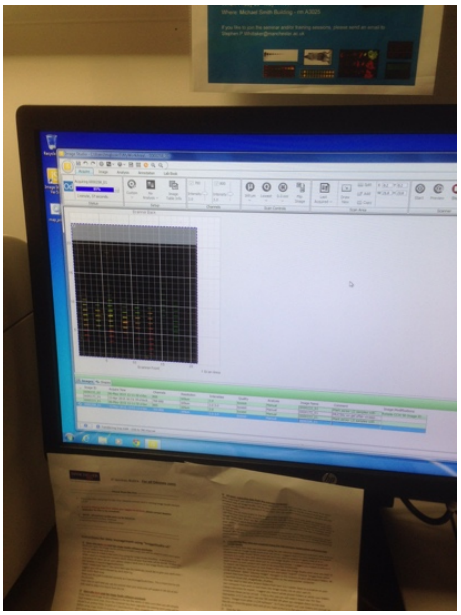

Figure 4.8b.

Figure 4.8: a) set-up of the nitrocellulose membrane on the Odyssey CLx LiCor machine. Care was taken to make sure there were no air bubbles beneath the membrane. This was performed quickly due to light exposure and the secondary antibodies. b) example of images that appear on the screen during the scanning process.

4.2.4. Growth studies in purified mucin

The growth culture was prepared by adding 2 mg/ml sample to an equal volume of double strength M9 to get a final concentration of 0.1% (w/v) sputum fraction in M9. Growth in 0.1% (w/v) DNase treated and non-DNase treated mucin-containing fractions 1-12 supplemented with M9 was assessed by CFU counts (Miles et al, 1938).

For further growth assays in purified mucin which had been 30 kDa filtered, growth assays were set up as described previously.

4.3. Results

4.3.1. Mucin purification

To investigate the role of mucin in promoting growth in CF, sputum samples collected at the LRI and Glenfield Hospital were separated via density gradient centrifugation. Respiratory mucins, MUC5B and MUC5AC, were then identified via PAS staining and Western blotting techniques. Mucins were run on SDS-agarose gels when it was necessary to assess how processing affected the structure.

4.3.1.1. Detection of MUC5B in sputum fractions

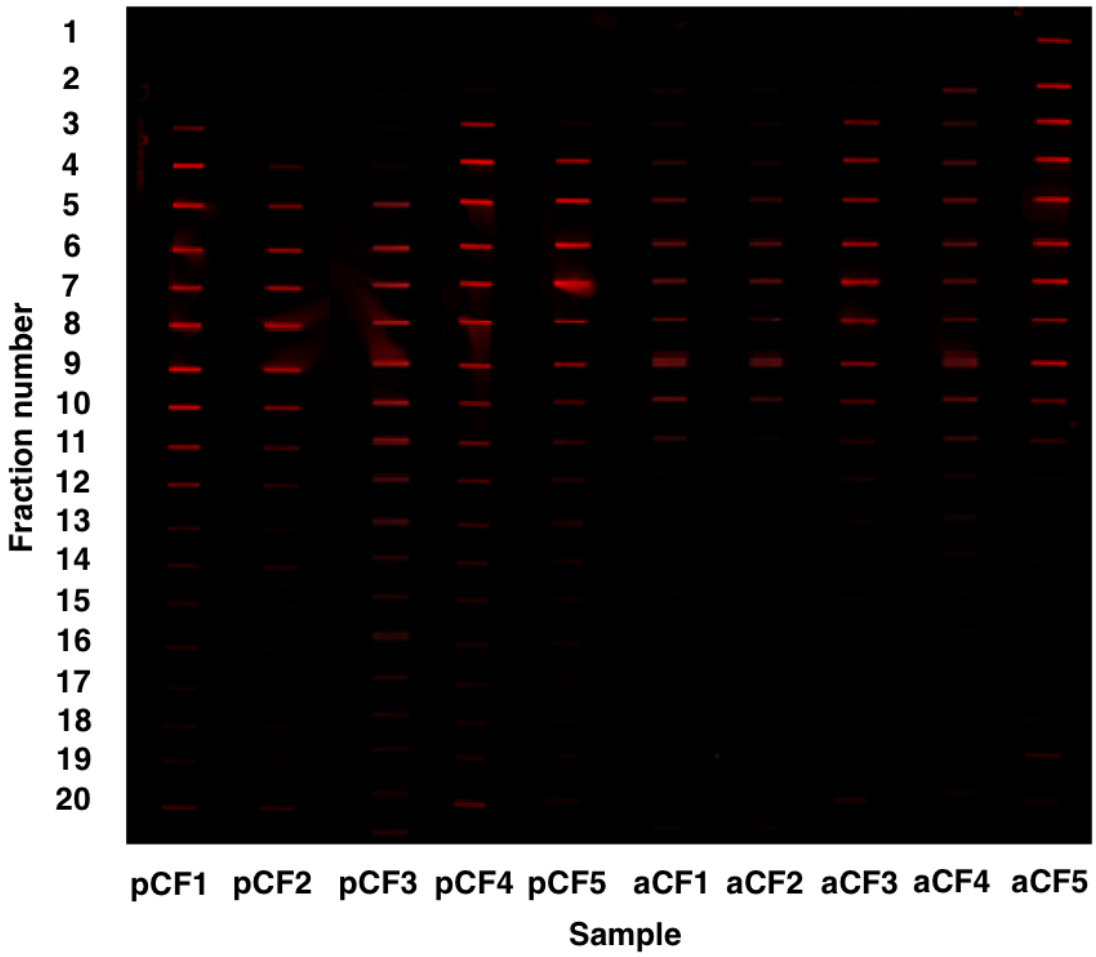

Figure 4.9a.

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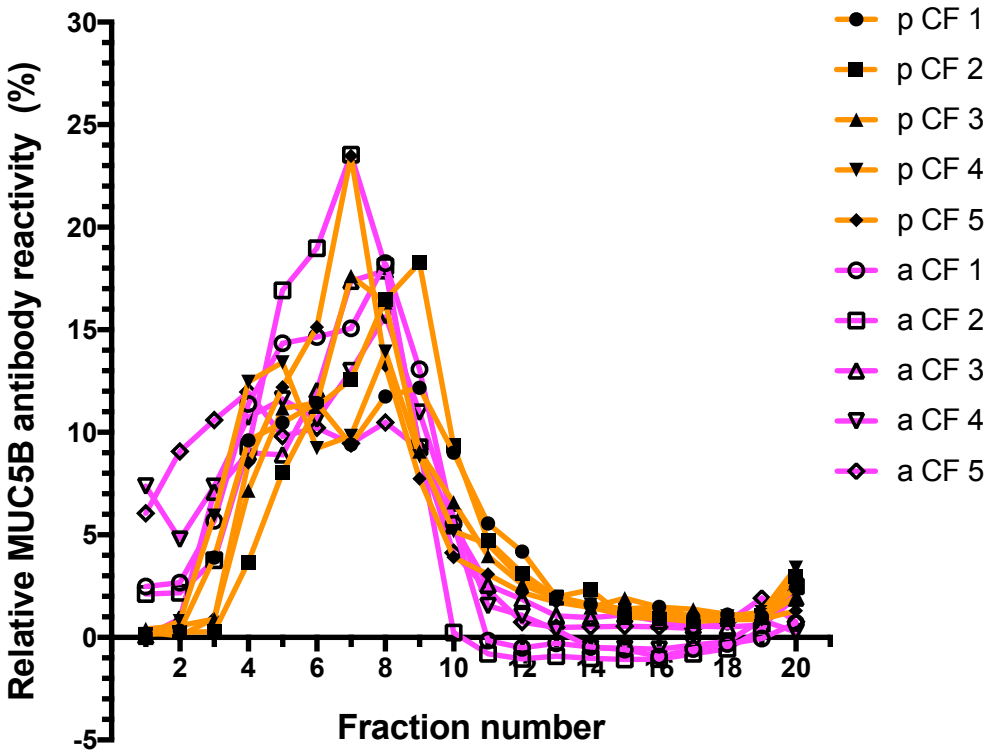

Figure 4.9b.

Figure 4.9: a) Slot blot of MUC5B reactivity using the primary monoclonal (mouse) antibody EU-MUC5Bb sequence of the immunisation peptide: RNREQVGKFKMC and the secondary Alexa Fluor® 680 goat anti-mouse IgG monoclonal antibody (H+L). The figure shows the fluorescence reactivity to MUC5B in 5 adult and 5 paediatric sputum samples separated into 20 fractions. Figure has been edited so that all samples are in order. 'pCF' represents the CF samples from paediatric patients and 'aCF' represents the CF samples from adult patients. b) Relative antibody reactivity to MUC5B and the intensity of the fluorescence measured using Image studio v5 software in adult (pink) and paediatric (orange) samples. Data are presented for each fraction as a percentage of reactivity relative to the total raw reactivity values for all 20 fractions in each individual sample.

Figure 4.9a. illustrates the slot blot antibody reactivity to MUC5B in each fraction and sputum sample. When the fluorescent bands were selected using the Image studio v5 software following scanning of the membrane, this generated a value for each fraction. This value was then divided by the sum of all the values for each sputum sample and multiplied by one hundred. These relative percentage reactivity values are plotted in figure 4.9b. for each sample. Figure 4.9a. demonstrates how there is greater reactivity as indicated by the increased intensity of the red bands between fractions 4-11. When

expressed as percentage reactivity, figure 4.9b. shows how the peak in MUC5B reactivity is in the higher fractions and drops after fraction 12.

4.3.1.2. Detection of MUC5AC in sputum fractions

The same observation was seen in figure 4.10 for detecting MUC5AC. Once again, mucins were found to be concentrated between fractions 1 and 12 (inclusive) as represented in figures 4.10a. and 4.10b.

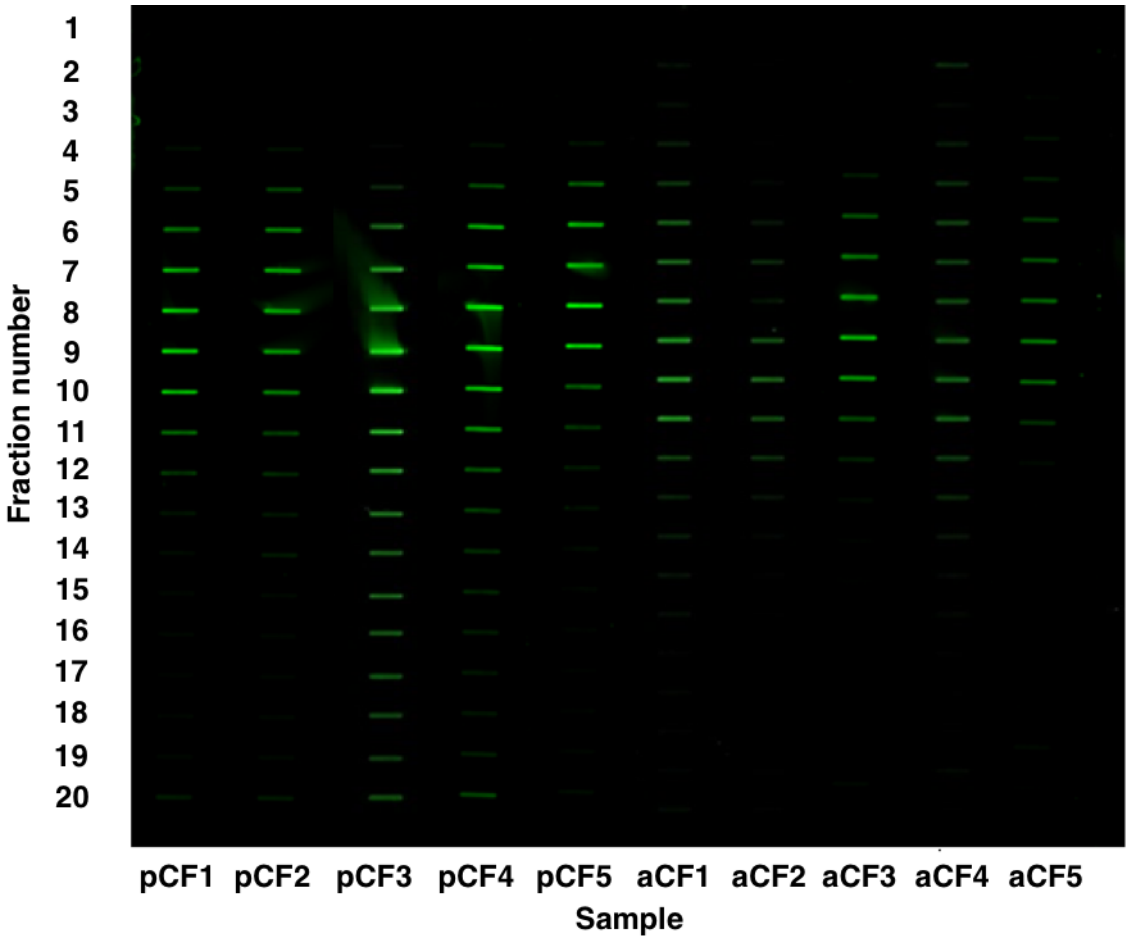

Figure 4.10a.

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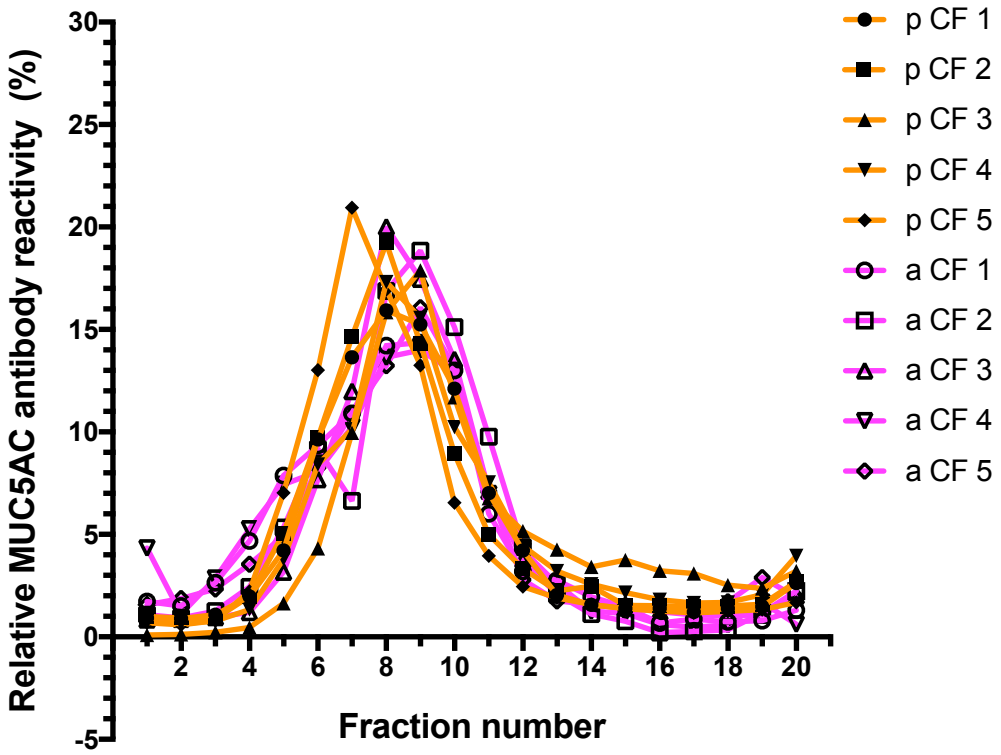

Figure 4.10b.

Figure 4.10: a) Slot blot of MUC5AC reactivity using the primary polyclonal (rabbit) antibody MAN5ACI sequence of the immunisation peptide: RNQDQQGPFKMC and the secondary IRDye® 800CW goat anti-rabbit (LI-COR®) polyclonal antibody. The figure shows the fluorescence reactivity to MUC5AC in 5 adult and 5 paediatric sputum samples separated into 20 fractions. The figure was edited so that all samples were in order. 'pCF' represents the CF samples from paediatric patients and 'aCF' represents the CF samples from adult patients. b) Figure shows the relative antibody reactivity to MUC5AC and the intensity of the fluorescence measured using Image studio v5 software in adult (pink) and paediatric (orange) samples. Data are presented for each fraction as a percentage of reactivity relative to the total raw reactivity values for all 20 fractions in each individual sample.

4.3.1.3. Detection via Periodic-acid Schiff staining

As a secondary measure, the fractions were loaded into slot blots, but rather than using specific antibodies detecting mucins, instead they were stained with Periodic acid – Schiff. This specifically detects polysaccharides and glycoproteins, and therefore would not only detect mucins, but also sugars in the samples (Hotchkiss, 1948).

Figure 4.11a. shows the scan of the slot blot membrane for each of the samples and reveals different intensities of the PAS stain in different fractions. Similarly to how the

relative percentage antibody reactivity was calculated, the relative percentage PAS intensity was calculated based on raw data intensity values generated from the Bio-Rad Chemidoc MP1. What appears evident as shown in figure 4.11b. is that there is not only a peak in intensity of the PAS stain between fractions 1 – 12, but also there is increased staining in the later fractions after fraction 16. This trend was not observed in the detection of MUC5B and MUC5AC. It was not assumed that this increase in PAS intensity in these later fractions was due to mucins since the sputum components would be separated based on their densities, and mucins are very large glycoproteins. For this reason, 1 ml of each fraction was weighed to ensure that the weight was greater in the lower fractions because this was where the mucins were expected to be located. The purple line represents the density gradient which was established in each sputum sample as shown in figures 4.12 and 4.13. Therefore, it appears that the increase in PAS intensity in the later fractions was due to the presence of sugars not associated with mucins.

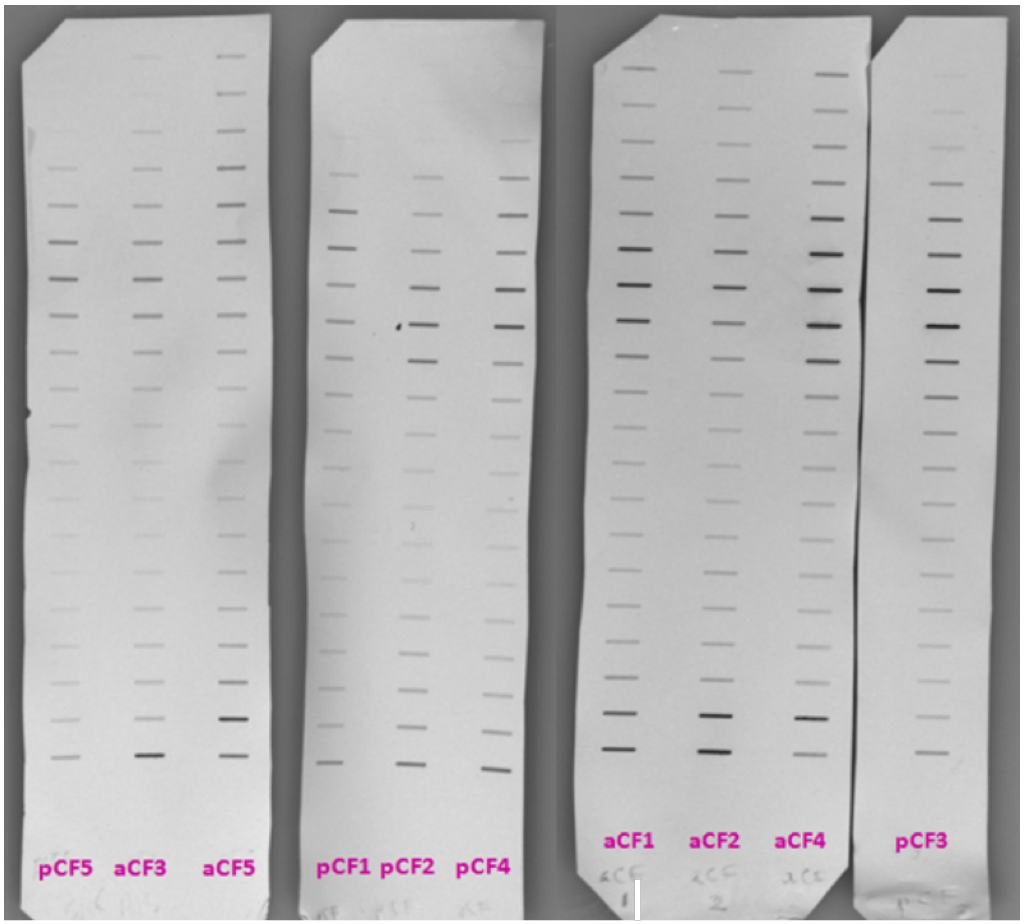

Figure 4.11a.

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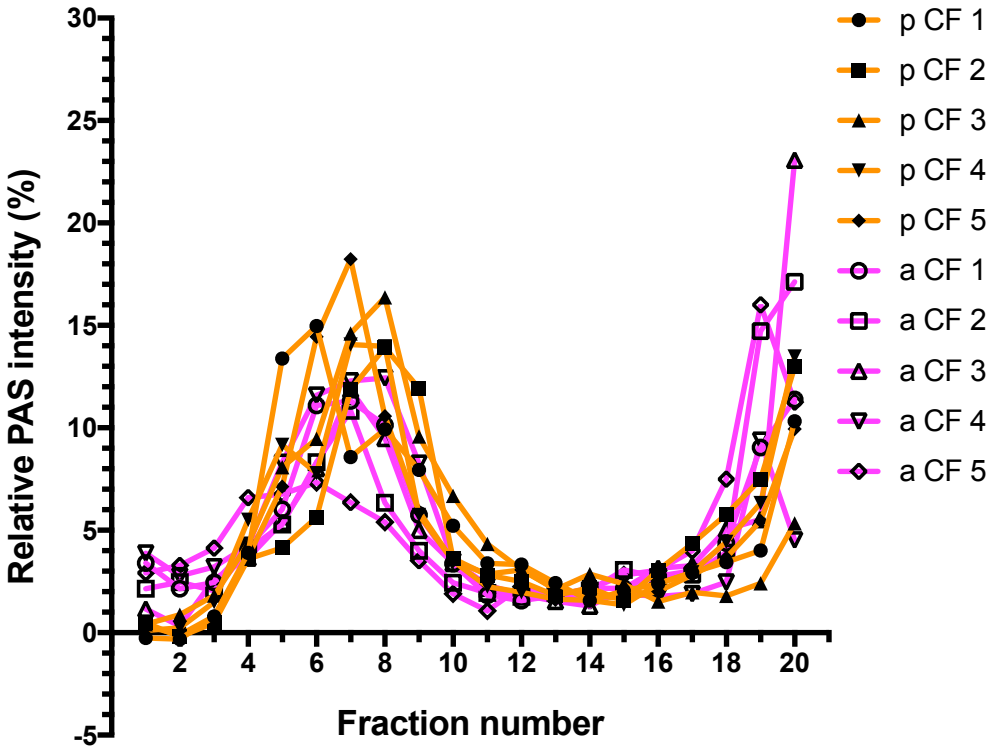

Figure 4.11b.

Figure 4.11: a) The figure shows the scan of the PAS stain of 5 adult and 5 paediatric sputum samples separated into 20 fractions. 'pCF' represents the CF samples from paediatric patients and 'aCF' represents the CF samples from adult patients. b) Relative PAS intensity (%) in each fraction for each of the 10 samples. Data are presented for each fraction as a percentage of intensity relative to the total raw intensity values for all 20 fractions in each individual sample. The pink line represents the change in intensity of the PAS stain in the adult samples whereas the orange line represents the change in intensity of the PAS stain in the paediatric samples.

4.3.1.4. Measurements of paediatric and adult CF sputum sample fractions

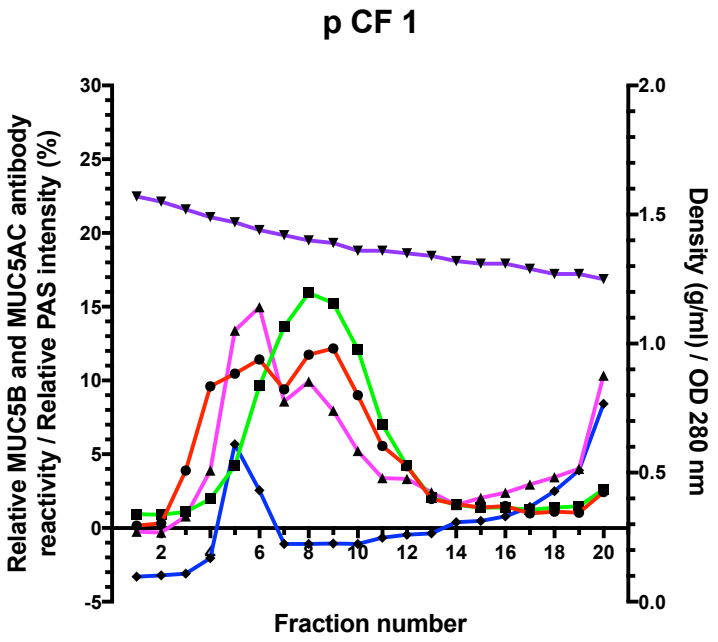

Figure 4.12a.

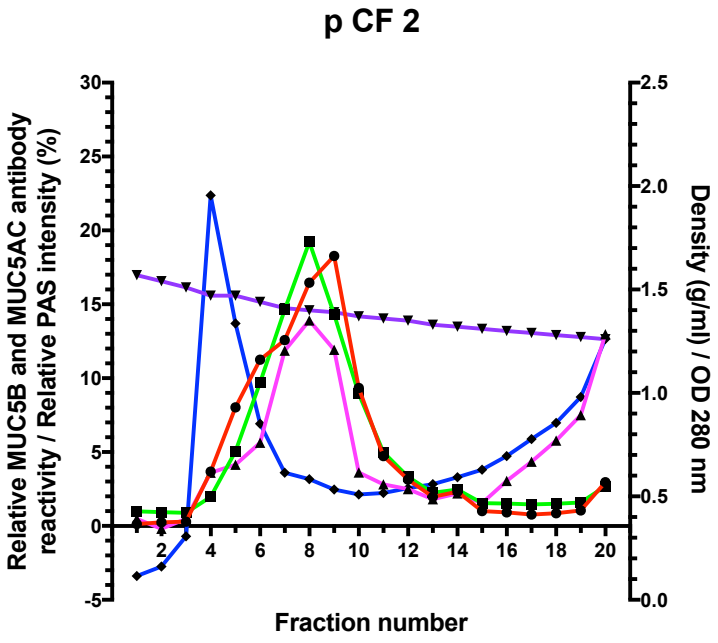

Figure 4.12b.

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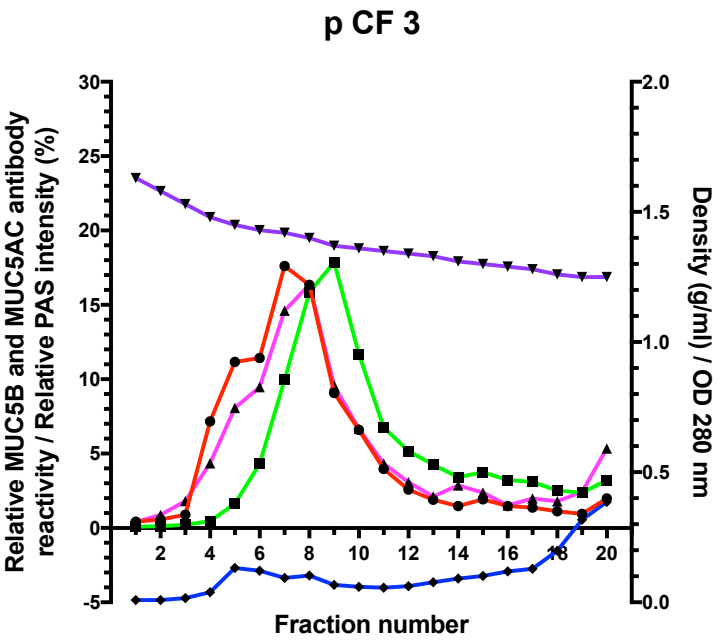

Figure 4.12c.

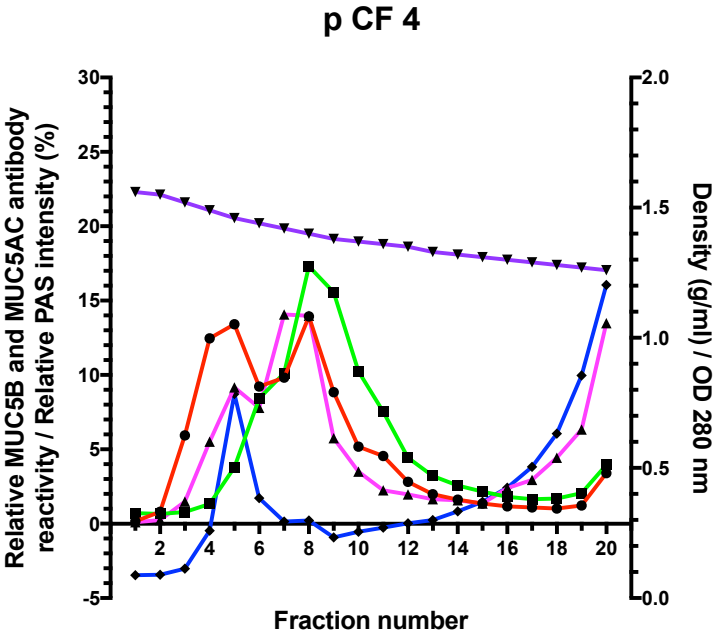

Figure 4.12d.

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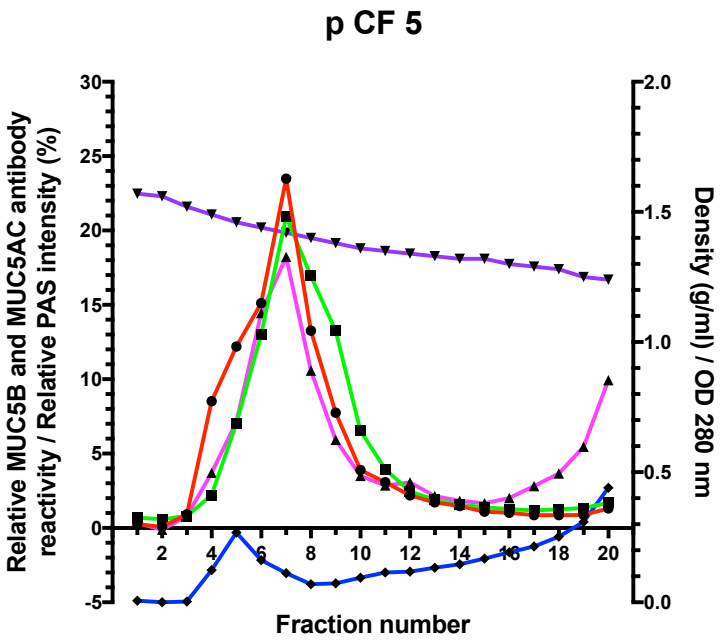

Figure 4.12e.

Figure 4.12: Graphs showing density (purple), OD_{280} (blue), relative percentage MUC5B antibody reactivity (red), relative percentage MUC5AC antibody reactivity (green), and relative percentage PAS intensity (pink) in paediatric CF samples 1 (a), 2 (b), 3 (c), 4 (d), and 5 (e). Density and OD_{280} were plotted on the secondary y – axis whereas the MUC5B, MUC5AC and PAS measurements were plotted on the primary y – axis.

Figures 4.12 and 4.13 show the relative MUC5B/MUC5AC antibody reactivity, density, relative PAS intensity, and absorbance at 280 nm plotted for each sample. All ten graphs show that the density gradient was maintained as shown by the purple line. If the density gradient was not maintained then the slot blot and PAS staining results would be misleading. It reveals how overall, the PAS staining correlates with MUC5B and MUC5AC reactivity in the sputum sample fractions. Absorbance at 280 nm was also measured as indicated by the blue line in these figures. Readings at OD_{280} come from tryptophan, tyrosine, and cysteine and serves as a measurement for protein (Thornton et al, 1991; Zhu et al, 2013). However, absorbance at 280 nm alone was not sufficient as a measurement of glycoproteins and for this reason MUC5B and MUC5AC specific antibodies were used. The figures demonstrate a peak in absorption at 280 nm in the earlier fractions 1 – 12, but this peak was not always shown to correlate with the mucin reactivity in the fractions.

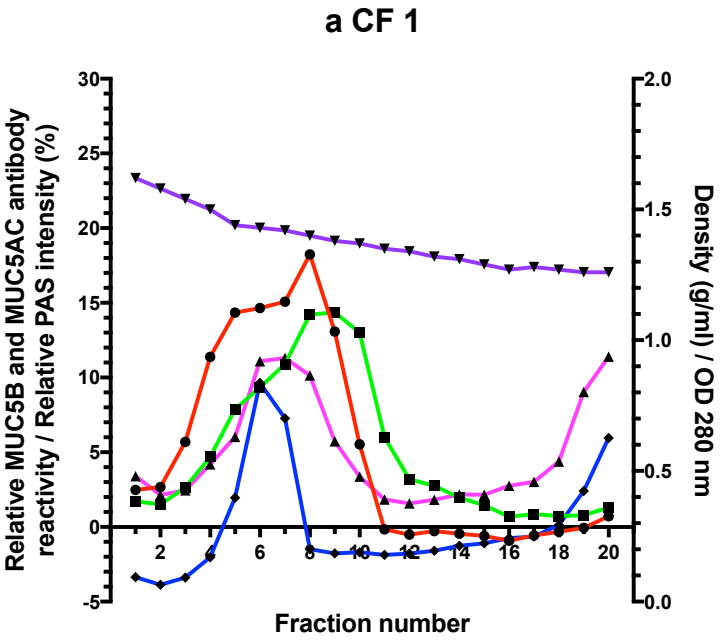

Figure 4.13a.

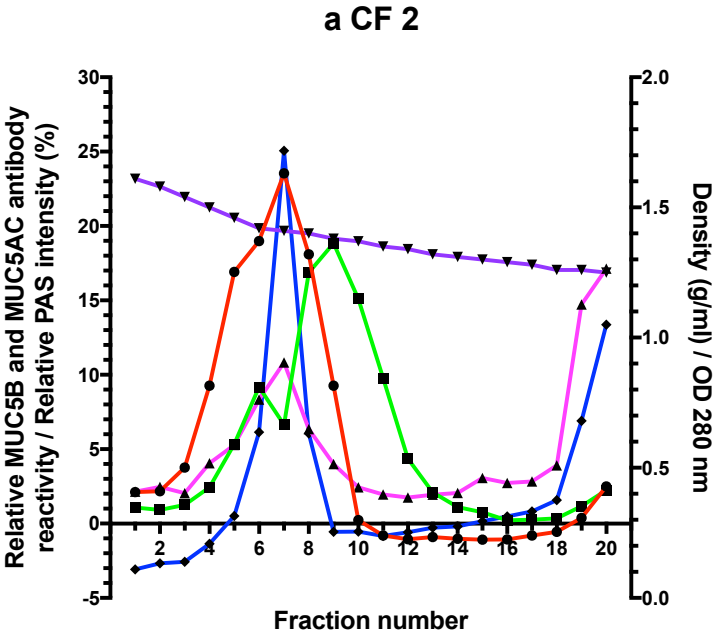

Figure 4.13b.

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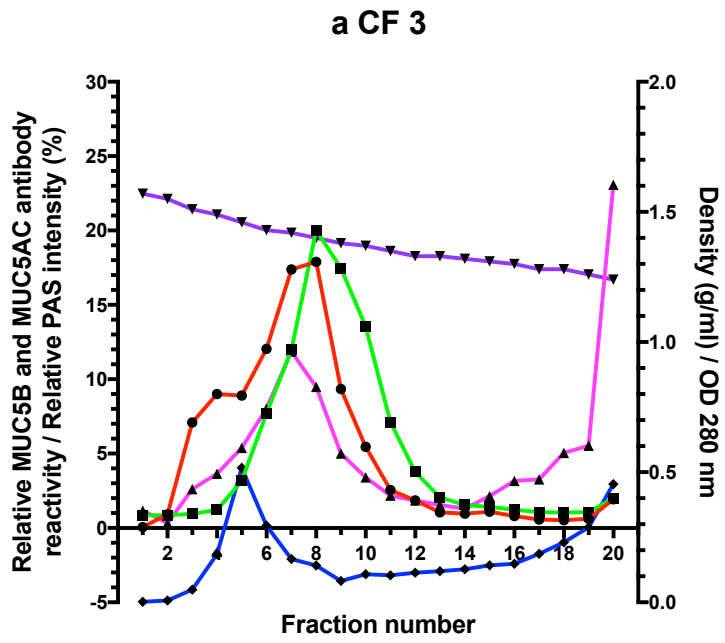

Figure 4.13c.

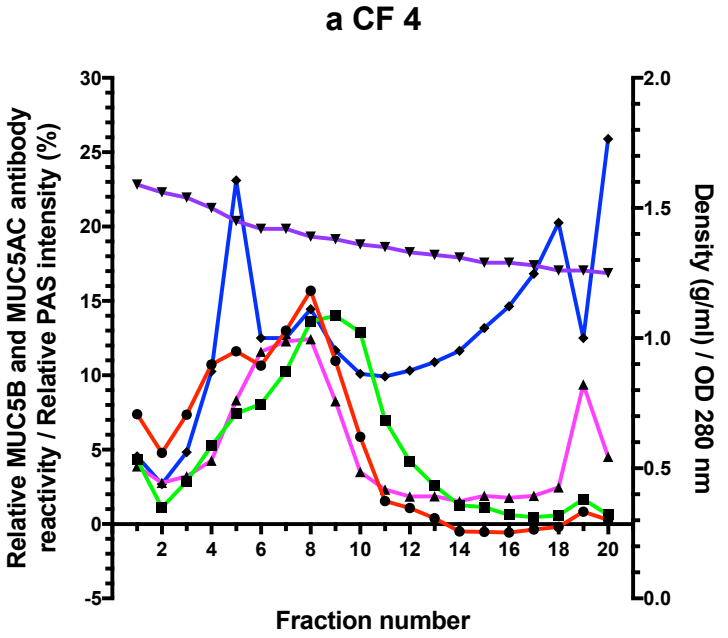

Figure 4.13d.

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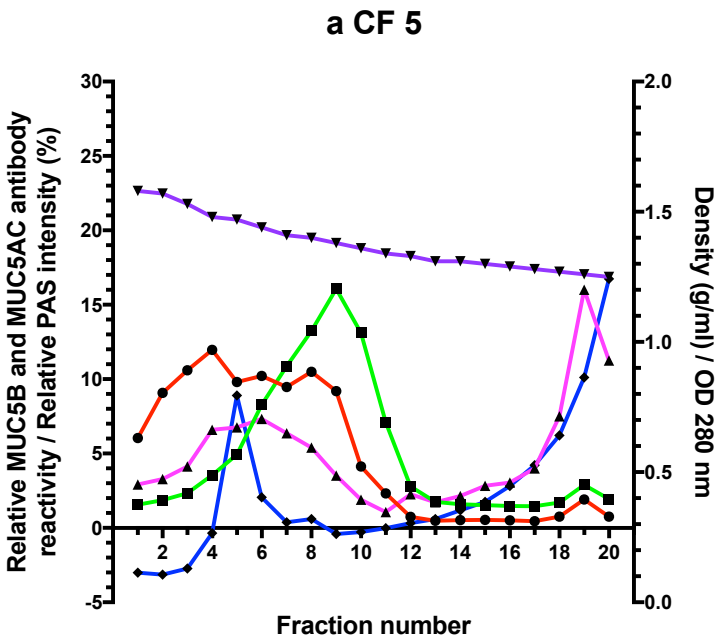

Figure 4.13e.

Figure 4.13: Graphs showing density (purple), OD_{280} (blue), relative percentage MUC5B antibody reactivity (red), relative percentage MUC5AC antibody reactivity (green), and relative percentage PAS intensity (pink) in adult CF samples 1 (a), 2 (b), 3 (c), 4 (d), and 5 (e). Density and OD_{280} were plotted on the secondary y – axis whereas the MUC5B, MUC5AC and PAS measurements were plotted on the primary y – axis.

4.3.2. Growth of *Pseudomonas aeruginosa* PAO1 in purified sputum fractions

Following density gradient centrifugation, based on the detection of mucin in fractions 1-12 it was decided to pool these fractions for growth assays to determine whether an increased growth rate in adult vs. paediatric CF mucin fraction was still observed when grown in purified mucin-containing fractions 1-12 similarly to when grown in whole sputum. Samples were prepared for growth studies as described in section 4.2.2.

4.3.2.1. Growth in mucin-containing fractions 1-12, and non-mucin containing fractions 13-20

Following growth of PAO1 in the mucin-containing fractions 1-12 it was once again observed, as found in whole sputum samples, that there was an increased growth rate in adult vs. paediatric CF fractions 1-12 (figure 4.14a.). This difference of 1.34 μ ($hour^{-1}$) $\pm$ 0.18 SEM vs. 0.83 μ ($hour^{-1}$) $\pm$ 0.08 SEM, respectively, represents a 1.6-fold increase

which was found to be significant (*p < 0.05) when analysed using a one-way ANOVA followed by a Tukey's multiple comparison test. However, when PAO1 was grown in the non-mucin-containing fractions 13-20, no difference in growth rate was observed between adult and paediatric fractions 13-20 (p > 0.05, figures 4.14b. and 4.14c.). Interestingly, it was found that there was an increased growth rate of 1.34 μ ($hour^{-1}$) ± 0.18 SEM in the adult mucin-containing fractions 1-12 vs. 0.77 μ ($hour^{-1}$) ± 0.11 SEM in the adult non-mucin-containing fractions 13-20 which was statistically significant (*p < 0.05) This suggests that the nutrient within adult CF sputum that causes an increased growth rate is present within the mucin-containing fractions and is either due to the mucin itself or associated growth promoting factors.

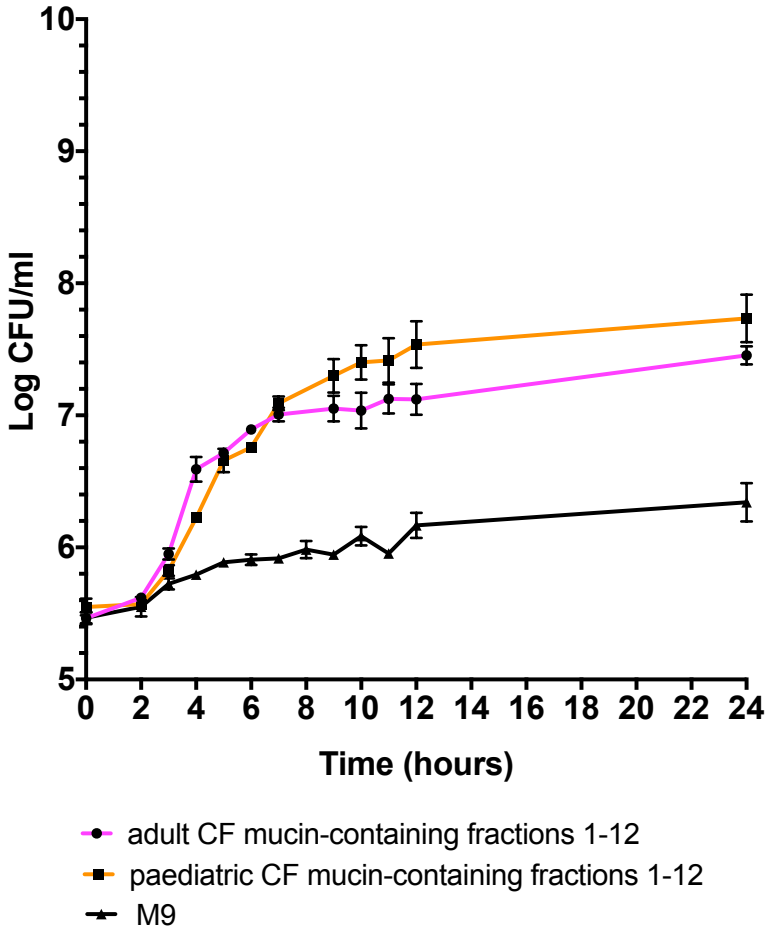

Figure 4.14a.

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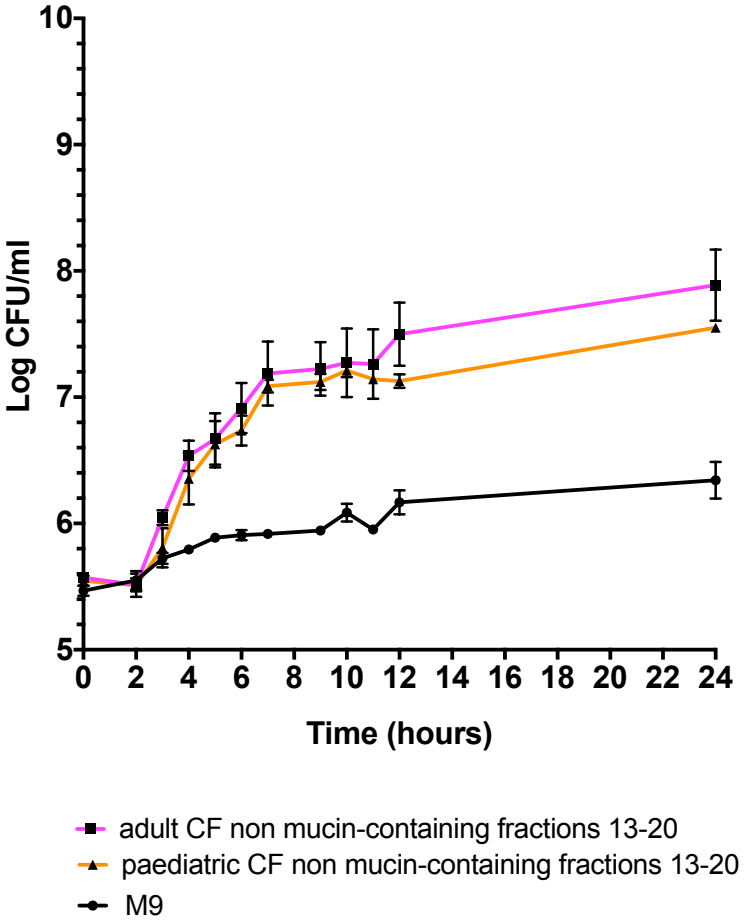

Figure 4.14b.

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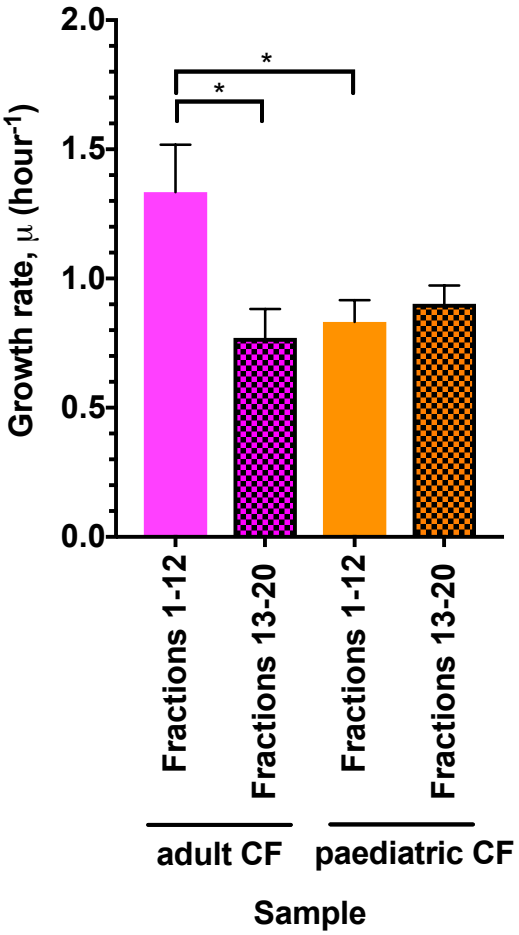

Figure 4.14c.

Figure 4.14: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) mucin-containing fractions 1-12 supplemented with M9. b) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) non-mucin-containing fractions 13-20 supplemented with M9. In graphs a) and b) the pink line is representative of mean log CFU/ml growth in adult CF fractions and the orange line is representative of growth in paediatric CF fractions from 5 adult and 5 paediatric biological replicates. All samples were supplemented with M9 (black line, 10 replicates). c) Mean specific growth rate, μ (hour^{-1}), of PAO1 calculated from graphs a) and b). Error bars show standard error of the mean (SEM). P values on the graph (*p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.2.2. Assessment of nucleic acid concentration in sputum fractions

Once PAO1 had been grown in CF sputum fractions 1-12 and fractions 13-20, it was questioned what could be causing this increased growth rate observed in the adult mucin-containing fractions 1-12. Although both adult and paediatric fractions 1-12 were known to contain mucins and were suspended based on their freeze-dried weights, it

was necessary to investigate whether factors other than mucin could be responsible for this increased growth rate. It was known from previous work conducted by Thornton and colleagues (1991) that in the earlier fractions plenty of DNA is present. This is normally removed by a secondary 0.2 M Gu-HCl density gradient centrifugation to further purify the mucin-containing fractions into DNA, high weight mucin, and low weight mucin fractions. It was decided not to proceed with this secondary purification step because the second step of mucin purification causes a considerable loss of mucin which would be detrimental to completing the growth assays in this study. Additionally, it was not expected that DNA would influence growth since *P. aeruginosa* is unable to uptake extracellular DNA (Chen and Dubnau, 2004). However, after measurements in sputum in this study revealed that DNA concentration was increased in unprocessed CF sputum vs. 'healthy' adult sputum (section 3.3.3.7), it was decided to measure the concentration in the sputum fractions which had been purified.

Therefore, since it was known that another component within these fractions could be DNA it was decided to measure the concentration of DNA/nucleic acid in the samples using NanoDrop One. In the process of doing this, only the corrected values for DNA were recorded in 0.1% (w/v) samples supplemented with M9 which were not used for growth studies. NanoDrop One revealed that whilst there were trace amounts of GuHCl following dialysis, carbohydrate and protein were also detected which was unsurprising considering mucin is a glycoprotein.

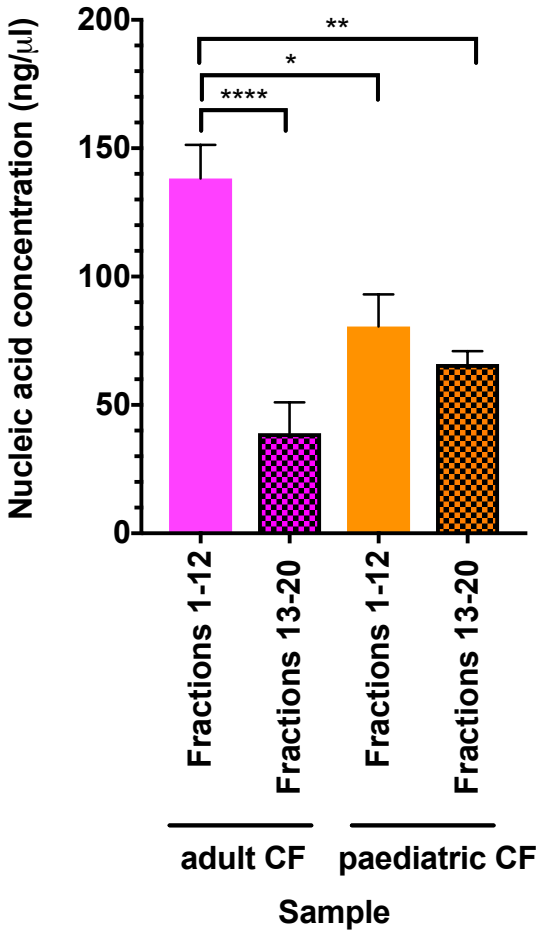

Figure 4.15.

Figure 4.15: DNA/nucleic acid concentration (ng/µl) measured using the corrected value on Nanodrop One for DNA before growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) mucin-containing fractions 1-12 and non-mucin-containing fractions 13-20 supplemented with M9. The pink bars are representative of the mean nucleic acid concentration (ng/µl) in adult CF fractions whilst the orange bars represent the concentration in paediatric CF fractions. The non-patterns bars represent the concentration in fractions 1-12 and the patterned bars show the concentration in fractions 13-20. Data are represented as the mean nucleic acid concentration from 5 adult and 5 paediatric biological replicates. Error bars show standard error of the mean (SEM). P values on the graph (**p < 0.0001, **p < 0.01, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

Measurements of the nucleic acid concentration revealed a significantly increased mean concentration in adult CF mucin-containing fractions 1-12 compared to the non-mucin-containing fractions 13-20 (****p < 0.0001, figure 4.15). For example, the nucleic acid concentration in adult CF fractions 1-12 was found to be 138.20 ng/µl ±13.08 SEM

compared to 38.98 ng/µl ±12.04 SEM in fractions 13-20. Notably there was also an increased concentration which was statistically significant in the adult fractions 1-12 vs. paediatric fractions 1-12 with the latter having a concentration of 80.54 ng/µl ±12.56 SEM (*$p < 0.05$).

4.3.2.3. Correlation of nucleic acid concentration with growth rate

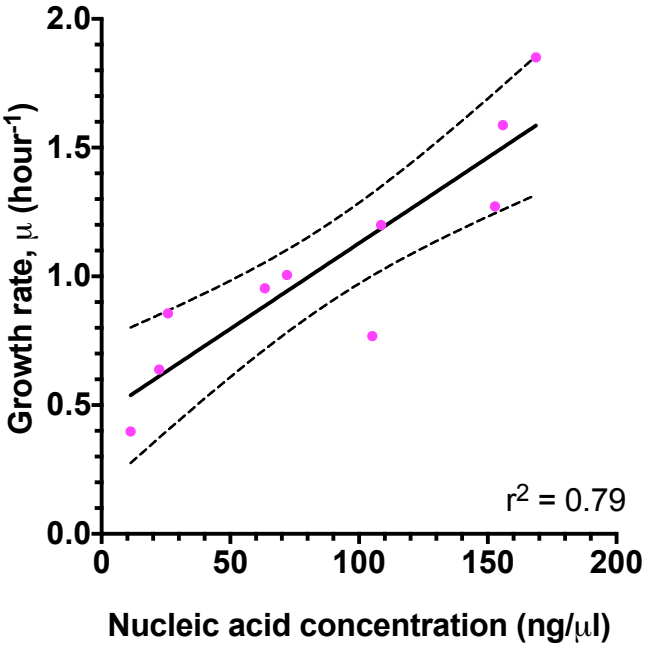

Figure 4.16a.

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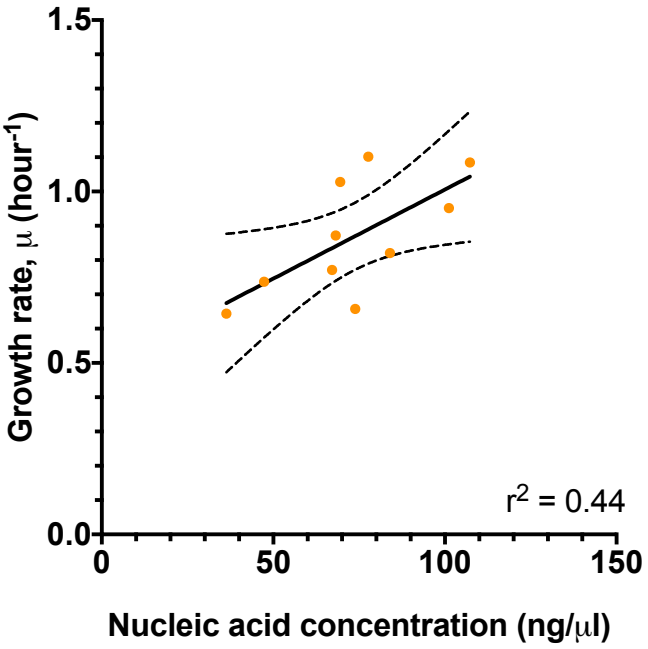

Figure 4.16b.

Figure 4.16: a) Growth rate plotted vs. nucleic acid concentration in adult (pink) CF fractions 1-12 and fractions 13-20. b) Growth rate plotted vs. nucleic acid concentration in paediatric (orange) CF fractions 1-12 and fractions 13-20. Linear regression reveals correlation of growth rate vs. nucleic acid concentration with 95% confidence bands.

Linear regression analysis of the growth rate plotted against the nucleic acid concentration for all samples revealed that there was strong correlation with an r^2 value of 0.75 (data not shown). This demonstrates that an increase in nucleic acid concentration strongly correlates with the growth rate observed when PAO1 was grown in the sputum fractions (figure 4.14a.). Interestingly, when analysis was performed on the adult samples this value increased to an r^2 value of 0.79 (figure 4.16a.) whilst in paediatric samples it was lower at 0.44 (figure 4.16b.). The data strongly suggests that nucleic acid concentration in adult samples influences the growth rate but not in paediatric samples.

4.3.2.4. Growth in mucin-containing fractions 1-12, 10 kDa filtered

Since it had been found that the amount of nucleic acid strongly correlates with the growth rate, it was decided to filter the mucin-containing fractions 1-12 since the highest concentration of nucleic acid had been previously measured in the adult mucin-

containing fractions 1-12 as shown in figure 4.15. By using a higher molecular weight cut-off of 10 kDa greater than 1 kDa which was previously used prior to growth in the fractions, excess DNA could be dialysed out of the sample, and the effect on growth rate could be assessed.

Figure 4.17a. shows the growth profile of *P. aeruginosa* PAO1 in mucin-containing fractions 1-12 which were filtered using a 10 kDa centrifugal filter. The data shows that there is no longer an increased growth rate in the same adult mucin-containing fractions 1-12 which was observed when only a 1 kDa MWCO was used. This was confirmed following analysis of the mean specific growth rate. An unpaired t-test revealed that there was no significant difference in the growth rate of PAO1 when grown in CF mucin-containing fractions 1-12 from adult and paediatric patients ($p > 0.05$). This data suggests that the removal of degraded DNA already present in the sample reduces the growth rate.

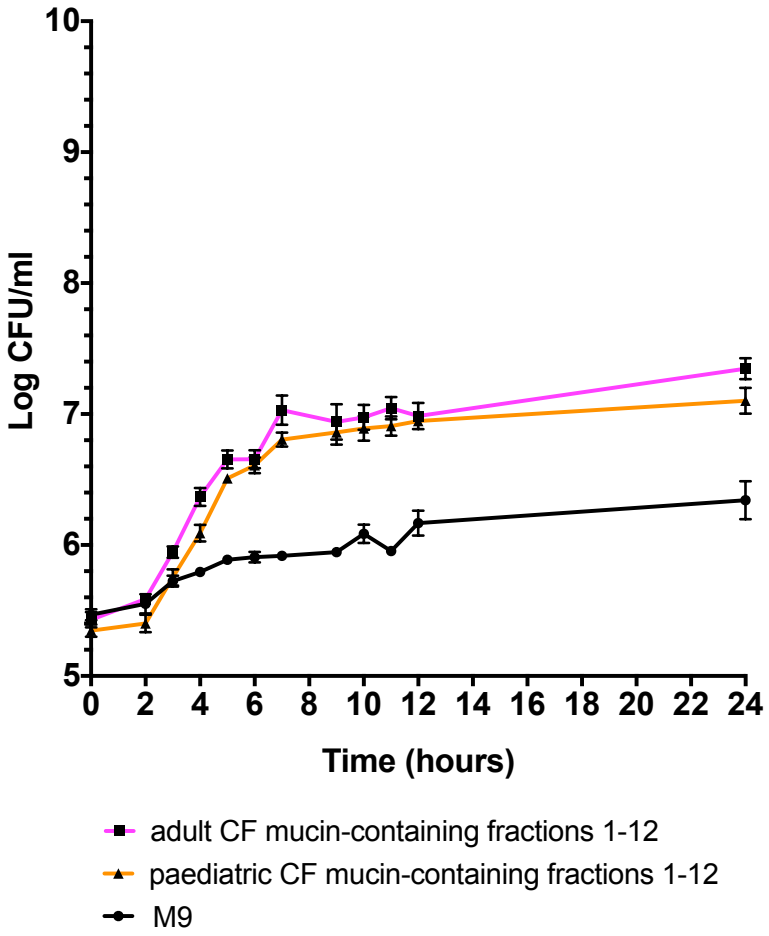

Figure 4.17a.

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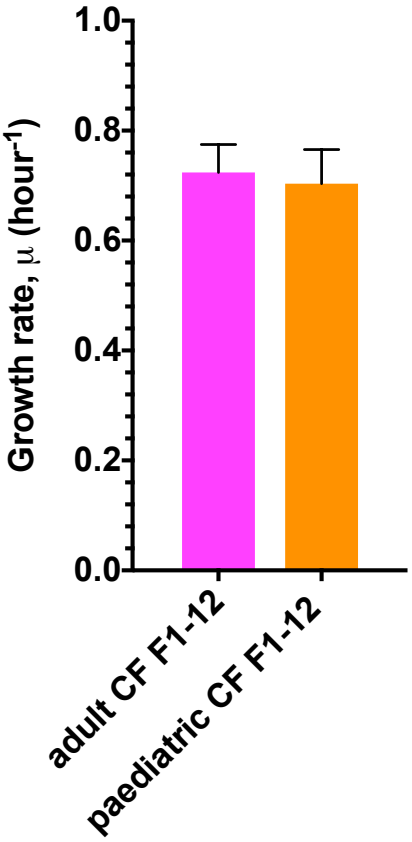

Figure 4.17b.

Figure 4.17: a) Growth (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) mucin containing fractions 1-12 supplemented with M9 which was filtered using a 10 kDa centrifugal filter. b) Mean specific growth rate, μ (hour^{-1}) of PAO1 calculated from graph a). Error bars show standard error of the mean (SEM). In graphs a) and b) the pink line/bar is representative of 5 adult CF fraction 1-12 biological replicates and the orange line/bar is representative of 5 paediatric CF fraction 1-12 biological replicates. All samples were supplemented with M9 (black line, 10 replicates). Data was analysed via a two-tailed, unpaired t-test.

4.3.3. Growth of *Pseudomonas aeruginosa* PAO1 in mucin-containing fractions 1-12 treated with DNase

The data suggests that the amount of nucleic acid measured in samples influences the growth rate of *P. aeruginosa* PAO1. Therefore, it was decided to DNase treat the adult and paediatric CF mucin-containing fractions 1-12 to identify whether the growth rate or yield is affected when more degraded DNA is produced. For the next stage of this project, the methods described in sections 4.2.2.2 and 4.2.2.3 were followed.

Figures 4.18a. and 4.18b. reveal that following DNase treatment, there was no significant difference in growth rate, when an unpaired, two-sided t-test was carried out on the mean specific growth rates. This was despite an increased nucleic acid concentration being measured in adult CF mucin-containing fractions 1-12 compared to the paediatric samples as shown in figure 4.18c. (*$p < 0.05$). For example, following dialysis after DNase treatment, the concentration in the adult samples was measured at 124.30 ng/µl ± 1.93 SEM compared to 51.15 ng/µl ± 8.88 SEM in the paediatric samples. Previously it was demonstrated in section 4.3.2.3 that the amount of nucleic acid in samples correlates with the growth rate. The data therefore indicates, as evidenced by the r^2 value of 0.002, that there is no longer a positive correlation when DNA is degraded.

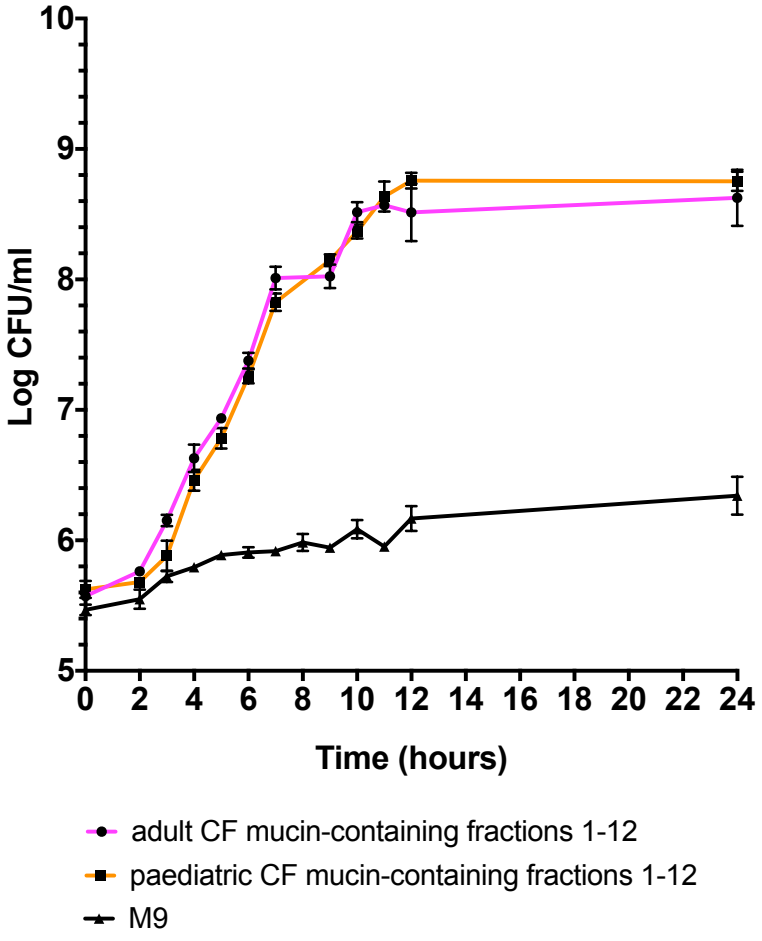

Figure 4.18a.

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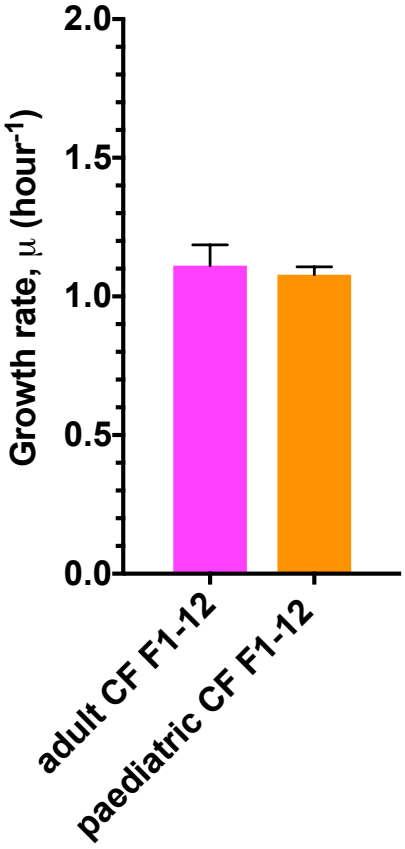

Figure 4.18b.

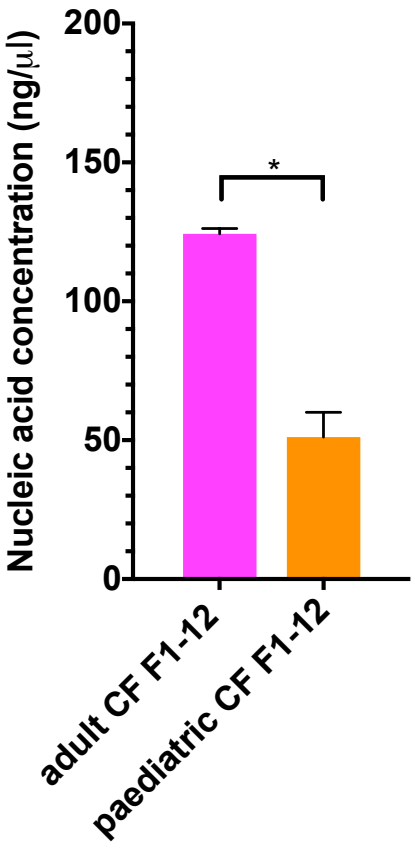

Figure 4.18c.

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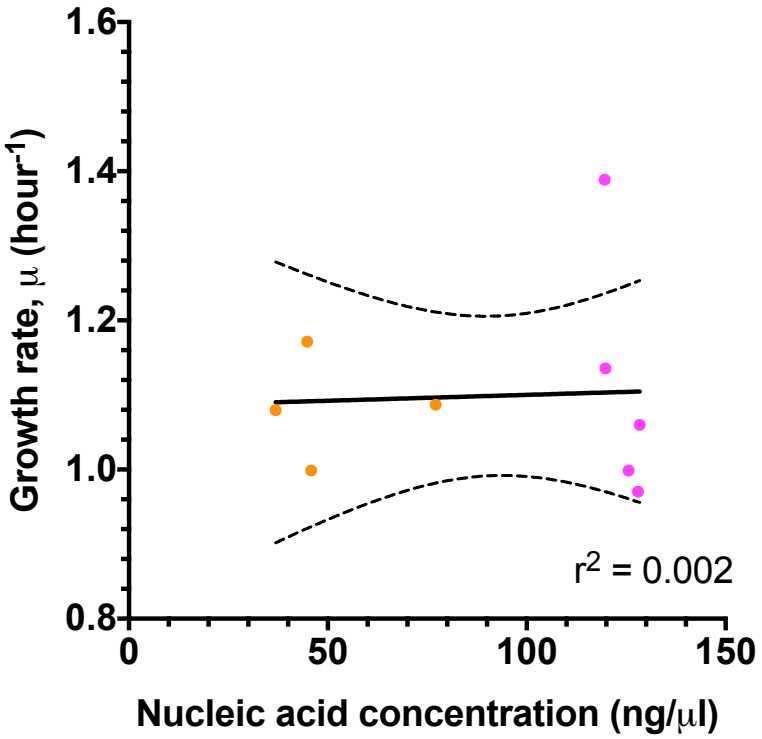

Figure 4.18d.

Figure 4.18: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/) adult and paediatric CF mucin fractions 1-12 which were treated with 60 U/ml DNase and dialysed using a 1 kDa membrane. b) Mean specific growth rate, μ (hour^{-1}) of PAO1 calculated from graph a). c) Mean nucleic acid concentration (ng/μl) in the samples prior to growth. Error bars show standard error of the mean (SEM). d) Growth rate plotted vs. nucleic acid concentration measured in each sample with 95% confidence bands. In all graphs, the pink line/bars/circles are representative of 5 adult CF biological replicates and the orange line/bars/circles are representative of 5 paediatric CF biological replicates, except for the paediatric samples in graphs c) and d) where an outlier was detected. All samples were supplemented with M9 (black line, 10 replicates). Graph b) was analysed using an unpaired, two-sided t-test and graph c) was analysed using a Mann-Whitney test (*p < 0.05).

Although the data reveals that the amount of available degraded DNA does not correlate with PAO1 growth rate, the data demonstrates that when DNA is degraded, the growth yield of PAO1 increases. Figure 4.19 demonstrates the growth profiles of PAO1 in non-DNase treated vs. DNase treated adult and paediatric CF mucin-containing fractions 1-12. When a one-way ANOVA was performed on the log CFU/ml growth yield after 24 hours (figure 4.19c.), it was revealed that by pre-treating the samples with DNase, the growth yield increases significantly in all ten CF mucin-containing fractions 1-12 (***p

< 0.001). For example, the data demonstrates a 22-fold increase in growth yield (CFU/ml) in adult CF mucin-containing fractions 1-12 from 2.99 x 10^7 CFU/ml to 6.65 x 10^8 CFU/ml when samples were DNase treated. A 7.48-fold increase in growth yield (CFU/ml) was reported in paediatric CF mucin-containing fractions 1-12 following DNase treatment. Therefore, it is evident from the data obtained in this study that *P. aeruginosa* can grow remarkably well when degraded DNA is provided as a nutrient source. To further consolidate the possible role of degraded DNA in providing a nutrient source for *P. aeruginosa* in the CF airway, it was considered appropriate to repeat DNase experiments in samples which are composed of pure DNA. For this reason, DNA experiments were further investigated using salmon sperm DNA. Salmon sperm DNA was chosen specifically because it is a readily available source of DNA.

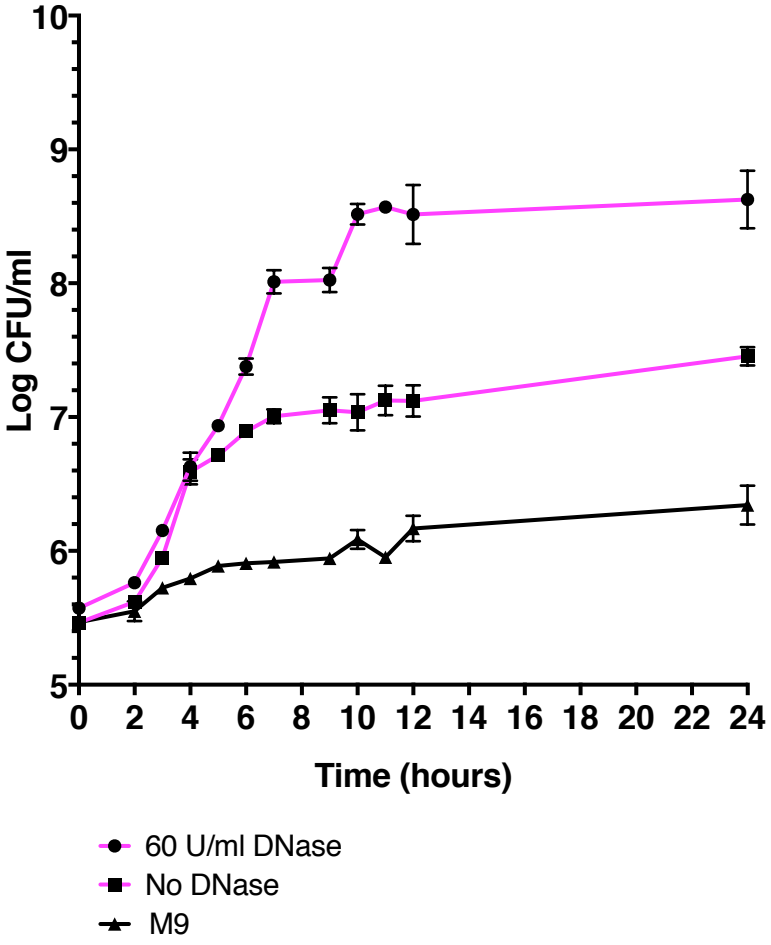

Figure 4.19a.

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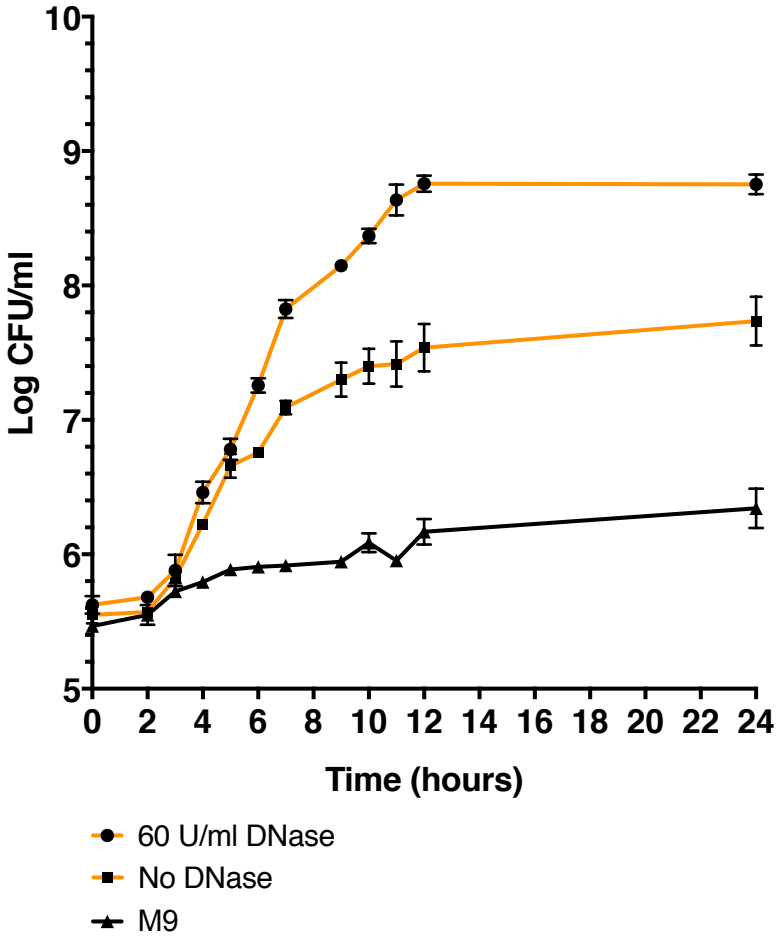

Figure 4.19b.

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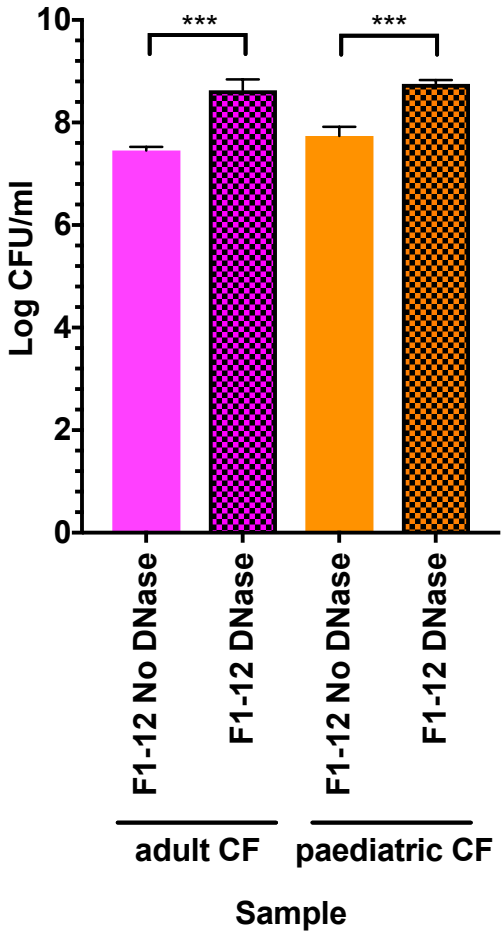

Figure 4.19c.

Figure 4.19: a) and b) Growth (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) adult (a, pink line) and paediatric (b, orange line) CF mucin-containing fractions 1-12 which were treated with 60 U/ml DNase (black circles) or were not treated (black squares). c) Log CFU/ml growth yield after 24 hours in non-DNase (non-patterned bars) and DNase treated (patterned bars) mucin fractions 1-12. Data represent the mean log CFU/ml from 5 adult CF biological replicates and 5 paediatric CF biological replicates. Error bars show standard error of the mean (SEM). Fractions were supplemented with M9 (black line, 10 replicates). P values on graph c) (*p < 0.001) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

4.3.4. Assessment of growth in degraded DNA

Since it had been demonstrated that *P. aeruginosa* was able to use degraded DNA as a nutrient source in mucin-containing fractions 1-12, known to contain abundant amounts of DNA (Thornton et al, 1991), it was decided to repeat DNase treatment growth assays in DNA considered to be pure and free of contaminants that would normally be found in

patient sputum samples. Salmon sperm was prepared to a final concentration of 400 ng/µl in nH_2O. The concentration of 400 ng/µl was selected so that when supplemented with an equal volume of double strength M9, the final concentration would be 200 ng/µl; the approximate concentration measured in unprocessed adult and paediatric CF sputum samples at 0.4% (w/v). The salmon sperm DNA was then checked to be pure via NanoDrop One analysis which revealed A260/280 ratios and A230/260 ratios of approximately 1.8.

4.3.4.1. Growth of *P. aeruginosa* PAO1 in salmon sperm DNA

P. aeruginosa PAO1 was grown in salmon sperm DNA which was not DNase treated to assess whether 'intact' DNA could be utilised as a nutrient source. PAO1 was also grown in salmon sperm DNA which was treated with 60 U/ml of DNase, and in salmon sperm DNA which was also 10 kDa filtered to attempt to remove the degraded DNA. The aim of this experiment was to firstly establish that PAO1 could indeed use degraded DNA as a nutrient source from a source that was known to be pure DNA. The second aim was to establish whether removal of degraded DNA via centrifugal filtration causes a reduction in growth yield of *P. aeruginosa*.

Firstly, prior to growth studies, it was confirmed that the concentration of degraded DNA in the DNase treated samples was 200 ng/µl in M9. Figure 4.20c. shows that following DNase treatment and filtration using a 10 kDa centrifugal filter that the concentration of DNA significantly reduced from 199.60 ng/µl ± 9.74 SEM to 89.27 ng/µl ± 18.65 SEM (**$p < 0.01$). This confirmed that centrifugal filtration was successful at removing from the sample DNA which was degraded. PAO1 was then grown in the different samples as shown in figure 4.20a.

Interestingly, in the samples which were DNase treated and filtered, it was found that PAO1 had a reduced growth rate compared to when grown in DNase treated salmon sperm DNA which was not filtered (figure 4.20b.). The data therefore suggests that to a certain extent that the amount of available degraded DNA can influence the growth rate since this difference of 1.04 µ ($hour^{-1}$) ± 0.08 SEM (DNase) vs. 0.66 µ ($hour^{-1}$) ± 0.05 SEM (DNase and filtered) was found to be statistically significant (*$p < 0.05$).

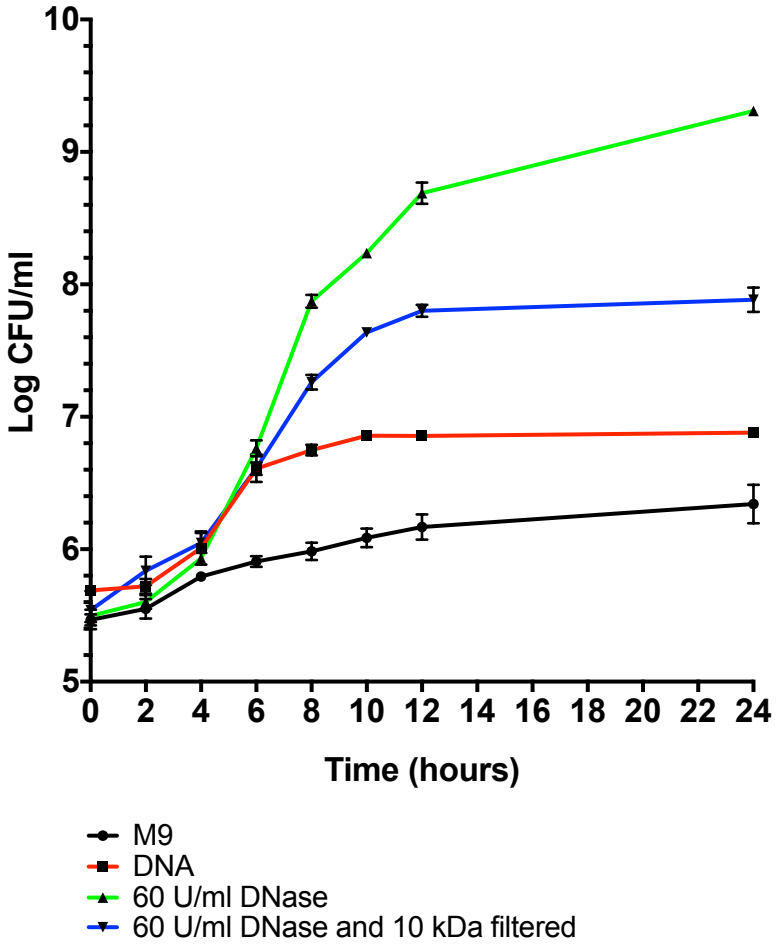

Figure 4.20a.

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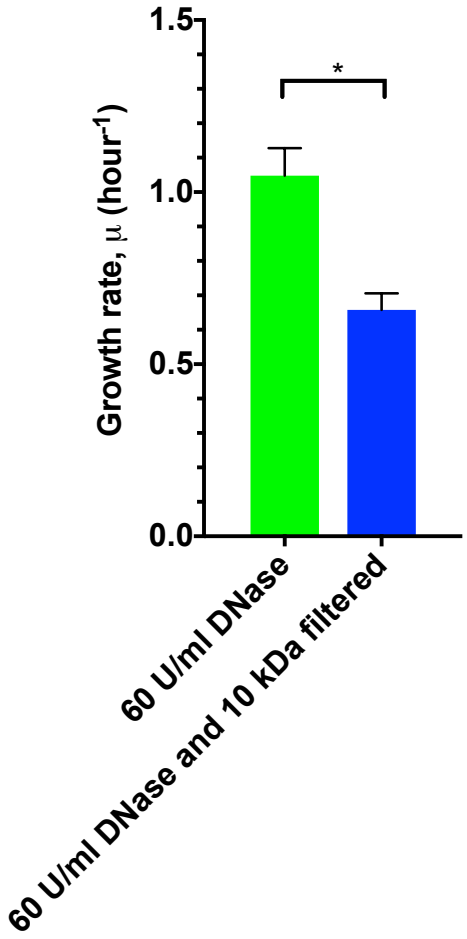

Figure 4.20b.

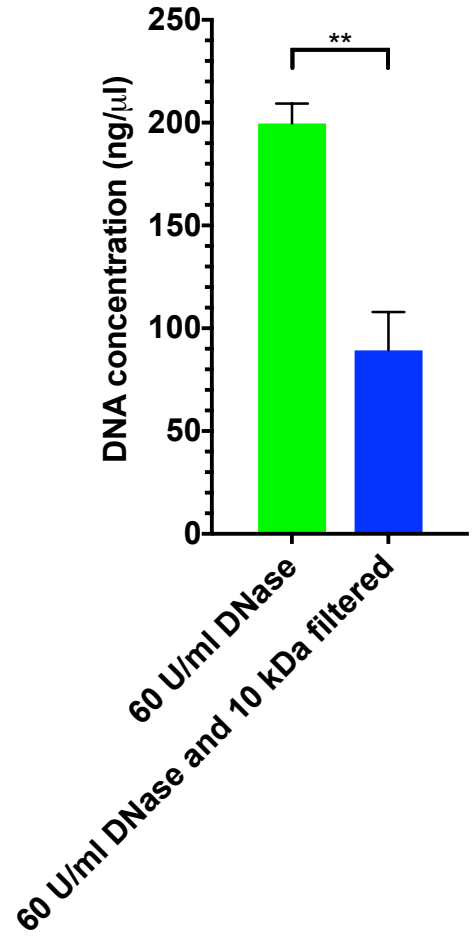

Figure 4.20c.

Figure 4.20: a) Growth of *Pseudomonas aeruginosa* PAO1 (log CFU/ml) over 24 hours in 200 ng/µl salmon sperm DNA (red line), 200 ng/µl DNase treated (60 U/ml) salmon sperm DNA (green line), and 200 ng/µl DNase treated (60 U/ml) and 10 kDa filtered salmon sperm DNA (blue line) supplemented with M9 (black line). b) Mean growth rates, µ (hour^{-1}) of PAO1 when grown in DNase treated salmon sperm DNA (green bar) and salmon sperm DNA which was DNase treated and 10 kDa filtered (blue bar). c) Mean DNA concentration (ng/µl) in the samples prior to growth. Data represent the mean from 3 biological replicates of separately prepared salmon sperm DNA. Error bars show standard error of the mean (SEM). All samples were supplemented with M9 (black bar, 3 replicates). P values on the graphs (*p < 0.05, **p < 0.01) were calculated using an unpaired, two-sided t-test.

Following growth studies, in addition to the standard serial dilutions that were performed after 24 hours of growth to determine the CFU/ml as part of the growth assay, the OD_{600} was also measured to assess growth yield as shown in figure 4.21.

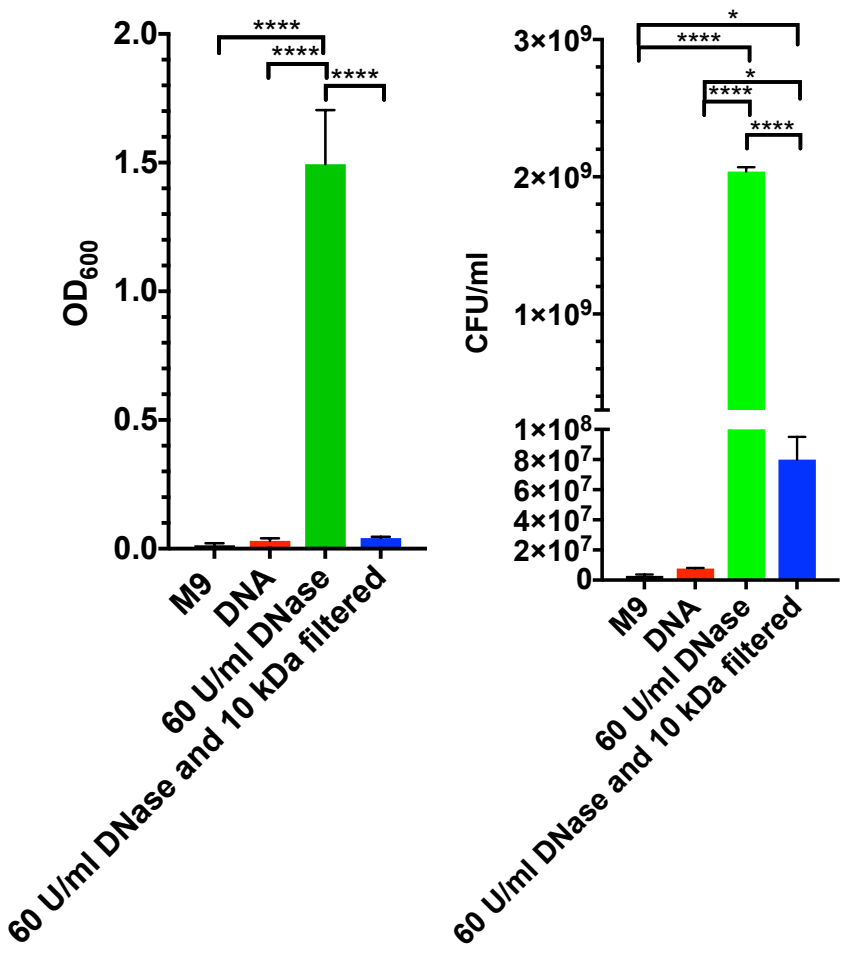

Figure 4.21a. *Figure 4.21b.*

Figure 4.21: Growth yield of *Pseudomonas aeruginosa* PAO1 after 24 hours of growth in 200 ng/µl salmon sperm DNA (red bar), 200 ng/µl salmon sperm DNA treated with 60 U/ml DNase (green bar), and 200 ng/µl DNase treated and 10 kDa filtered salmon sperm DNA (blue bar). a) Mean optical density at 600 nm (OD_{600}) after 24 hours. b) Mean CFU/ml after 24 hours. Data represent the mean OD_{600} / CFU/ml from 3 biological samples of prepared salmon sperm DNA. Error bars show standard error of the mean (SEM). All samples were supplemented with M9 (black bar, 3 replicates). P values (*p < 0.05, ****p < 0.0001) were calculated from a one-way ANOVA followed by Tukey's multiple comparisons test.

The data in figures 4.21a. and 4.21b. demonstrate that PAO1 can grow to a significantly higher growth yield in samples that were treated with DNase (****p < 0.0001). This supports the finding which was also observed in CF patient mucin-containing fractions 1-12 which were treated with DNase. It was found that when PAO1 was grown in DNase treated salmon sperm DNA there was not only a substantial increase in growth

yield compared to when grown in M9 alone (****$p < 0.0001$), but also the increase in yield was highly significant compared to the growth yield when grown in salmon sperm DNA which was not treated with DNase (red bars, ****$p < 0.0001$). This clearly demonstrates that the presence of DNase and degraded DNA promotes growth of *P. aeruginosa*. For example, the growth yield in terms of OD_{600} was measured at 0.03 ± 0.01 SEM in 'intact' DNA vs. 1.49 ± 0.21 SEM when DNase treated. This represents a 268-fold increase in CFU/ml of PAO1 when DNA is degraded. Removal of the degraded DNA via a filter was shown to reduce the growth yield significantly from 2.04×10^9 CFU/ml to 8.00×10^7 CFU/ml representing a 25-fold decrease in growth yield of PAO1 (****$p < 0.0001$). These data successfully demonstrate that the availability of degraded DNA influences growth yield.

DNA that was not treated with DNase is referred to as 'intact'. It must be noted that the degree of degradation in the samples is unknown, but since these samples were not treated with DNase it was assumed that they would not be degraded. Interestingly in these samples, although there appears to be some growth as shown in the growth profile in figure 4.20a., it was very little compared to when grown in M9 alone (black line). Analysis of the growth yields in figure 4.21 shows that there was no significant difference in yield after 24 hours in 'intact' DNA vs. when grown in M9 ($p > 0.05$). For this reason, the data reveals that *P. aeruginosa* is less able to use 'intact' DNA as a nutrient source for growth.

Data was then analysed by plotting the growth yield and rate against the DNA concentration measured in each respective sample. This was done to determine whether the concentration of degraded DNA correlates with the growth yield and/or rate. Linear regression analysis of the data shown in figure 4.22 demonstrates that there is strong positive correlation which confirms that with an increased concentration of degraded DNA there is both an increase in growth rate ($r^2 = 0.89$, $p = 0.0044$) and growth yield ($r^2 = 0.89$ $p = 0.0046$).

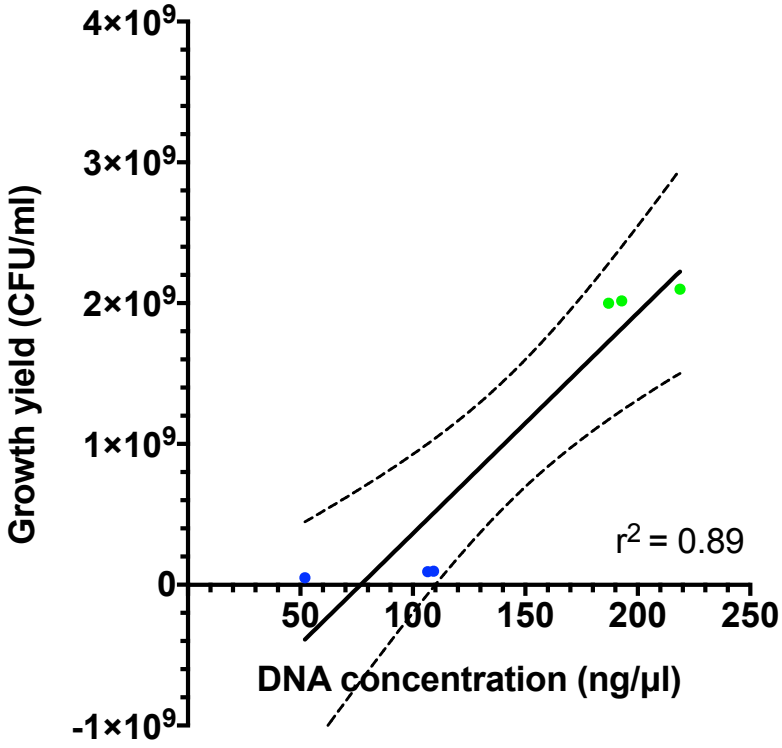

Figure 4.22a.

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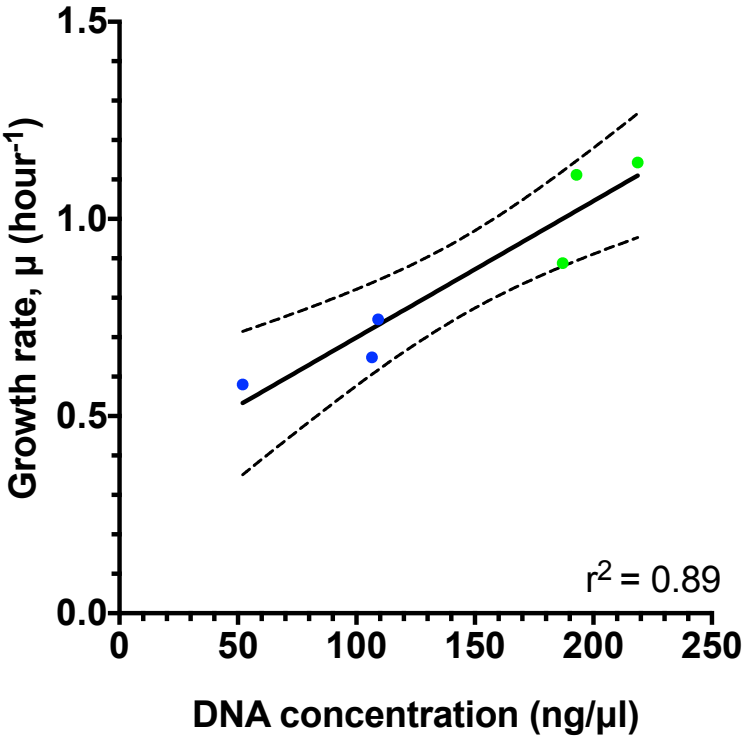

Figure 4.22b.

Figure 4.22: a) Growth yield (CFU/ml) plotted vs. DNA concentration (ng/µl) in 60 U/ml DNase treated salmon sperm DNA (green circles, 3 biological replicates) and salmon sperm DNA which was treated with 60 U/ml DNase and 10 kDa filtered (blue circles, 3 biological replicates). b) Growth rate, µ (hour^{-1}) plotted vs. DNA concentration (ng/µl). Linear regression reveals correlation with 95% confidence bands.

4.3.4.2. Growth of *P. aeruginosa* in deoxyribose or thymine

The next aim of the study was to determine what *P. aeruginosa* PAO1 was using within degraded DNA as a nutrient source. It is well documented that *P. aeruginosa* can use glucose as a source of carbon and for this reason it was included as a substrate in the growth assay to compare growth against (Garnett et al, 2013). Ideally, growth needed to be assessed in all components which make up DNA. The enzyme DNase is known to degrade DNA via the hydrolysis of phosphodiester links in the DNA backbone. TURBO DNase, which was used in this study, specifically breaks down DNA to 5' phosphorylated oligodeoxynucleotides according to the manufacturer (Ambion).

Based on the data obtained in this study, *P. aeruginosa* can therefore utilise nucleic acid in the form of oligodeoxynucleotides. Furthermore, it must have the ability to further degrade it to a point that its components can be used as a nutrient source. However, in DNA there are four types of nucleotides, whereby a nucleotide is composed of a phosphate group, deoxyribose sugar, and a nucleobase such as adenine, thymine, guanine, and cytosine (Chargaff et al, 1951). It would therefore be advantageous to discover whether *P. aeruginosa* prefers one nutrient over another. To determine whether *Pseudomonas* can grow in these components, *P. aeruginosa* PAO1 was grown in deoxyribose and one nucleobase. Thymine was selected as it could easily be suspended in water without the need for acid. Guanine is insoluble in water. Despite reports that adenine is soluble in water, when 0.1 g was suspended in 100 ml to get 10% (w/v), it did not dissolve. A recent publication by Ghoshdastidar and colleagues (2016) has addressed this difficulty is dissolving nucleobases in water.

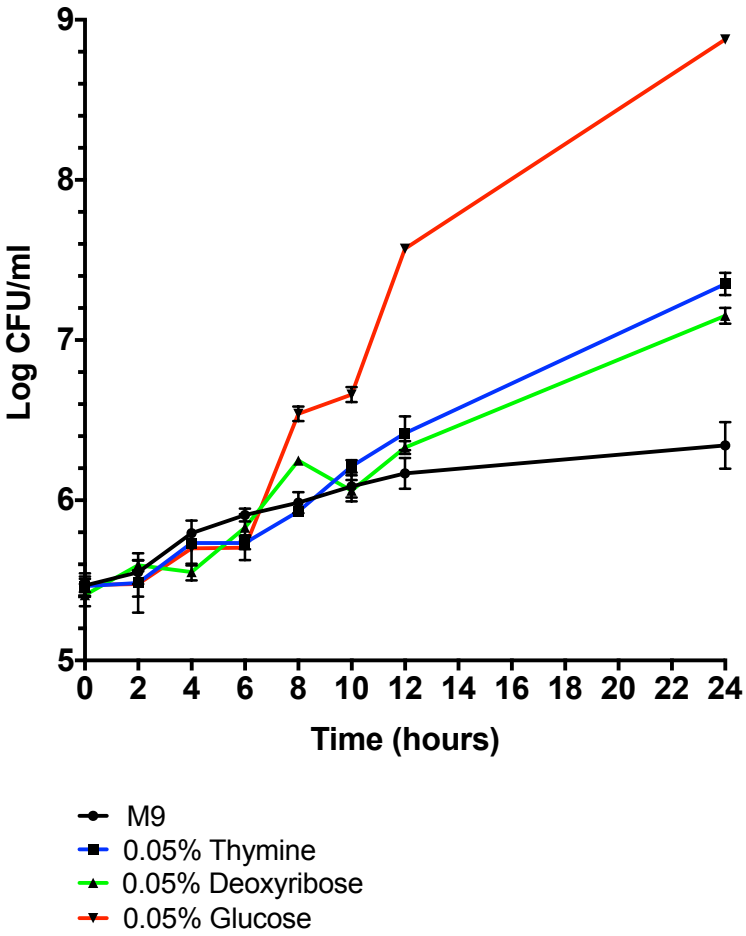

Figure 4.23a.

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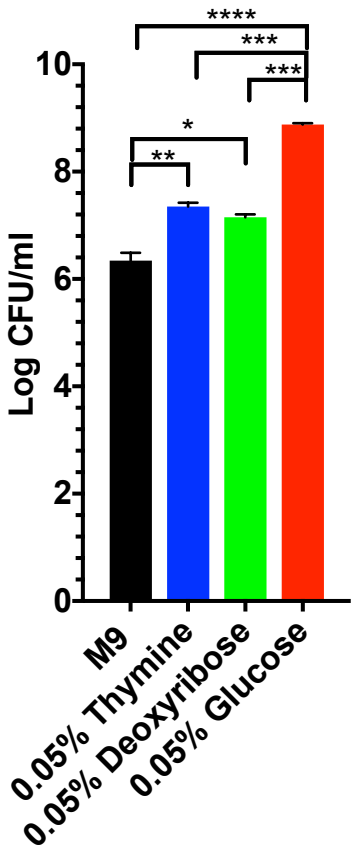

Figure 4.23b.

Figure 4.23: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.05% (w/v) glucose (red), deoxyribose (green), and thymine (blue), supplemented with M9 (black). b) Growth yield of PAO1 after 24 hours of growth. Data are represented as the mean log CFU/ml from 3 biological replicates. Error bars show standard error of the mean (SEM). All samples were supplemented with M9 (black bar, 10 replicates). P values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) were calculated by a one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 4.23a. demonstrates the growth profile of PAO1 when grown in 0.05% (w/v) of glucose, thymine, or deoxyribose supplemented with M9. Ideally a higher concentration would have been selected for growth studies, but the concentration was limited due to the solubility of thymine. Substrates were suspended to a final concentration of 0.1% (w/v) with nH_2O. An equal volume of double strength M9 was then added to 0.1% (w/v) of substrate. Growth studies were performed as previously described.

The data reveals that PAO1 can grow very well in 0.05% (w/v) glucose compared to growth in M9 alone as shown by the growth profile in figure 4.23a. However, until 10

hours of growth there appeared to be no difference in the growth profile of PAO1 in deoxyribose and thymine compared to M9 alone. When a one-way ANOVA was performed on the raw CFU/ml data, it was found that there were significant differences between the variances. A one-way ANOVA was therefore performed on the log CFU/ml data. Figure 4.23b. reveals that after 24 hours of growth of PAO1 the log growth yields in both thymine (**$p < 0.01$) and deoxyribose (*$p < 0.05$) were significantly increased. Notably, there was no significant difference between the growth yields in the two substrates. For example, the log CFU/ml growth yield in thymine was 7.35 ± 0.07 SEM compared to 7.15 ± 0.05 SEM in deoxyribose. The difference in growth yield compared to glucose, however, was highly significant (***$p < 0.001$) which demonstrated a log CFU/ml growth yield of 8.88 ± 0.03 SEM compared to the growth yields measured in thymine and deoxyribose. This therefore demonstrates how *P. aeruginosa* can grow 'better' as determined by growth yield after 24 hours in glucose compared to deoxyribose or thymine at the same concentration of 0.05% (w/v).

Based on this data it is evident that *P. aeruginosa* readily uses glucose as studies by Garnett and colleagues (2013) have also shown. However, the data in this project reveals that PAO1 has the potential to use deoxyribose and thymine present within DNA when degraded as a nutrient source for growth. However, the role of mucin in promoting growth still needed to be determined. For this reason, the next aim of the study was to investigate the growth of *P. aeruginosa* PAO1 in sugars found within mucins, and compare the growth to that in glucose.

4.3.5. Assessment of growth in mucin sugars

To investigate the role of mucin in promoting growth of *P. aeruginosa*, it was necessary to assess whether *P. aeruginosa* can utilise mucin sugars as a nutrient source. This was compared to growth in glucose since glucose is known to support growth in the CF airway as previously discussed.

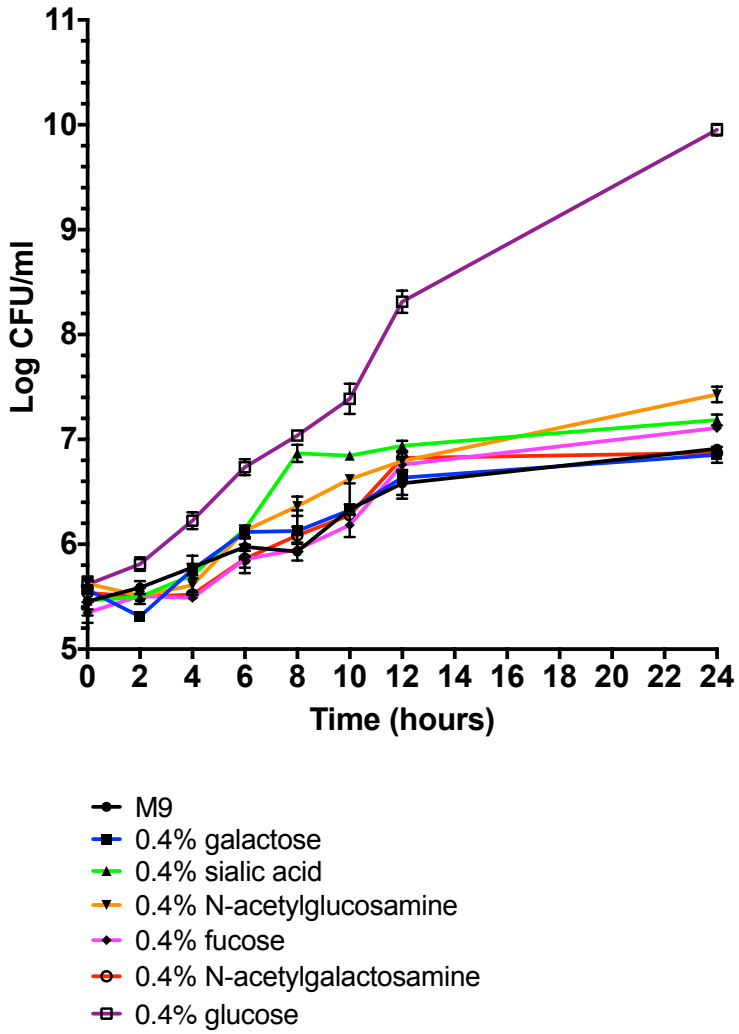

Figure 4.24a.

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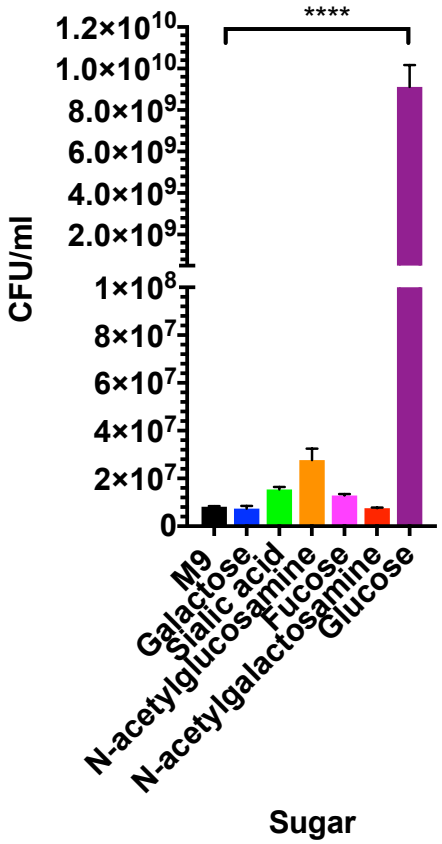

Figure 4.24b.

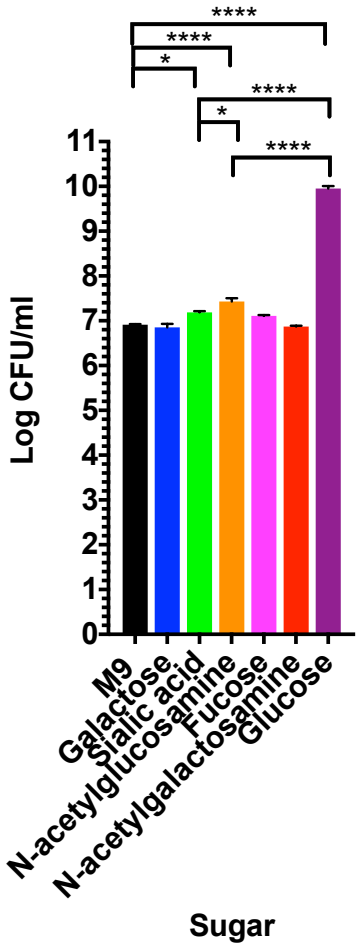

Figure 4.24c.

Figure 4.24: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.4% (w/v) galactose (blue), sialic acid (green), N-acetylglucosamine (orange), fucose (pink), N-acetylgalactosamine (red), and glucose (purple), supplemented with M9 (black). Data are represented as the mean log CFU/ml growth over 24 hours at each time point from 3 biological replicates. b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth in the mucin sugars. c) Mean growth yield (log CFU/ml) of PAO1 after 24 hours of growth in the mucin sugars. Error bars represent standard error of the mean (SEM). P values (*p < 0.05, **p < 0.01, ****p < 0.0001) on graphs were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test. P values have only been stated on the graph between sugars if there was a significant difference of the sugar compared to M9.

The growth profile in figure 4.24a. reveals that *P. aeruginosa* PAO1 was able to grow very well in 0.4% (w/v) glucose supplemented with M9 but there was little growth in the mucin sugars. There does appear to be an increased growth rate in sialic acid compared to growth in M9 alone, as seen in figure 4.24a, but the data in figures 4.24b.

and 4.24c. revealed that this did not amount to a growth yield that was comparable to the growth yield when grown in glucose.

Figures 4.24b. and 4.24c. show the maximum growth yield after 24 hours. Following a one-way ANOVA followed by a Tukey's multiple comparisons test, statistical analysis revealed that there was no difference in growth yield of PAO1 in mucin sugars compared to M9 whilst there was a difference when grown in glucose (****$p < 0.0001$). However, when the raw data was transformed to its logarithm as presented in figures 4.22a. and 4.22c., differences in the growth yield of PAO1 in sialic acid (*$p < 0.05$) and N-acetylglucosamine (****$p < 0.0001$) were found to be significant compared to the log growth yield in M9 alone. The data in figure 4.22c. also reveals that the increased growth yield in glucose compared to sialic acid and N-acetylglucosamine was statistically significant (****$p < 0.0001$). This demonstrates that glucose is more preferred as a substrate compared to the mucin sugars.

This study has found that *P. aeruginosa* has a limited ability to utilise mucin sugars within mucin when growth is compared to growth in glucose. It remains to be determined however, whether mucin sugars are clinically relevant as a nutrient source, since it could be that *P. aeruginosa* uses these mucin sugars to an extent that allows favourable growth due to their abundance in the CF airway rather than at the concentration of 0.4% (w/v) investigated in this experiment.

4.3.6. Assessment of sample processing on mucin structure

The next aim of this study was to establish whether the processing of the mucins in preparation for growth studies affects the mucin structure in a way that the favourable growth observed is due to a structural change. Therefore, two adult CF samples were selected to assess differences in mucin structure before and after processing. MUC5B and MUC5AC were assessed in their whole (polymeric) and reduced form by initially separating the mucins via SDS-agarose gel electrophoresis followed by western blot methods as described in section 4.2.3.

4.3.6.1. Whole mucins from adult CF sputum run similarly to reduced mucins after processing

Unprocessed and processed samples were run on the gel either whole or reduced, with the prior treatment of DTT as discussed in section 4.2.3.2. The unprocessed samples were concentrated using 10 kDa centrifugal filters and run at either 1X or 3X concentrated. The processed samples were either run 'neat' and is representative of the mucin in 0.1% (w/v) growth assays, or were further diluted as follows; 1:2; 1:4; 1:8. The 1:8 dilution was equal to the 3X concentrated, unprocessed mucins so they could be compared. Following addition of the primary and secondary antibodies, the membrane was scanned by the Odyssey CLx LiCor using Image studio v5 software. The images of these scans are presented in figures 4.25a. and 4.25b.

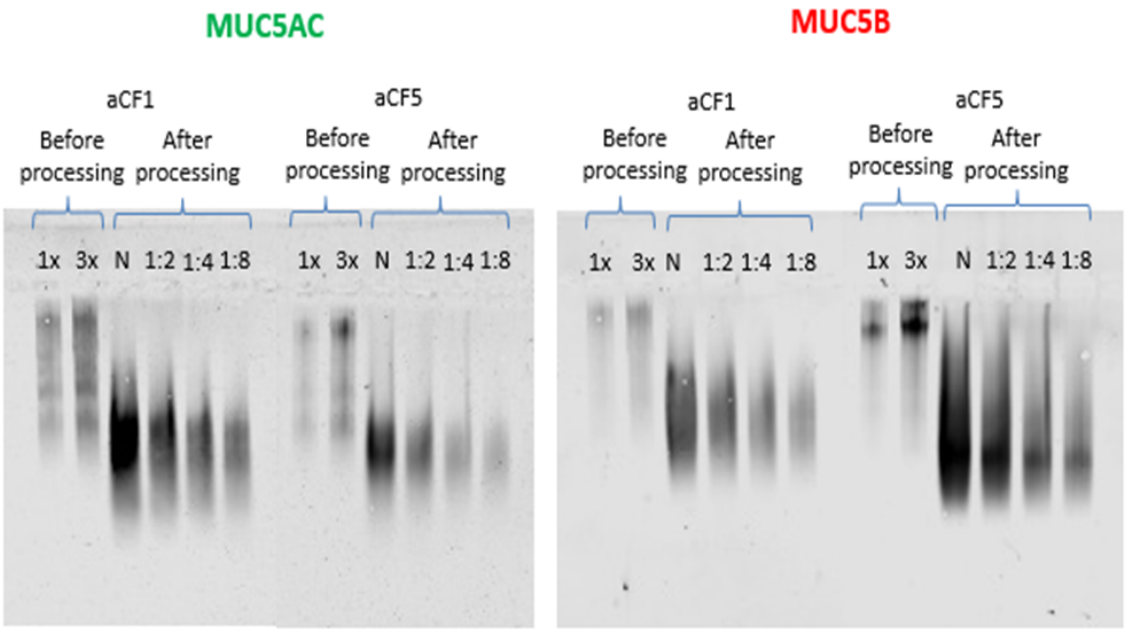

Figure 4.25a.

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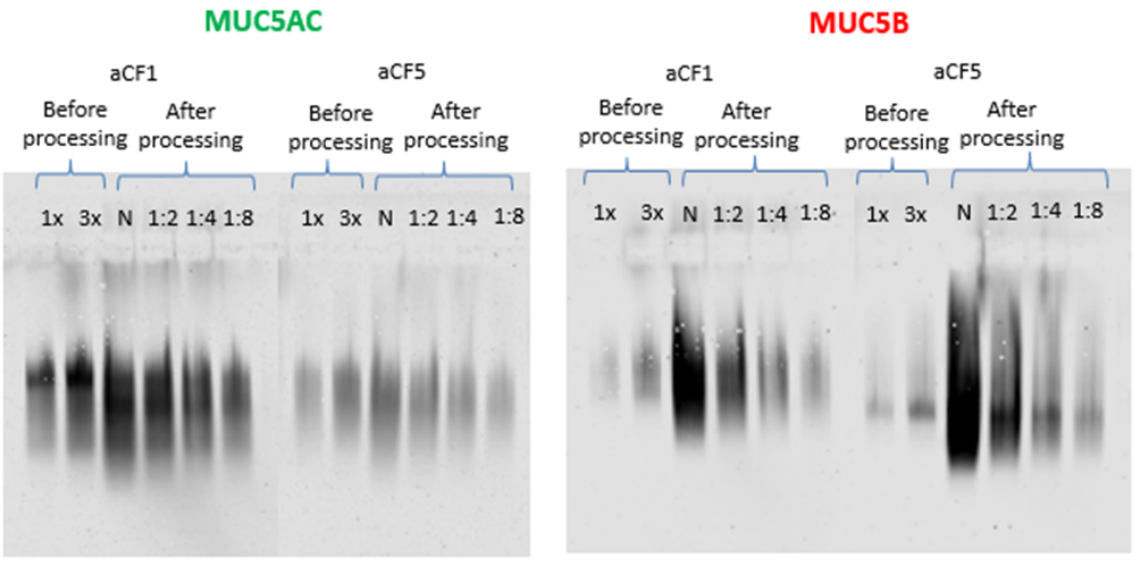

Figure 4.25b.

Figure 4.25: SDS-agarose electrophoresis gel followed by transfer to a nitrocellulose membrane and western blotting of 2 adult CF mucin-containing fractions 1-12 (aCF1 and aCF5) before and after processing in preparation for growth assays. Images are of whole (a. and b.) and reduced (c. and d.) mucins, MUC5AC (a. and c.) and MUC5B (b. and d.). 1x = sample was not concentrated using a 10 kDa centrifugal filter, 3x = sample was concentrated 3x using a 10 kDa centrifugal filter, N = the processed sample was suspended in water at a concentration of 2 mg/ml and run on the gel as the neat sample, 1:2 = 1 in 2 dilution of the neat sample, 1:4 = 1 in 4 dilution of the neat sample, 1:8 = 1 in 8 dilution of the neat sample which is the same concentration of the unprocessed 3x concentrated sample.

The gel images in in figures 4.25a. and 4.25b. reveal that there were notable changes in the structure of respiratory mucins after processing. Processing included dialysis, freeze-drying, DNase treatment, dialysis, freeze-drying, and then autoclaving. The images in figure 4.25a. reveal that the whole mucin structure is changed following processing as evidenced by the mucins appearing further down the gel compared to the unprocessed mucins. Not only does it run further down the gel but it has lost its polymeric structure as evidenced by the loss of the bands normally present in unprocessed, whole MUC5AC (figure 4.25a.).

Following processing, separation of the mucins via SDS-agarose electrophoresis revealed that the processed ‘whole’ mucins ran on the gel more like mucins which were reduced. The image in figure 4.25b. demonstrates minimal degradation since there does

not appear to be much difference between the 3X unprocessed and the 1:8 diluted processed MUC5AC. However, when the 3X unprocessed MUC5B is compared to the 1:8 diluted and processed MUC5B, the mucins are shown to have travelled further down the gel. Overall, this data demonstrates that the polymeric structure is destroyed following processing.

At this point in the study it was not known whether this observation was unique to adult CF samples or the processing procedure. It was thought to be highly likely that the polymeric structure would be affected by autoclaving the samples. Since it had not been previously assessed how processing affects mucin structure it was therefore necessary to establish if autoclaving affects the mucins and whether paediatric samples are affected to the same degree. This was necessary to determine because differences between the sample structures could be argued to influence bacterial growth.

4.3.6.2. Autoclaving identified to be responsible for change in structure

Because it had been found that processing affects the whole mucin structure and that processed mucins run similarly on a gel compared to unprocessed mucins, it was also necessary to assess the change in whole mucin structure in paediatric samples.

Figures 4.26a. and 4.26b. reveal that the polymeric structure was conserved up until autoclaving. Although it reveals that some mucin was lost following dialysis as shown by a reduction in intensity, the structure itself was conserved up until autoclaving. This is evident in figure 4.26b. when observing the whole MUC5AC structure for the sample labelled as ‘pCF 3’. This image demonstrates the classic ladder pattern structure for MUC5AC. However, this structure was shown to be lost following autoclaving.

Since mucins were lost following dialysis, for further growth assays, centrifugal filters were used like in previous mucin studies (Ridley et al, 2016; Welsh et al, 2017).

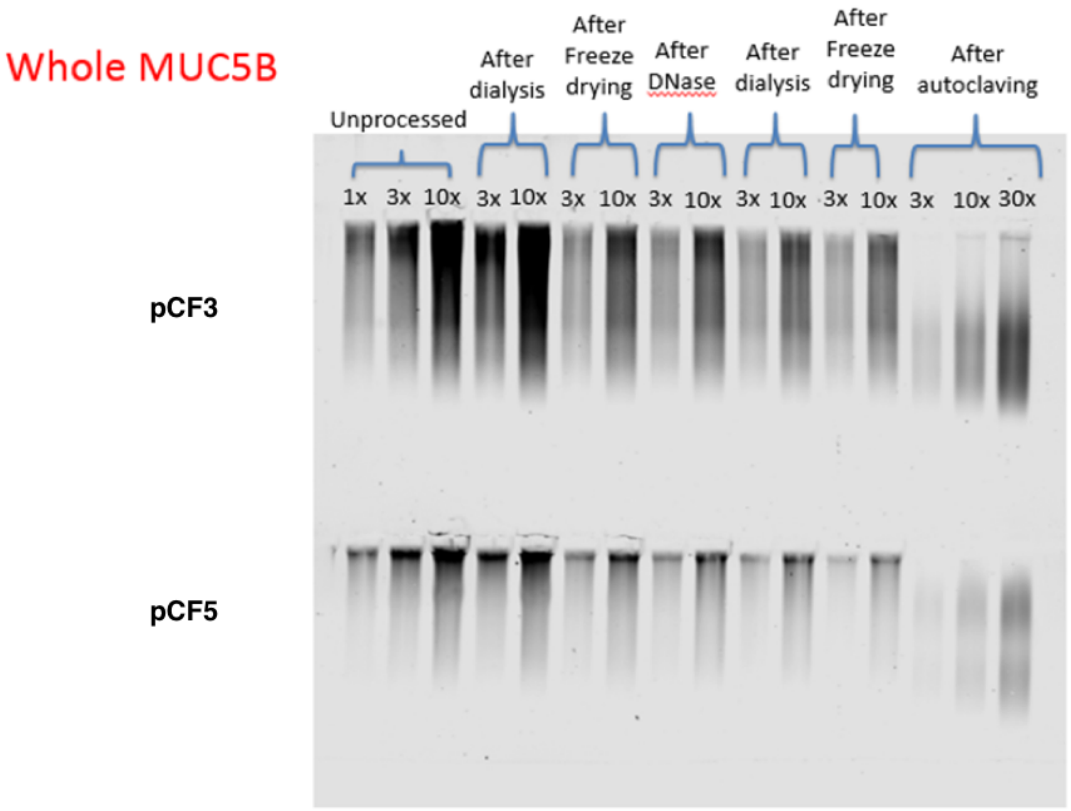

Figure 4.26a.

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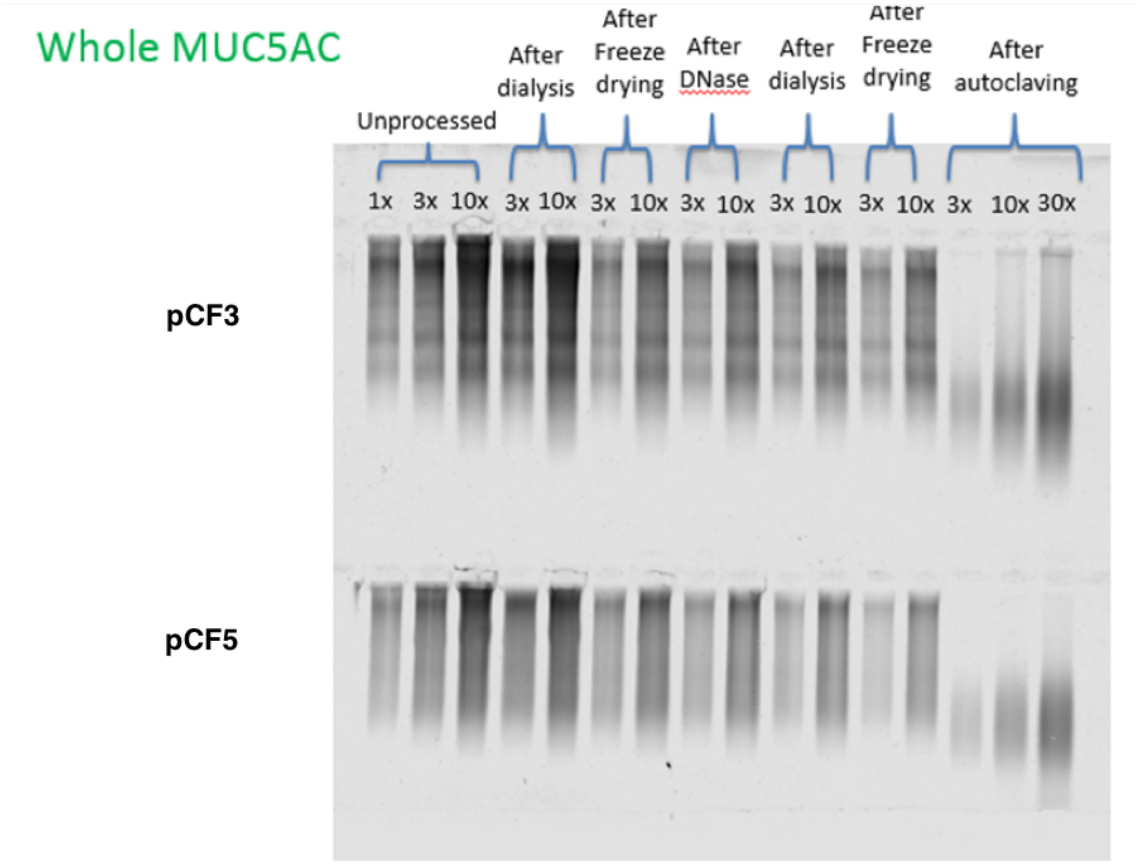

Figure 4.26b.

Figure 4.26: SDS-agarose electrophoresis gel followed by transfer to a nitrocellulose membrane and western blotting of 2 paediatric CF mucin-containing fractions 1-12 (pCF3 and pCF5) before and after processing in preparation for growth assays. Images are of whole mucins, MUC5B (a.) and MUC5AC (b.). 1x = sample was not concentrated, 3x = sample was concentrated 3 times using a 10 kDa centrifugal filter, 10x = sample was concentrated 10 times, 30x = sample was concentrated 30 times.

4.3.7. Assessment of growth in mucins

The next aim of this project was to determine the involvement of mucins in promoting growth in the CF airway. In this study, it was shown that *P. aeruginosa* PAO1 grows 'better' as determined by growth yield in glucose vs. mucin sugars at the same concentration. Other studies have also suggested that glucose drives respiratory pathogen growth (Garnett et al, 2013). However, it is unknown to what extent mucin affects growth, or, indeed if there is any involvement of mucin in promoting growth.

My study has shown that *P. aeruginosa* grows to a limited extent in sugars found within mucin (section 4.3.5). However, it could be that when mucins are highly concentrated, as is the case in CF due to the inability to effectively clear the mucus, that utilisation of these sugars is then favourable for the pathogens due to the hyper-concentration of the mucins in the airway (Henderson et al, 2014).

4.3.7.1. Removal of degraded DNA from mucin

To assess the role of mucins in promoting growth it was necessary to remove as much degraded DNA from the CF mucin-containing fractions 1-12 as possible. Since SDS-agarose electrophoresis gel analysis revealed that the combination of using dialysis tubing and freeze drying caused a reduction in the concentration of mucins, it was decided to use different MWCO centrifugal filters to filter out the degraded DNA. Using centrifugal filters for processing of mucins has been utilised in many other studies (Thornton et al, 1991; Luo et al, 2014; Shea et al, 2015).

Due to the requirement of conserved patient sample usage, PGM was used as a control sample to test which filter should be used to remove degraded DNA from samples. The 10 kDa filter was used as a starting point based on the salmon sperm DNA growth experiments. The DNA/nucleic acid concentration in three separately processed PGM samples was measured using the corrected value given by NanoDrop One. At the time of this experiment it was not known whether PGM contained DNA, however a recent publication by Schömig and colleagues (2016) has confirmed the presence of DNA in PGM.

PGM was suspended to a concentration of 2 mg/ml and then treated with 60 U/ml DNase. The samples were then filtered through a 10, 30, and 50 kDa centrifugal filters with washes of nH_2O between each cycle. Following four cycles of centrifugation the volume was adjusted to that of the pre-filtered sample with nH_2O. Samples were then measured for their DNA/nucleic acid concentration.

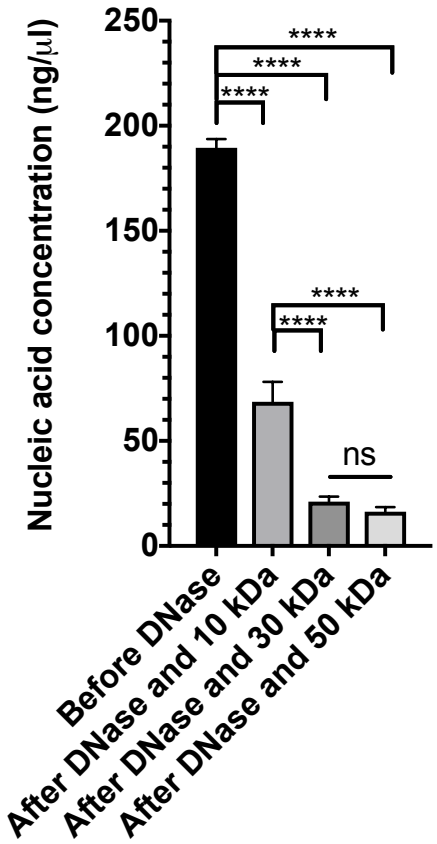

Figure 4.27.

Figure 4.27: Nucleic acid/DNA concentration (ng/µl) in 2 mg/ml PGM before treatment with 60 U/ml DNase, and following DNase treatment and centrifugation using a 10 kDa, 30 kDa, and 50 kDa centrifugal filters. Data are represented as the mean concentration from 3 separately processed PGM samples. Error bars show standard error of the mean (SEM). P values on the graph (**p < 0.0001) were obtained using a one-way ANOVA followed by a Tukey's multiple comparisons. No statistical difference is noted as 'ns' on the graph.**

Figure 4.27. shows that following degradation of DNA in PGM samples, that centrifugal filtration even with the lowest MWCO (10 kDa) significantly reduced the mean concentration of nucleic acid in PGM samples. For example, this reduced from 189.50 ng/µl ± 4.15 SEM to 68.63 ng/µl ± 9.46 SEM (****p < 0.0001). Compared to the nucleic acid concentration measured in samples which were 10 kDa filtered, the reduction in concentration following use of the 30 and 50 kDa filters was also highly significant, reducing to 21.51 ng/µl ± 1.73 SEM and 16.31 ng/µl ± 1.18 SEM, respectively (****p < 0.0001). However, there was no significant difference between the concentrations measured following use of the 30 kDa and 50 kDa filters. Therefore,

the 30 kDa filter was selected for growth assays using adult and paediatric CF mucin-containing fractions 1-12 because there was no further significant reduction in the nucleic acid concentration measured. However, for preliminary experiments using PGM, the samples which had been filtered using the 50 kDa centrifugal filter were used for growth assays. To confirm presence of sugars, a PAS stain was carried out on the samples. The presence of magenta bands confirmed that sugar was still present following filtration (data not shown).

4.3.7.2. Growth of *Pseudomonas aeruginosa* PAO1 in PGM with degraded DNA removed

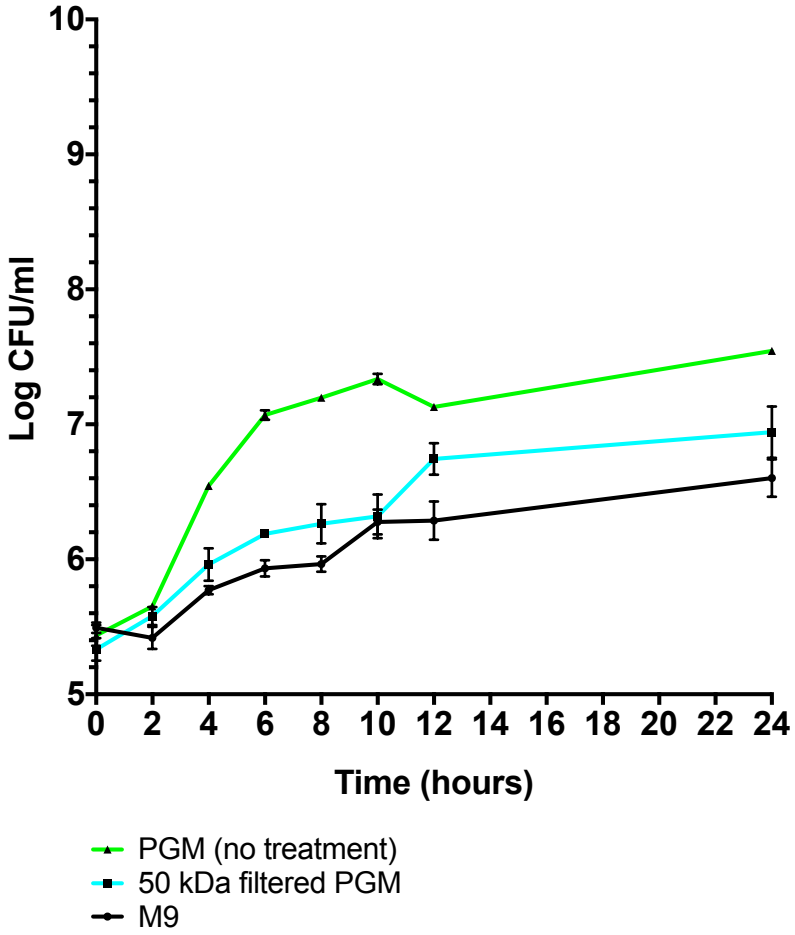

Figure 4.28a.

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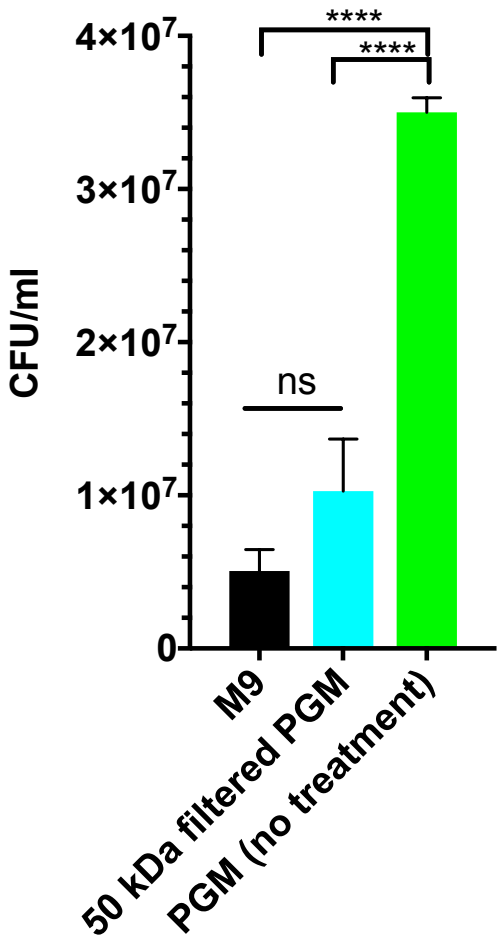

Figure 4.28b.

Figure 4.28: Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) PGM supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at different time points over 24 hours in PGM. Samples were either not treated with DNase (green) or treated with 60 U/ml DNase and filtered using a 50 kDa centrifugal filter (light blue). Error bars represent standard error of the mean (SEM) of 3 separately processed PGM samples. All samples were supplemented with M9 (6 replicates, black). b) Growth yield (CFU/ml) of PAO1 after 24 hours of growth. P values on graph (**p < 0.0001) were obtained by a one-way ANOVA followed by a Tukey's multiple comparisons test. No statistical difference is noted as 'ns' on the graph.**

Figure 4.28a. shows that when PAO1 was grown in 0.1% (w/v) PGM which was not treated with DNase, there appeared to be an increased growth rate compared to when grown in M9 alone and PGM which was DNase treated and filtered to remove degraded DNA. In terms of the growth profile it appears that PAO1 can grow in non-DNase treated PGM to a limited extent as observed by the growth yield in figure 4.28a.

However, there appeared to be no difference between growth of PAO1 in the 50 kDa filtered PGM sample vs. M9.

Analysis of the growth yield after 24 hours revealed that whilst the growth yield of PAO1 in PGM which was not treated with DNase was significantly increased compared to M9 (****$p < 0.0001$), there was no significant difference in the growth yield of PAO1 when grown in PGM which was DNase treated and 50 kDa filtered compared to M9. For example, the mean growth yield of PAO1 in M9 alone was found to be 5.06×10^6 CFU/ml $\pm$ 1.39×10^6 SEM compared to 1.03×10^7 CFU/ml $\pm$ 5.90×10^6 SEM in PGM which was DNase treated and filtered. Although this represents a 2-fold increase in yield compared to M9, this was not found to be statistically significant ($p > 0.05$). Therefore, these data suggest that PAO1 is unable to utilise mucin within PGM as a nutrient source when degraded DNA is removed.

Figure 4.29a. shows the growth of PAO1 in DNase treated PGM which was filtered using a 50 kDa filter to remove degraded DNA. However, in this figure growth is compared to growth in DNase treated PGM which was not filtered. PAO1 was also grown in the collected flow-through of the sample following DNase treatment and filtering using a 50 kDa centrifugal filter. Consequently, the flow-through contains degraded DNA and other possible nutrients which can pass through the filter. The nucleic acid concentration was measured in the flow-through and was diluted to 8 ng/µl – the approximate concentration which was also measured in the 50 kDa filtered PGM at 2 mg/ml. Hence, when added to an equal volume of M9, this concentration became 1 mg/ml, or 0.1% (w/v) PGM. In diluting the PGM sample by 1 in 2, the concentration of nucleic acid measured in the filtered PGM sample therefore reduced from 16.31 ng/µl to 8.16 ng/µl. The flow-through sample was therefore diluted to approximately 8 ng/µl nucleic acid to match the amount of nucleic acid measured in the PGM sample. It was assumed that because the PGM was treated with DNase, that logically there would be degraded DNA present as a nutrient in the flow-through. Therefore, growth of PAO1 in PGM which was DNase treated and 50 kDa filtered (containing 8 ng/µl DNA) was compared to growth in the flow-through sample containing 8 ng/µl of degraded DNA. However, in the DNase treated and 50 kDa filtered PGM it was assumed that this remaining nucleic acid was not degraded since it was not removed via filtration.

Salmon sperm DNA was used as a positive control for assessing growth in the flow-through samples and PGM which was DNase treated. Prior to use, the salmon sperm was checked for purity using NanoDrop One. The salmon sperm DNA samples were also treated with 60 U/ml DNase. The concentration of degraded DNA was then adjusted so that the degraded salmon sperm DNA concentration matched the concentrations measured in DNase treated PGM (68 ng/µl) and the flow-through samples (8 ng/µl) for each 50 kDa filtered PGM sample.

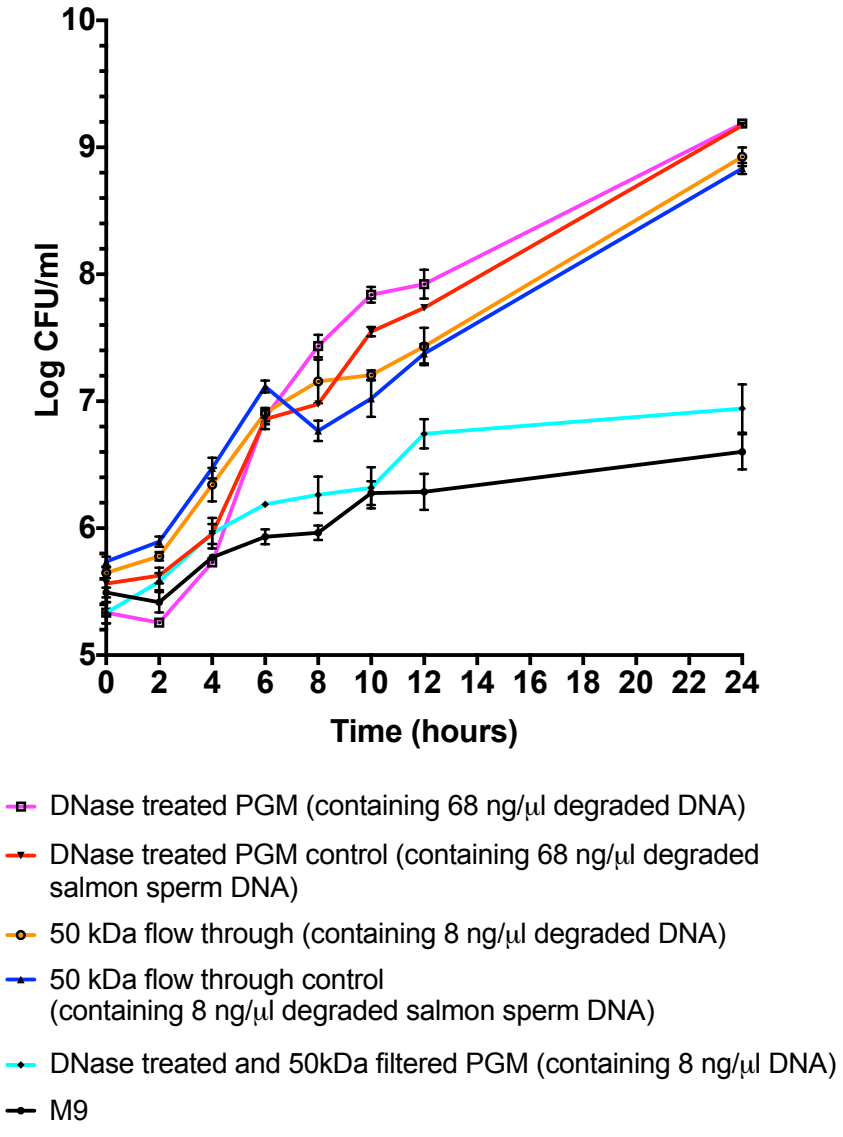

Figure 4.29a.

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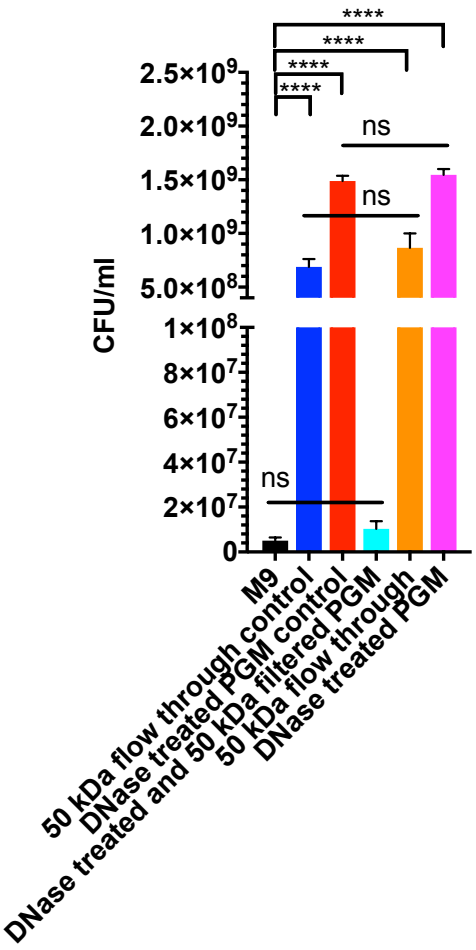

Figure 4.29b.

Figure 4.29: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) PGM supplemented with M9. Data are represented as the mean log growth (log CFU/ml) over 24 hours in PGM treated with DNase, treated with DNase and filtered using a 50 kDa filter, and the 50 kDa flow-through sample. Error bars represent standard error of the mean (SEM) from 3 biological replicates. All samples were supplemented with M9 (6 replicates). b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth. P values (**p < 0.0001) on the graph were obtained by a one-way ANOVA followed by a Tukey's multiple comparisons test. No statistical difference is noted as 'ns' on the graph. Degraded salmon sperm DNA was used as the control for samples.**

Figure 4.29a. reveals the growth profile when PAO1 was grown in the different samples. As presented in the previous figure (4.28a.), PAO1 appeared unable to grow in PGM which was DNase treated and 50 kDa filtered to remove degraded DNA (containing 8 ng/µl DNA). However, when grown in DNase treated samples which contained 68 ng/µl degraded DNA it was found that PAO1 could grow very well in PGM which was DNase treated. For example, a mean growth yield of 1.54 x 10^9 CFU/ml in DNase treated PGM was found to be comparable to 1.49 x 10^9 CFU/ml in the DNase treated salmon sperm DNA control at the same concentration of DNA (68 ng/µl) ($p > 0.05$).

Moreover, whilst it was shown that PAO1 was unable to grow in PGM when degraded DNA was mostly removed, PAO1 was also shown to grow extremely well in the collected flow-through containing 8 ng/µl of degraded DNA. In support of the argument that it is indeed solely degraded DNA in these samples is the growth profile of PAO1 when grown in degraded salmon sperm DNA control at the same concentration of 8 ng/µl. Once again, no significant difference was found between the growth yield in the flow-through sample and the control when grown in a comparable amount of degraded salmon sperm DNA. These data therefore suggest that any growth observed in PGM samples is influenced by the extent of DNA degradation.

4.3.7.3. Growth of *Pseudomonas aeruginosa* PAO1 in adult CF mucin-containing fractions 1-12 with degraded DNA removed

Once it had been established that *P. aeruginosa* PAO1 was unable to grow in PGM which was DNase treated and filtered to remove degraded DNA, the experiment was repeated using the mucin-containing fractions 1-12 which were purified via density gradient centrifugation. The aim was to determine whether *P. aeruginosa* has the capability to utilise mucins from CF patient sputum when free of degraded DNA. However, on this occasion it was decided to use the 30 kDa centrifugal filter since there was no statistically significant reduction in the nucleic acid concentration following use of the 50 kDa centrifugal filter ($p > 0.05$, figure 4.27).

The graph in figure 4.30a. shows the growth profile of PAO1 when grown in M9 alone, untreated adult CF mucin-containing fractions 1-12, and adult CF mucin-containing

fractions 1-12 which were DNase treated and 30 kDa filtered. Salmon sperm DNA was used as a control DNA. It was not included in the PGM experiment in figure 4.28a, but was included because nucleic acid was measured in the sample following centrifugal centrifugation. It was assumed that this remaining DNA was intact since it was unable to pass through the 30 kDa filter. Therefore, 'intact', non-DNase treated salmon sperm DNA at the same concentration was used as a control to assess for any differences in growth of PAO1 in the two growth conditions.

The data revealed that *P. aeruginosa* was unable to grow in adult CF mucin-containing fractions 1-12 when degraded DNA was removed. Figure 4.30b. demonstrates that there was no significant difference in the growth yield of PAO1 when grown in 30 kDa filtered mucin compared to M9 ($p > 0.05$). There was also no difference compared to the respective salmon sperm DNA control containing the same concentration of intact salmon sperm DNA at 10 ng/µl. The data therefore suggest that even when DNA is present, when intact, there is little growth of PAO1.

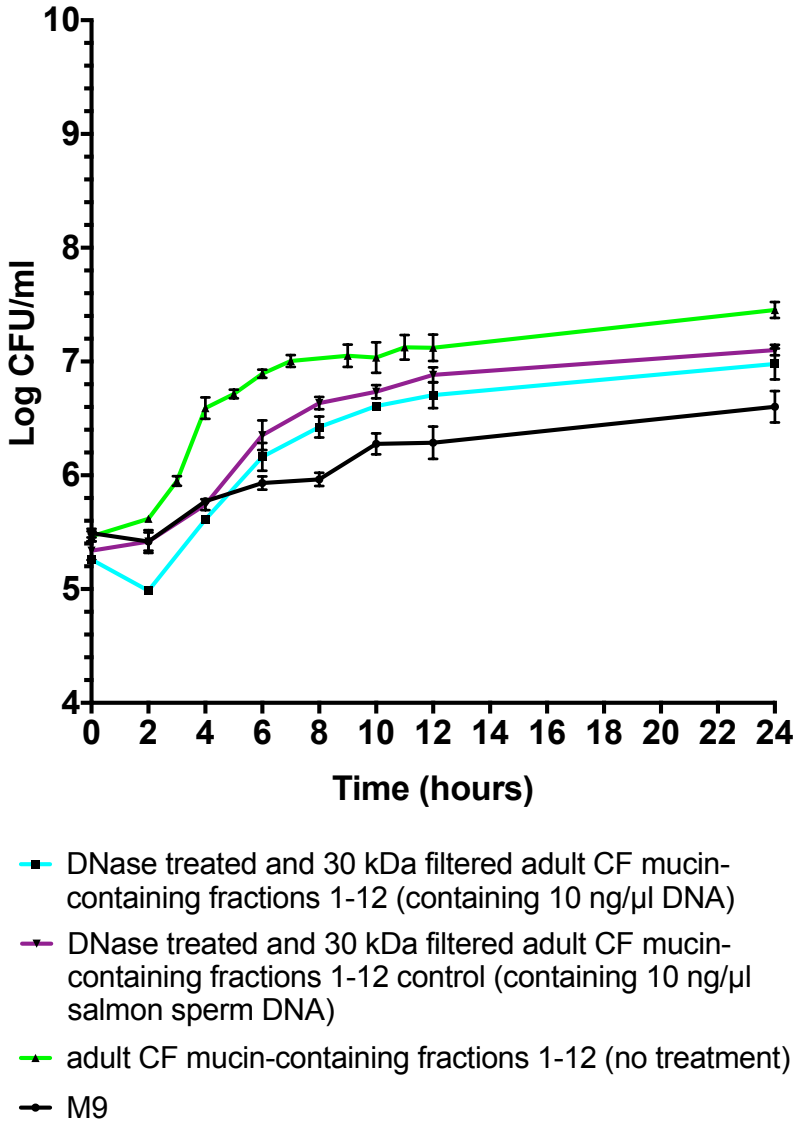

Figure 4.30a.

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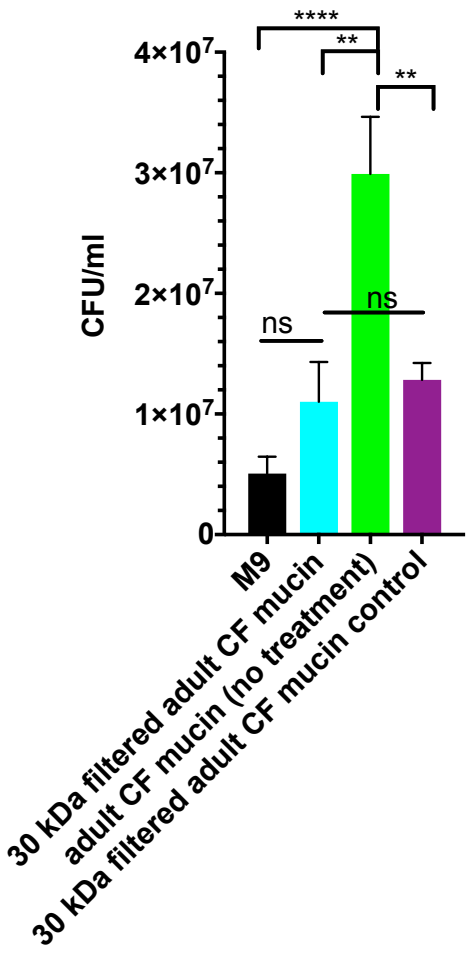

Figure 4.30b.

Figure 4.30: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) adult CF mucin-containing fractions 1-12 supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at different time points over 24 hours in adult CF mucin-containing fractions 1-12 samples which were not treated with DNase (green) or were treated with 60 U/ml DNase and filtered using a 30 kDa centrifugal filter (light blue). All samples were supplemented with M9 (6 replicates, black). b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth. Salmon sperm DNA was used as a control (purple). Error bars represent standard error of the mean (SEM) of 4 'adult CF mucin-containing fractions 1-12' replicates. Data was analysed by a one-way ANOVA followed by a Tukey's multiple comparisons test. No statistical difference is noted as 'ns' on the graph.

Once it had been determined that *P. aeruginosa* was unable to utilise adult CF mucins within sputum as a nutrient source for growth, it was decided to investigate the growth in DNase treated adult CF mucin-containing fractions 1-12. Degraded salmon sperm DNA was once again used as a positive control for samples containing degraded DNA.

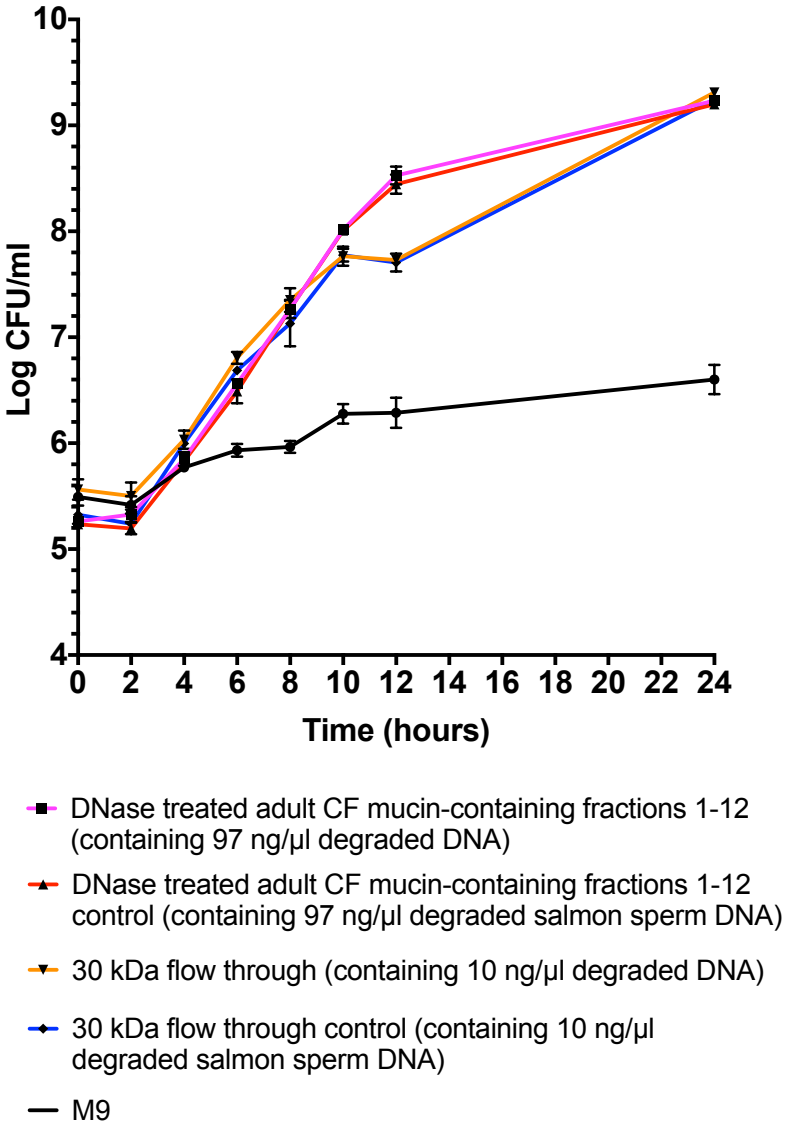

Figure 4.31a.

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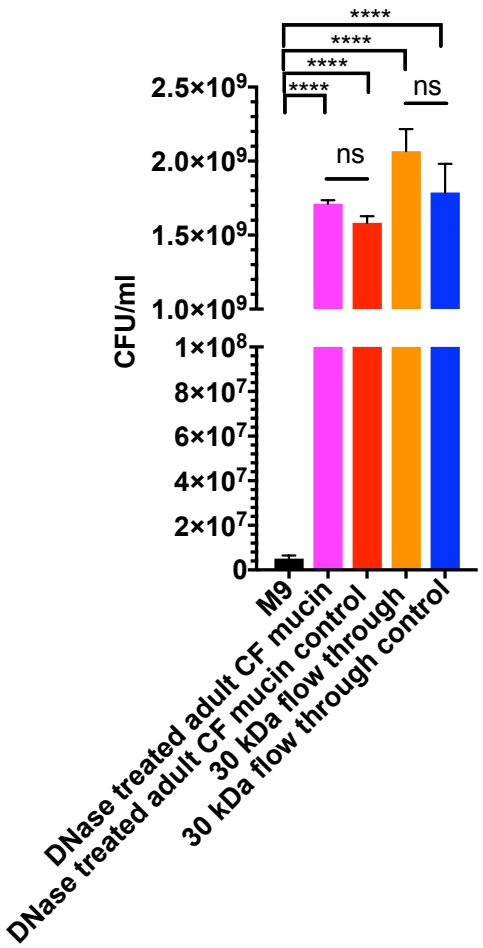

Figure 4.31b.

Figure 4.31: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) adult CF mucin-containing fractions 1-12 supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at different time points over 24 hours in adult CF mucin-containing fractions 1-12 samples treated with DNase, treated with DNase and filtered using a 30 kDa filter, or in the 30 kDa flow-through samples. Error bars represent standard error of the mean (SEM) of 4 'adult CF mucin-containing fractions 1-12' replicates. All samples were supplemented with M9 (6 replicates). Degraded salmon sperm DNA was used as a positive control. b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth. Data was analysed by a one-way ANOVA followed by a Tukey's multiple comparisons test. No statistical difference is noted as 'ns' on the graph. All other interactions had p-values < 0.0001.

The data in figures 4.31a. and 4.31b. reveal that following DNase treatment of the adult CF mucin-containing fractions 1-12 that once again, PAO1 was able to grow to a highly significant growth yield above M9 (****$p < 0.0001$). The growth profile in 4.31a.

shows the growth of PAO1 in the samples compared to their respective controls. Notably the growth profiles are similar in appearance to one another. This suggests that PAO1 was indeed using the degraded DNA as a nutrient source in both the DNase treated adult CF mucin-containing fractions 1-12 and the 30 kDa flow-through sample.

Statistical analysis using a one-way ANOVA revealed that there was no significant difference in growth yield of PAO1 after 24 hours of growth when grown in DNase treated adult CF mucin-containing fractions 1-12 (containing 97 ng/µl degraded DNA) vs. its respective DNase treated control (containing 97 ng/µl degraded salmon sperm DNA). There was also no difference in growth yield when grown in the 30 kDa flow-through (containing 10 ng/µl degraded DNA) vs. the 30 kDa flow-through control (containing 10 ng/µl degraded salmon sperm DNA) ($p > 0.05$). Once again, the data strongly suggests that PAO1 was using degraded DNA as a nutrient source for growth.

4.3.7.4. Growth of *Pseudomonas aeruginosa* PAO1 in paediatric CF mucin-containing fractions 1-12 with degraded DNA removed

Next it was necessary to establish whether *P. aeruginosa* was also unable to grow in paediatric CF mucin-containing fractions 1-12 when degraded DNA was removed.

The green line in figure 4.32a. shows the growth in paediatric CF mucin-containing fractions 1-12 which were not treated with DNase. It appears that it was able to grow relatively well in these samples compared to when grown in M9 alone as determined by the growth yield after 24 hours (**$p < 0.01$, figure 4.32b.). However, due to the large error bars, this was perhaps less significant than expected. The graph however, demonstrates that when degraded DNA was removed from the mucin fractions, there was no significant difference in growth yield after 24 hours in the filtered mucin (containing 2.5 ng/µl salmon sperm DNA) compared to its respective control containing 2.5 ng/µl of intact salmon sperm DNA. There was also no difference when compared to the growth yield in M9 alone ($p > 0.05$, Kruskal-Wallis test, Dunn's multiple comparisons test). Once again, the data suggests that PAO1 is unable to grow in paediatric CF mucin when degraded DNA is removed.

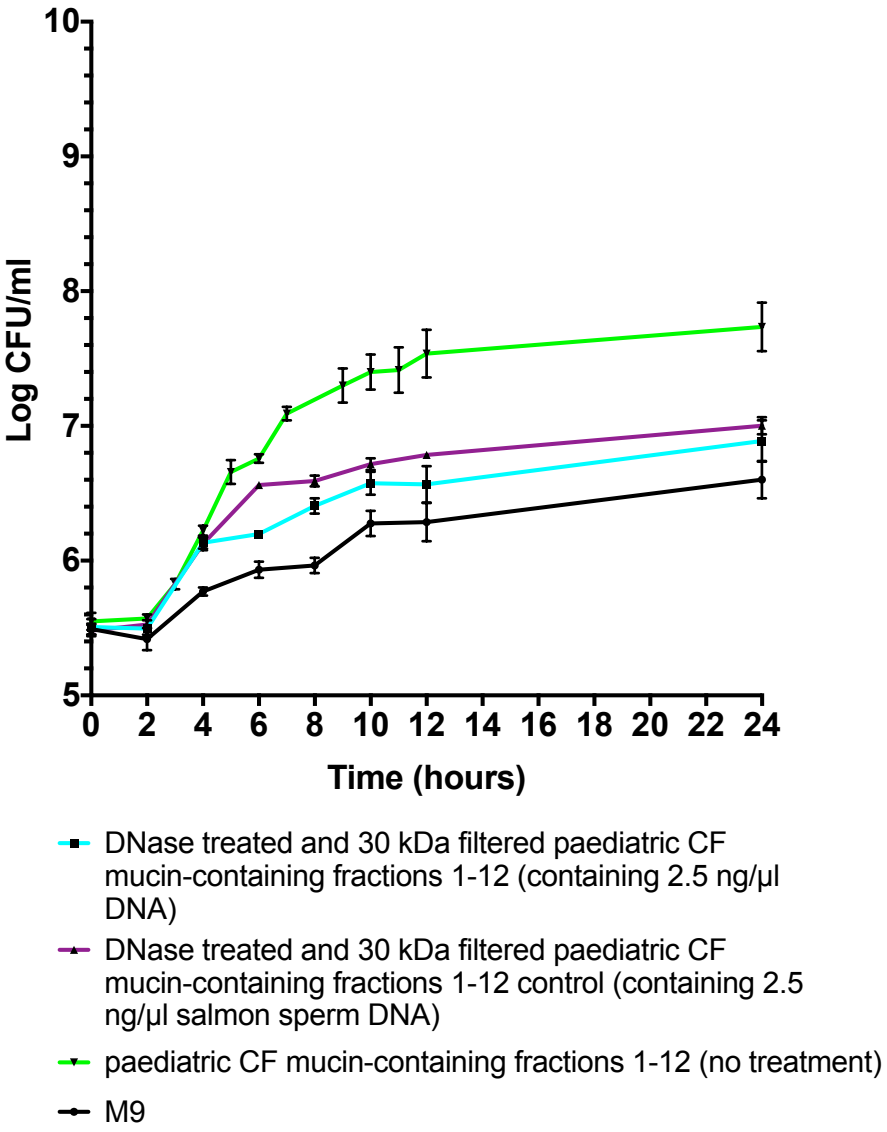

Figure 4.32a.

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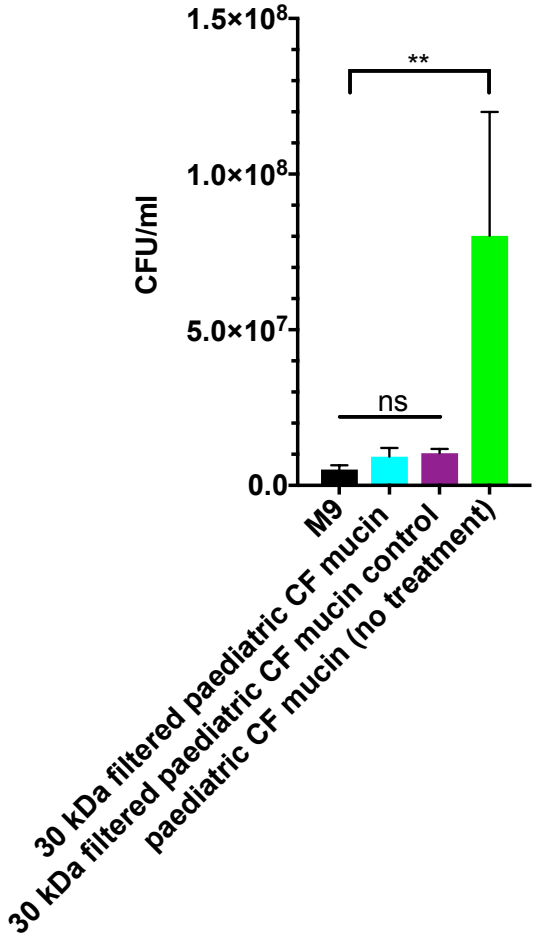

Figure 4.32b.

Figure 4.32: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) paediatric CF mucin-containing fractions 1-12 supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at different time points over 24 hours in paediatric CF mucin-containing fractions 1-12 which were not treated with DNase (green) or were treated with 60 U/ml DNase and filtered using a 30 kDa centrifugal filter (light blue). All samples were supplemented with M9 (6 replicates). Salmon sperm DNA was used as a control (purple). Error bars represent standard error of the mean (SEM) of 4 'paediatric CF mucin-containing fractions 1-12' replicates. b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth. P value on graph (p < 0.01) was obtained by a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. No statistical difference is noted as 'ns' on the graph.**

The data in figure 4.33. demonstrates the growth profile of PAO1 when grown in DNase treated paediatric CF mucin-containing fractions 1-12, the 30 kDa flow-through sample, and their respective degraded salmon sperm DNA controls. Once again, PAO1 was shown to grow well in both the DNase treated sample (containing 22.5 ng/µl degraded

DNA) and the 30 kDa flow-through sample (containing 2.5 ng/µl degraded DNA) compared to M9 (*$p < 0.05$). Statistical analysis revealed that the mean growth yield after 24 hours of PAO1 was 1.98 x 10^9 CFU/ml in DNase treated paediatric CF mucin-containing fractions 1-12 (containing 22.5 ng/µl degraded DNA) was comparable to its respective degraded salmon sperm DNA control with a mean CFU/ml value of 1.92 x 10^9 ($p > 0.05$, Kruskal-Wallis test). This demonstrates that PAO1 was able to utilise degraded DNA within paediatric CF sputum as a nutrient source for growth.

The data in figure 4.33b also demonstrates an increase in growth yield of PAO1 when grown in the 30 kDa flow-through compared to when grown in M9 alone (**$p < 0.01$). However, when the growth yield in the 30 kDa flow-through control (containing 2.5 ng/µl of degraded salmon sperm DNA) was compared to M9, statistical analysis revealed there was no difference ($p > 0.05$). However, on this occasion there also appeared to be a difference between the yield in the 30 kDa flow-through sample compared to its respective degraded salmon sperm DNA control. Since the variances were shown to be significantly different, a Kruskal-Wallis test was performed. Although this revealed there was no significant difference in growth yield between the flow-through sample and its control, the visual appearance of the graph suggests otherwise. For example, there was a mean growth yield in the flow-through sample at 2.19 x 10^9 CFU/ml vs. 5.25 x 10^8 CFU/ml in the degraded salmon sperm DNA control. This represents a 4.2-fold increase in the flow-through sample compared to its control. Although a Kruskal-Wallis revealed that there was no significant difference between these growth yields, when an unpaired, two-sided t-test was performed this difference was found to be highly significant (****$p < 0.0001$). Therefore, this suggests that perhaps there is some other nutrient within paediatric CF mucin-containing fractions 1-12 that can promote growth other than degraded DNA.

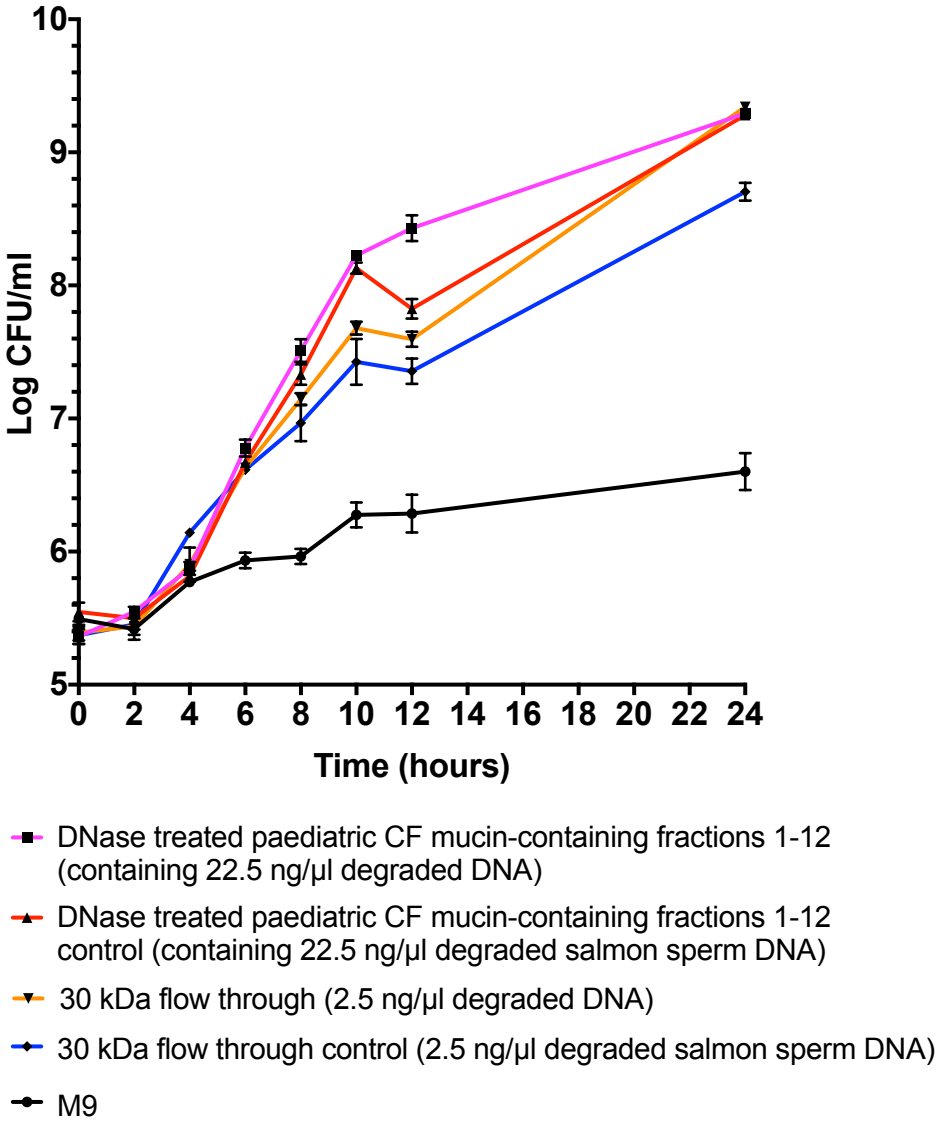

Figure 4.33a.

See legend on the next page

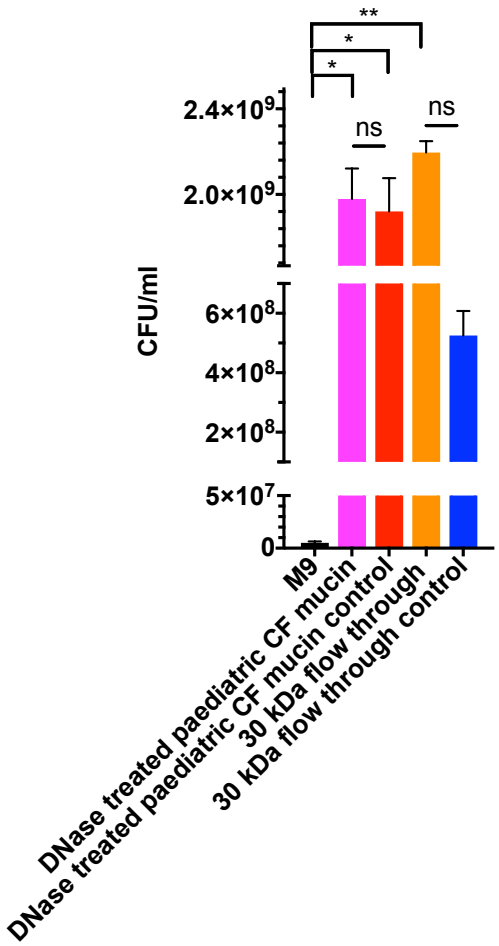

Figure 4.33b.

Figure 4.33: a) Growth of *Pseudomonas aeruginosa* PAO1 in 0.1% (w/v) paediatric CF mucin-containing fractions 1-12 supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at different time points over 24 hours in paediatric CF mucin-containing fractions 1-12 samples treated with DNase, treated with DNase and filtered using a 30 kDa filter, or in the 30 kDa flow-through samples. Error bars represent standard error of the mean (SEM) of 4 'paediatric CF mucin-containing fractions 1-12' replicates. All samples were supplemented with M9 (6 replicates). Degraded salmon sperm DNA was used as a positive control. b) Mean growth yield (CFU/ml) of PAO1 after 24 hours of growth. P values on the graph (*p < 0.05, **p < 0.01) were obtained by a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. No statistical difference is noted as 'ns' on the graph.

4.4. Discussion – Nutrient sources in CF sputum

Since previous work revealed that other components within sputum could affect growth, it was decided to purify the mucin within the sputum samples, so that growth could be assessed in the mucin-containing fractions 1-12. This was done by density gradient centrifugation in Prof. David Thornton's laboratory at the University of Manchester using his methods for purification of the mucin (Thornton et al, 1991).

4.4.1. *Pseudomonas aeruginosa* PAO1 grows 'better' as determined by growth rate in the mucin-containing sputum fractions from adult patients vs. the non-mucin-containing sputum fractions from adult patients and grows better compared to paediatric mucin-containing fractions. Nucleic acid concentration measurements strongly correlate with the growth rate observed in these samples.

Consistent with previous whole sputum growth assay results, once again *P. aeruginosa* PAO1 was demonstrated to have an increased growth rate in adult vs. paediatric CF mucin-containing fractions 1-12, whilst there was no difference in growth rate when grown in the non-mucin-containing fractions 13-20. This data therefore provides evidence that nutrients affecting growth rate in adult samples are associated with the mucins in the sputum sample. Interestingly, there was an increased growth rate in the adult mucin-containing fractions vs. non-mucin-containing fractions which was not observed in the paediatric samples. This strongly suggests that adult and paediatric sputum composition differs since growth is reproducibly different. To date, no growth assays have been conducted on purified mucin fractions from sputum samples from adult and paediatric CF patients, but attempts have been made to mimic the CF sputum for growth assays using a synthetic sputum medium (Sriramulu et al, 2005; Fung et al, 2010; Palmer et al, 2007).

Notably, DNA has not previously been implicated as an important nutrient source for *P. aeruginosa* in the CF airway. However, DNA is credited with supporting biofilm formation, by not only promoting its formation by allowing the cross-linking of sputum, but also indirectly protecting it from the action of neutrophil proteases (Whitchurch et al, 2002). For example, DNA is released in the form of neutrophil extracellular traps (NETs) from infiltrating neutrophils. Therefore, treatment with DNase such as dornase

alfa degrades the DNA within the NETs allowing for the liberation of proteases to target pathogens (Dubois et al, 2012). However, DNA in the biofilm does not just originate from neutrophils, although evidence has shown that most does originate from the host, it is also produced by *P. aeruginosa* itself (Lethem et al, 1990; Walker et al 2005; Allesen-Holm et al, 2006). In addition to its undeniable presence within sputum, it is consistently found at high concentrations meaning it is high enough in abundance to be recognised as a potential nutrient source of concern.

4.4.2. Degraded DNA is a nutrient in CF sputum samples

For the first-time, it has been shown that degraded DNA originating from CF sputum can efficiently promote growth of *P. aeruginosa*. In this study mucin-containing fractions which were treated with DNase had a significantly higher growth yield compared to the mucin fractions which were not treated with DNase. Growth was also assessed in DNase treated salmon sperm DNA.

Interestingly, a previous study by Mulcahy and colleagues (2010) revealed that when grown in fish sperm DNA, expression of PA3909 was induced which encodes the secreted extracellular DNA degradation protein, EddB, in *P. aeruginosa*. Based on these findings, it is surprising that experiments in this thesis demonstrated limited growth in salmon sperm DNA which was not DNase treated. Although it must be noted that this previous study used a higher concentration of DNA which was two thirds greater than the concentration of DNA used for the growth studies in this thesis. It therefore may be that higher concentrations of DNA are required to stimulate growth due to expression of EddB. Therefore, whilst it has been demonstrated that *P. aeruginosa* can produce its own extracellular DNase, it appears that it is the pre-existing degraded DNA which is likely to be used more efficiently as an immediate nutrient source in the CF airway as evidenced by the growth assays. Therefore, although some literature has addressed DNA being able to provide nutrients to *P. aeruginosa,* there is more focus on DNase being used to destroy the biofilm (Gnanadhas et al, 2015). This is rather than addressing what underlying role degraded DNA has on *P. aeruginosa* survival in the CF airway in the long-term because of dornase alfa treatment.

Furthermore, it is unsurprising that the data in this study reveals that *P. aeruginosa* is unable to utilise intact, non-degraded DNA as well as degraded DNA. The reasons for this are because *P. aeruginosa* is not able to uptake extracellular DNA. Though this may explain why the extracellular DNase, EddB, is produced by *P. aeruginosa* (Mulcahy et al, 2010). Therefore, the reason why intact DNA may not be utilised as well as degraded DNA is likely to be because *P. aeruginosa* does not exhibit natural competence via transformation unlike other pathogens such as *H. influenzae* and *S. pneumoniae*, in which it was first discovered (Chen and Dubnau, 2004; Griffith, 1928).

Natural competence via transformation is the process by which exogenous DNA in the surrounding environment is taken up by a bacterial cell and incorporated into its genome, thereby allowing it to secure favourable genetic traits which promote survival in challenging environments (Lorenz and Wackernagel, 1994; Chen and Dubnau, 2004). For this to occur in the many pathogens in which this phenomenon has been identified, bacteria have conserved genes shared amongst distant bacterial relatives. For example, the type VI pili, interestingly used by *P. aeruginosa* to initially adhere in the airway and form a biofilm, are needed for the formation of the channel comEC/Rec 2 for the uptake of DNA in *S. pneumoniae* and *H. influenzae* – pathogens which are also present in the CF airway environment (Woods et al, 1980; Chen and Dubnau, 2004). Because *P. aeruginosa* also possesses type VI pili, this alludes to some involvement with DNA. In support of this, based on the current literature, perhaps *P. aeruginosa* is present within the mucus layer as described by Lau and colleagues (2005), because the type VI pili can bind to DNA associated within sputum. This could subsequently allow for biofilm formation as observed by O'Toole and Kolter (1998) due to Type VI twitching which then causes the formation of tight *Pseudomonas* microcolonies (Schaik et al, 2005; Mattick, 2002). Therefore, because DNA is closely associated with *P. aeruginosa*, when it is degraded by inhaled dornase alfa, degraded DNA could potentially serve as an immediate source of energy for growth for not only *P. aeruginosa*, but other respiratory pathogens when not used for transformation. Interestingly, DNA has been regarded to be a viable nutrient for *E. coli* whereby it was shown that the uptake of extracellular DNA does not serve solely for genetic transformation, but also acquisition of nutrients and therefore could also serve as a nutrient for other bacteria in the CF airway as previously mentioned (Finkel and Kolter, 2001).

This argument of DNA being a nutrient source is further strengthened by data showing that degraded salmon sperm DNA also promotes growth, whilst intact DNA does not. Salmon sperm DNA used in this study was selected because in a study by Schaik and colleagues (2005) their work revealed that *P. aeruginosa* binds to salmon sperm DNA, of which binding was demonstrated to be in a concentration-dependent manner with preferential binding to pyrimidines via PAK pili of the type VI pili family. Because of this previously demonstrated interaction, in this study, growth was assessed in the pyrimidine, thymine. This study has confirmed that *P. aeruginosa* could use thymine at low concentrations as a nutrient source for growth, as it was also shown that it could grow on deoxyribose – another component of DNA. A limitation of this experiment was that further growth studies could not be assessed using other purines or pyrimidines since they could not be solubilised in water, and could only be solubilised in acid which kills the organism.

Once it had been established that *P. aeruginosa* PAO1 could grow very well in degraded DNA present in CF sputum and salmon sperm DNA, further experiments in this study focused on purifying the mucins to identify whether mucins could still grow in mucin-containing fractions in the absence of degraded DNA.

4.4.3. Autoclaving destroys the polymeric structure of mucins

In these further purification experiments, analysis revealed that it was the process of autoclaving that affects the mucin structure. Processing procedures were considered to ensure that processing of the sputum did not manipulate the mucins in any way that would favour growth. Since autoclaved mucins ran similarly on gels to reduced mucins, autoclaving was considered appropriate, although this process was shown to destroy the quaternary structure of the mucin, which had not previously been demonstrated.

4.4.4. *Pseudomonas aeruginosa* was unable to use CF patient mucin as an immediate nutrient source

When degraded DNA was removed from the mucin from eight different CF patient sputum samples and three independently processed PGM samples, growth seen in 'pure' mucin was no different to that observed in M9. Therefore, although mucins have been demonstrated to be degraded in CF as discussed in section 3.4.2.3., it has been demonstrated that *P. aeruginosa* PAO1 was unable to grow even when mucins were degraded and autoclaved (Henderson et al, 2014). This was not an unexpected outcome at this stage in the study because when PAO1 was previously grown in mucin sugars, it found that there was very little growth in the mucin sugars compared to glucose. Furthermore, whilst in this study I found that *P. aeruginosa* was unable to utilise CF patient mucin as a nutrient source, interestingly this has also been shown in a more recent study using PGM, which I also used. The study conducted by Flynn and colleagues (2016) revealed that *P. aeruginosa* PA14 was unable to efficiently utilise PGM itself, but required anaerobic bacteria originating from the oral cavity to initially degrade mucins into the subcomponents: acetate and propionate, which *P. aeruginosa* could then metabolise. This study therefore supports my findings that *P. aeruginosa* cannot utilise PGM and mucin originating from CF sputum as a nutrient source, and instead, it is other subcomponents which support growth. Based on the data in this study, I have found that PAO1 is able to utilise degraded DNA in CF patient sputum samples.

However, the data did reveal interesting results when growth assays were performed in the 30 kDa flow-through samples originating from the paediatric CF samples. Following processing of the paediatric samples as presented in figure 4.26., this difference in growth yield when grown in the flow-through (containing 2.5 ng/µl degraded DNA) vs. the degraded DNA salmon sperm control (containing 2.5 ng/µl of degraded salmon sperm DNA) could be due to liberation of amino acids following autoclaving. Further work is therefore needed to assess what other components are within the flow-through which could be promoting growth other than degraded DNA.

4.5. Chapter conclusions

The second and third aims of this project were as follows:

2) To identify whether mucin is a potential airway nutrient for *P. aeruginosa* and *S. pneumoniae.*
3) To identify other potential nutrients, present within airway sputum.

The findings in this study are novel because it was demonstrated that *P. aeruginosa* was unable to utilise airway mucins purified from patient samples as a nutrient source for growth. However, PAO1 was found to be able to utilise degraded DNA within CF sputum as a nutrient source, but was less able to utilise intact DNA. Therefore, because the amount of DNA was shown to correlate with the growth rate in adult CF mucin, but not in paediatric CF mucin, and because increased DNA was measured in adult vs. paediatric CF mucin, the data suggests the increased growth rate of PAO1 observed in adult vs. paediatric CF sputum was due to the availability of degraded DNA in the samples. The data also suggests there may be other nutrients in paediatric samples which may be promoting growth. Nevertheless, the data suggests an important need for CF patients to quickly expel mucus from the airway following dornase alfa inhalation so that degraded DNA can be removed from the airway as quickly as possible before it can be utilised by *P. aeruginosa* as a nutrient source.

Chapter 5. Gene expression of *Pseudomonas aeruginosa* in adult and paediatric CF sputum in the presence and absence of DNase

5.1. Introduction and aims

The final aim of the study was to identify how treatment with DNase affects the expression of genes involved in purine and pyrimidine metabolism with the aim of identifying a mechanism by which *P. aeruginosa* PAO1 favours growth when in the presence of degraded DNA. To date there is little understanding of *P. aeruginosa* nutritional requirements in the CF lung environment. As previously discussed in section 4.1, amino acids constitute an important nutrient source for pathogens in CF, together with glucose and lipids. DNA, however, has never been attributed with having a prominent role in promoting growth in the CF airway, though a previous study has shown that *P. aeruginosa* can produce an extracellular DNase which allows for utilisation of fish sperm DNA as a nutrient source (Mulcahy et al, 2010). Additionally, DNA has been shown to be a nutrient for *E. coli.* In a study by Finkel and Kolter (2001), it was hypothesised that natural competence, which is known to occur in many bacterial species, not only occurs for the sole purpose of transformation, but also for acquisition of extracellular DNA for the nutritional demand of the bacterium. Whether DNA serves as a nutrient for other organisms in the CF airway remains to be determined.

5.2. Methods

5.2.1. Sputum processing for growth of *Pseudomonas aeruginosa* in the presence and absence of dornase alfa

Because previous work demonstrated that *P. aeruginosa* was unable to utilise mucins as a nutrient source, but could efficiently grow in the presence of DNase and degraded DNA, it was questioned what effect dornase alfa has on the growth of *P. aeruginosa*. The concentration of dornase alfa in sputum was measured in eighteen CF patients after 15 minutes following inhalation. The concentration of dornase alfa was reported to be

approximately 3 μg/ml (FDA, 2014). This concentration was used to assess the effect of dornase alfa on the growth of *P. aeruginosa*.

CF patient sputum samples were processed as described in 3.2.2.2, but rather than being suspended to 8 mg/ml, they were suspended to 2 mg/ml due to limited availability at this point in the project. Notably, the sputum originated from the same patient samples used for density gradient centrifugation and growth studies in mucin-containing fractions 1-12. However, it must be noted that on this occasion whole sputum samples were used and not the pooled fractions.

Samples to be treated with 3 μg/ml dornase alfa containing 3 U/ml DNase (Pulmozyme, Genetech) were re-suspended to 2 mg/ml before being lyophilised. Sputum was then re-suspended to a volume that would allow a volume of 1 mg/ml dornase alfa (1000 U/ml) stock to be added to a final concentration of 6 μg/ml (6 U/ml) in 2 mg/ml sputum. Therefore, when added to an equal volume of double strength M9, the final concentration of dornase alfa and sputum would be 3 μg/ml (3 U/ml) in 1 mg/ml (0.1% w/v) CF sputum, respectively. Dornase alfa was sterilised via passing through a 0.2 μm acrodisc® before adding to sterilised sputum. Following addition of dornase alfa at a concentration found in CF sputum 15 minutes after dosage, all samples were incubated at 37^{o}C for 15 minutes prior to inoculation with *P. aeruginosa* PAO1.

5.2.2. Sputum processing for *Pseudomonas aeruginosa* gene expression in the presence and absence of DNase

For gene expression studies, CF patient sputum was processed as described in section 3.2.2.2. It was then re-suspended to 2 mg/ml with nH_2O and treated with TURBO DNase as described in section 4.2.2.2. Samples were then sterilised via autoclaving in the presence of 10 mM potassium phosphate buffer.

5.2.3. Primer design and preparation

Genes selected for study were chosen from purine and pyrimidine metabolism KEGG pathways. Although many genes were initially selected for optimisation with the aim of determining a preferred metabolism pathway, the table in appendix 2 summarises the

sixteen genes and the proteins they encode, which were eventually selected following optimisation for qRT-PCR.

Primers were designed to amplify a small region of the genes of interest using OligoPerfect^{TM} designer. Forward and reverse primers were selected to have approximately 50% GC content, a length of 19-21 bp, annealing temperature of 57-63^{o}C, and a product size of 140-160 bp. Please see appendix 2.

Forward and reverse primers were suspended to a final concentration of 100 pmol/µl using sterile RNase/DNase-free dH_2O. The volume added was based on the volumes described in the 'Oligonucleotide Synthesis Report' sheet for each primer. In a sterile microtube, 10 µl of forward (100 pmol/µl) and 10 µl of reverse primers (100 pmol/µl) were added to 80 µl RNase/DNase-free dH_2O to obtain a stock of forward and reverse primers at 10 pmol/µl (10 µM).

5.2.4. Bacterial genomic DNA extraction

Genomic DNA (gDNA) was extracted from *P. aeruginosa* PAO1 using the PureLink® Genomic DNA mini kit (Invitrogen). Cultures were grown to 2 x 10^9 CFU/ml overnight before being centrifuged. Next, the supernatant was discarded and the bacterial pellet was re-suspended in 180 µl of PureLink® genomic digestion buffer. The cells were then lysed with the addition of 20 µl Proteinase K and mixed well by vortexing. The sample was then incubated at 55^{o}C with occasional mixing until lysis was complete. After 4 hours, 20 µl of RNase A was added to the lysate and mixed again by brief vortexing, before incubating at room temperature. After 2 minutes, 200 µl of the PureLink® genomic lysis/binding buffer was added and mixed. Following this, 200 µl of 96-100% ethanol was added to the lysate and the sample was once again mixed via vortexing to obtain a homogenous solution.

5.2.5. DNA purification

To purify the DNA, instructions as part of the PureLink® Genomic DNA Mini Kit were followed. The lysate containing ethanol and the PureLink® genomic lysis/binding buffer were added to a spin column which was centrifuged at 10000 x g for 1 minute at room temperature. The previous collection tube was then discarded and the spin column was

placed in a new collection tube before adding 500 µl of wash buffer 1 prepared in ethanol. This column was once again centrifuged. Following this, the collection tube was discarded, replaced with a new collection tube, and 500 µl of wash buffer 2 was added to the column. The column was then centrifuged at maximum speed for 3 minutes at room temperature. The spin column was then placed in a sterile 1.5 ml microtube, and 25-200 µl of PureLink® genomic elution buffer was added to the column. After 1 minute of incubation at room temperature, the tube was then centrifuged at maximum speed for 1 minute to collect the purified gDNA. DNA for short-term storage was kept at 4^{o}C and for long-term storage at -20^{o}C. Genomic DNA was used for assessing annealing temperature of primers by conventional PCR and determining primer efficiency.

5.2.6. Determining PCR efficiency

5.2.6.1. Conventional PCR

Conventional end-point PCR was performed to determine that following PCR, the amplicon product was the expected size. The HotStarTaq® *Plus* mastermix 2X reagent (Qiagen) was added to a final concentration of 1X in a final reaction volume of 20 µl. To this, 6 µl of DNase/RNase-free dH_2O was added, 2 µl of gDNA from PAO1 at a concentration of 20 ng/µl, and 2 µl of 10 µM F and R primer stock. This was then pipette mixed, before starting the PCR. The BioRad-T100™ Thermo cycler was set to the following settings as shown in table 5.1.

Table 5.1: Conventional PCR protocol using HotStarTaq® *Plus* kit

Step	Temperature (°C)	Duration (mm:ss)
Check lid	**105**	∞
1	95	10:00
2	95	00:45
3	55	00:15
4	72	01:00
5	• Repeat steps 2, 3, 4 x 29 further cycles	
6	72	10:00
7	4	∞

Agarose gel electrophoresis was then performed to confirm product size against the oligonucleotide synthesis report (Eurofins).

5.2.6.2. Standard curve and melt-point analysis

Purified gDNA was diluted to 20 ng/µl and 10-fold serial dilutions were performed to get 2, 0.2, 0.02, 0.002, and 0.0002 ng/µl respectively in RNase/DNase-free dH_2O. For each gene and dilution, tubes were prepared in duplicate. A non-template control (NTC) with no DNA was added to determine formation of primer-dimers or contamination. The following was added to each reaction tube in the Rotor-Gene style 4-strip tubes: 10 µl of SensiMix™ SYBR® Hi-ROX (Bioline), 7 µl of RNase/DNase-free dH_2O, 2 µl of gDNA, and 1 µl of primer stock (10 µM) to get a final concentration of 0.5 µM of forward and reverse primers in the final reaction mixture. The tubes were then covered in foil once the SYBR dye had been added. Gene expression was quantitated on the Rotor-Gene™ 6000 instrument (Corbett) using the protocol described in table 5.2.

Table 5.2: Rotor-Gene™ 6000 settings for qPCR

Step	Temperature (^{o}C)	Duration (mm:ss)
1	95	10:00
2	95	00.15
3	55.5/56	00.15
4	72	00.15
5	83	00.15
6	• Acquire to cycling A on green	
7	• Repeat steps 2-7 x 39 further cycles	
8	• Hold for 90 seconds of pre-melt conditioning on first step. • Ramp temperature from 60 → 99 rising by 1^{o}C with each step. Hold for 5 seconds between each step.	

A standard curve was generated for each primer to determine primer efficiency under the reaction conditions. Generally, primers were only selected with an efficiency greater than 90%. In the case that primers had efficiencies ranging from 80 to 89% these were only selected upon identification of a single peak following melt-point analysis. Primers which did not satisfy the selection criteria were not investigated further. Analysis of relative gene expression was assessed via the Pfaffl method as described in section 5.2.10.

5.2.7. Bacterial RNA extraction for investigating gene expression

Bacterial cultures were prepared as discussed and were exposed for 3 hours to sputum samples which were either treated with 60 U/ml DNase or left untreated. After 3 hours, the 1.5 ml culture was centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded before adding 500 µl of TRIzol™ reagent and vortexed. Chloroform (100 µl) was then added and vortexed for 15 seconds before transferring the contents to a Lysing Matrix B tube. The sample was sonicated for 45 seconds at 6.5 power setting using the ribolyser. The sample was then left for 1 minute at room temperature before centrifuging at 12000 x g for 15 minutes at 4^{o}C. Next, the aqueous upper phase containing the RNA was carefully removed and transferred to a fresh microtube. Isopropanol (250 µl) was added to precipitate the RNA and vortexed for 15 seconds

before leaving at room temperature for 15 minutes. The tube was then centrifuged at 12000 x g for 10 minutes at 4°C. The supernatant was then discarded and 500 µl of 75% ethanol (in DEPC-treated dH_2O) was added and vortexed. The tube was centrifuged again at 12000 x g for 5 minutes at 4°C and the supernatant once again discarded. The RNA pellet was then left to air dry at room temperature. Once dry, the pellet was re-suspended in 50 µl RNase/DNase-free dH_2O and mixed. The RNA was then aliquoted for NanoDrop, Bioanalyzer 2100 analysis, and first-strand cDNA synthesis. To avoid repeated freeze-thawing, RNA was kept on ice during assessment of the RNA quality and quantity until needed for cDNA synthesis. Residual samples were only stored at -20°C once the other stages were complete.

5.2.8. Assessment of RNA quantity and quality

5.2.8.1. NanoDrop One

RNA quantity and quality was assessed using NanoDrop One. NanoDrop measures the concentration (ng/µl) and absorption at 260/280 nm and 260/230 nm. This was performed to determine whether the RNA extracted was free of contaminants. A ratio of 260/280 nm of approximately 2.0 is generally accepted as 'pure' for RNA whereas a ratio of 260/230 nm which is used as a secondary measure, commonly ranges between 1.8-2.2.

5.2.8.2. DNase treatment of RNA samples

After analysis via NanoDrop, the TURBO DNA-free™ kit (Ambion) was used to degrade any residual gDNA present. A volume of 20 µl of freshly extracted RNA was treated with 2 µl TURBO DNase and 2 µl 10X buffer (0.1X volume). After the addition of these components, the solution was pipette mixed before incubation at 37°C. After 20-30 minutes, the DNase inactivating reagent was vortexed before use and 4 µl (0.2X volume) was added to the sample. This was then incubated at room temperature and mixed occasionally for 5 minutes before centrifuging at 10000 x g for 15 minutes. The RNA supernatant was then carefully removed from the inactivated DNase pellet and transferred to a new microtube. This DNase treated RNA was then diluted by taking 5

µl and adding an equal volume (5 µl) of RNase/DNase-free dH_2O. This diluted RNA was then assessed using the Bioanalyzer 2100.

5.2.8.3. Bioanalyzer 2100

The Agilent RNA 6000 Nano kit was used to measure the integrity of the RNA in samples. The chip set up station was prepared and the electrode decontaminated.

Following arrival of the kit, the RNA ladder was dispensed into an RNase/DNase-free microtube. It was then denatured for 2 minutes at 70^{o}C followed by incubation on ice. Aliquots of the ladder were then prepared and stored at -80^{o}C. Upon thawing of an aliquot, care was taken to not allow excessive warming so that degradation of the ladder could be avoided.

In preparation for making the gel, 30 minutes before required, the reagents were given time to reach room temperature. Once at room temperature 550 µl of Agilent RNA 6000 nanogel matrix was added to a spin filter which was centrifuged at 4000 rpm for 10 minutes. Once filtered, 65 µl of the gel was transferred to 0.5 ml RNase-free microtubes supplied with the kit. These were then stored as aliquots at 4^{o}C. Following this, the RNA 6000 nanodye concentrate was vortexed for 10 seconds and 1 µl was added to the filtered gel. This was mixed by vortexing and then centrifuged at 14000 rpm for 10 minutes. After being spun down, the tube was covered in foil to prevent exposure to light during preparation of the RNA nanochip on the priming station. Gel-dye mix (9 µl) was then dispensed into the well indicated as 'G'. The plunger was then positioned to 1 ml and the chip priming station closed. This was left for 30 seconds before pushing the plunger of the syringe until it was held by the clip of the station. This was left for another 30 seconds before releasing the plunger from the mechanism and positioned back at 1 ml. This was necessary to pressurise the chip. The chip priming station was then opened and 9 µl of the gel-dye was added to the two wells above the wells indicated as 'G'. Next, 5 µl of the RNA 6000 Nanomarker was added to twelve further wells and the well indicated by a ladder symbol. Following from this, the ladder and samples could be added to the chip. To the well indicated as the 'ladder,' 1 µl was dispensed in this well. In the other twelve wells to which the RNA 6000 Nanomarker was added, 1 µl of extracted RNA was then added to these wells. The chip was then

vortexed at 2000-2400 rpm in the IKA vortex mixer for 1 minute. Finally, the chip was then inserted into the Bioanalyzer and the run was started (Agilent Technologies, 2013).

5.2.8.4. Agarose gel electrophoresis

RNA quality was additionally assessed by agarose gel electrophoresis. RNA samples were diluted based on the concentrations measured by the Bioanalyzer 2100. This is included in this section in addition to the method described in chapter 2 due to differences when running an agarose gel with RNA.

The 1% agarose gel was prepared by dissolving 1 g agarose in 100 ml TAE (1X) buffer. Ethidium bromide was then added to a final concentration of 0.5 µg/ml. DNA ladder (6X concentrated, 1 kbp) was prepared by adding to an equal volume of loading dye and mixing with four times the volume of RNase/DNase-free dH_2O to get a final ratio of 1:1:4. RNA (4 µl) containing approximately 120 ng RNA was then mixed with 3 µl loading dye and 3 µl RNase/DNase-free dH_2O. Prior to loading, the samples were incubated at 70^{o}C for 3 minutes and put on ice. The gel was then run at 90 V.

Following agarose gel electrophoresis, RNA samples were assessed via NanoDrop again and diluted to 30 ng/µl for first-strand cDNA synthesis.

5.2.9. cDNA production

The reverse transcriptase (RT) reaction was carried out using Superscript™ III Reverse Transcriptase (Invitrogen) and set up in a reaction volume of 20 µl containing 5 µl of RNA (150 ng), 1 µl random primers, 1 µl dNTP (10 mM) and 13 µl RNase/DNase-free dH_2O. PCR tubes were initially heated to 65^{o}C in the BioRad-T100™ Thermo cycler for 5 minutes. The samples were then left on ice for 1 minute before adding 4 µl of 5X first-strand buffer, 1 µl of 0.1 M DTT, and 1 µl of Superscript™ III Reverse transcriptase. The thermo cycler was then programmed to heat to 25^{o}C for 5 minutes, 50^{o}C for 35 minutes, and 70^{o}C for 15 minutes to synthesis cDNA from the RNA samples. This was performed in duplicate with one tube lacking Superscript™ III. These samples provided the RT control for qPCR. Following from this, the cDNA was diluted by taking 10 µl and adding to 90 µl RNase/DNase-free dH_2O and leaving on ice, before performing another 1:10 dilution so that the final amount of cDNA used for

qPCR was a 1:100 dilution of the neat cDNA. The remaining stock cDNA was stored at -20^{o}C. Before use in qPCR, the cDNA was assessed by NanoDrop and freshly diluted to 4 ng/µl.

5.2.10. qPCR

For qPCR, a mastermix containing 10 µl SensiMix™ SYBR Hi-ROX, 2 µl diluted cDNA, and 7 µl RNase/DNase-free dH_2O per sample was mixed. To the Rotor-Gene style 4-strip tubes, 19 µl of this mastermix was added to each of the tubes before adding 1 µl of the primer stock mix for each gene of interest. For each gene, this was performed in duplicate. The protocol was followed as detailed previously in table 5.2, section 5.2.6.2. Analysis of relative gene expression was assessed via the Pfaffl method (2001), which normalises the CT values against that of a stable housekeeping gene (HKG). This method also allows for correction of primer efficiency values which fall below 90%.

The following calculation was used to determine a ratio of a target gene expression in a sample vs. the expression in the control sample. This was then compared to the expression in a reference gene (Pfaffl, 2001):

$$\text{Ratio} = \frac{(\text{E target})^{\Delta \text{CP target (control – sample)}}}{(\text{E ref})^{\Delta \text{CP ref (control – sample)}}} \quad ,$$

where, 'CP' is the crossover point at the set threshold, and 'E' is the qPCR efficiency calculated from the following equation:

$$\text{E} = 10^{(-1/\text{slope})} \quad ,$$

where, the 'slope' is calculated from the standard curve generated from each primer pair.

5.3. Results

5.3.1. Growth of *Pseudomonas aeruginosa* PAO1 in whole CF patient sputum

As detailed in the previous chapter, it was shown that the addition of 60 U/ml of DNase positively influenced the growth yield of *P. aeruginosa* PAO1 in adult and paediatric CF mucin-containing fractions 1-12. It was found that mucin does not serve as a

nutrient source and instead it is highly likely that any growth observed in these fraction samples was due to the presence of any previously present degraded DNA. Since it was determined that mucin has a minimal role in providing carbon as an immediate nutrient source under the conditions in this study, it was decided to repeat the growth assays of *P. aeruginosa* PAO1 in whole sputum and compare it to growth in whole sputum which was DNase treated.

5.3.1.1. Growth of *Pseudomonas aeruginosa* PAO1 in whole CF patient sputum treated with dornase alfa

Although not a focus of this study, it was decided to investigate whether dornase alfa present at a concentration of 3 μg/ml in CF sputum can influence the growth yield of *P. aeruginosa* PAO1. This concentration of dornase alfa was selected as it was the mean concentration detected in airway sputum after 15 minutes following administration (FDA, 2014).

Figure 5.1 demonstrates no difference in growth rate of *P. aeruginosa* PAO1 when grown in whole CF sputum which was pre-treated with 3 μg/ml dornase alfa compared to growth in sputum which was not treated. However, figure 5.1b. demonstrates a 3-fold increase in mean growth yield after 24 hours from 8.03 x 10^7 CFU/ml to 2.54 x 10^8 CFU/ml when treated with dornase alfa (****$p < 0.0001$, paired, two-sided t-test).

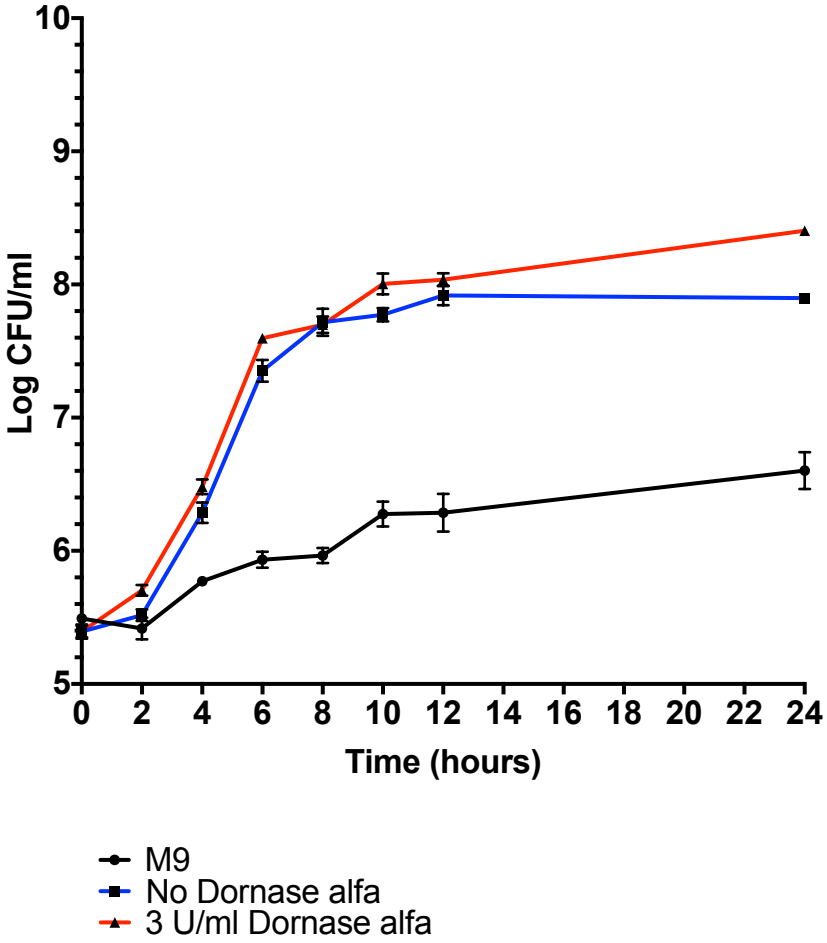

Figure 5.1a.

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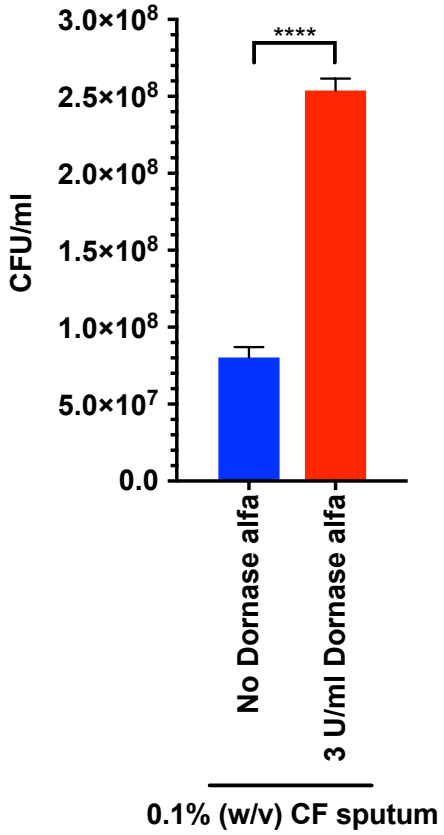

Figure 5.1b.

Figure 5.1: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) adult and paediatric CF whole sputum supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at the different time points in M9 (black line, 6 replicates), CF sputum which was not treated with dornase alfa (blue line, 4 replicates: 2 adult and 2 paediatric), and CF sputum which was treated with dornase alfa containing 3 U/ml DNase (red line, 4 replicates: 2 adult and 2 paediatric). b) Growth yield (CFU/ml) of PAO1 after 24 hours in whole CF sputum which was either treated with dornase alfa containing 3 U/ml DNase (red bar) or was not treated with dornase alfa (blue bar). Error bars show standard error of the mean (SEM). P value on the graph (**p < 0.0001) was obtained by a paired, two-sided student t-test.**

5.3.1.2. Growth of *Pseudomonas aeruginosa* PAO1 in whole CF patient sputum treated with DNase

For gene expression experiments, it was decided to investigate the expression of genes when exposed to DNase rather than dornase alfa. Although this was a novel finding, before further work to assess how dornase alfa affects the CF microbiome in the long

term, it was necessary to firstly understand mechanisms by which *P. aeruginosa* utilises degraded DNA as a nutrient source.

In the previous chapter of this thesis, it was described how sputum fractions purified via density gradient centrifugation were subjected to thorough DNase treatment to remove contaminating DNA in mucin-containing fractions. Consequently, it was determined that *P. aeruginosa* was unable to use mucins as a nutrient source but because samples were treated with 60 U/ml of DNase it was decided to use the same concentration for investigation of gene expression in *P. aeruginosa* PAO1 in whole sputum rather than mucin-containing CF fractions.

Growth assays were repeated in whole sputum because it was necessary to identify mid-exponential phase of growth so that RNA could be extracted from PAO1. Figure 5.2 shows the log growth of PAO1 over 24 hours in six whole sputum samples. Once again, the data shows an increase in growth yield after 24 hours which was found to be statistically significant when treated with DNase (****$p < 0.0001$). The graph also shows that PAO1 is in mid-exponential phase of growth when grown in sputum treated with 60 U/ml DNase after 5 hours, whereas at 5 hours this is mid-late exponential phase when grown in the absence of DNase. Following RNA extraction after 5 hours it was found that the RNA extracted yielded an insufficient quantity of RNA necessary for analysis and cDNA synthesis. By this point in the project, there was very little remaining sample that remained following conserved usage throughout the project. Therefore, the growth assay was repeated using a higher starting inoculum (~ 1×10^8 CFU/ml). Growth was assessed to determine that a significant increase in growth yield after 24 hours was still observed following addition of a higher starting inoculum.

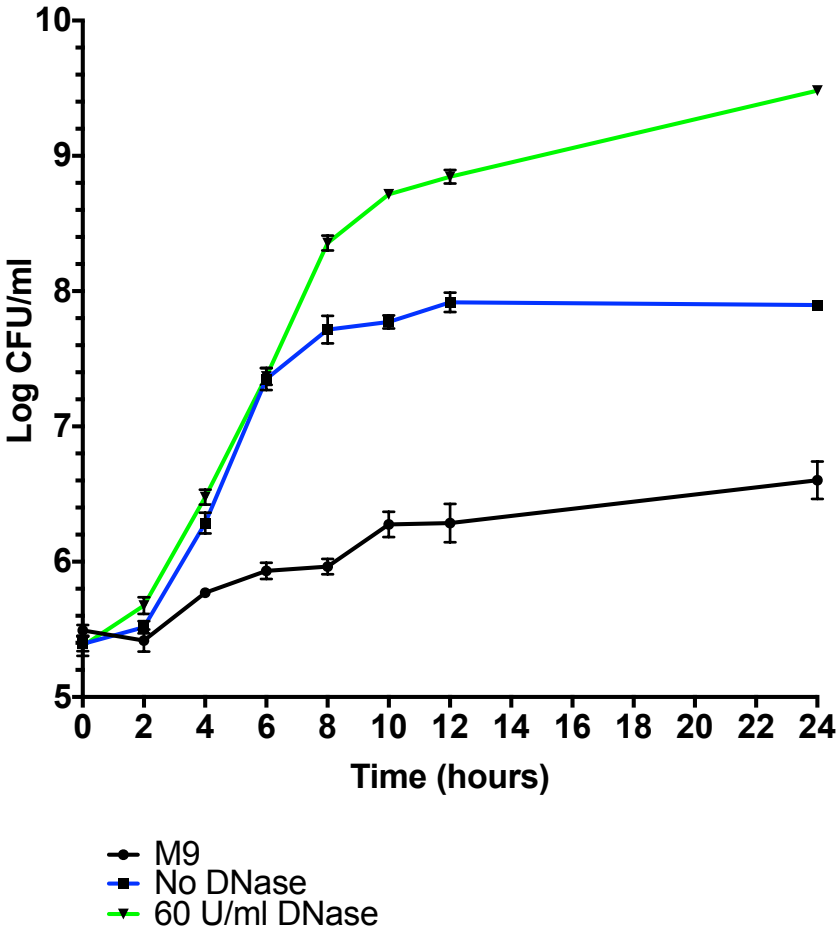

Figure 5.2a.

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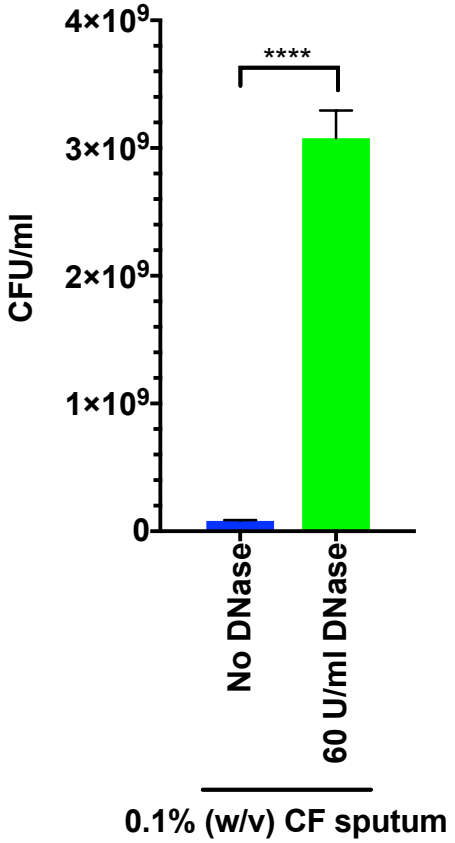

Figure 5.2b.

Figure 5.2: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) adult and paediatric CF whole sputum supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at the different time points in M9 (black line, 6 replicates), CF sputum which was not treated with DNase (blue line, 6 replicates: 3 adult, 3 paediatric), and CF sputum which was treated with 60 U/ml DNase (green line, 6 replicates: 3 adult, 3 paediatric). b) Mean growth yield (CFU/ml) after 24 hours of growth of PAO1 in CF sputum which was treated with 60 U/ml DNase (green bar) or was not treated with DNase (blue bar). Error bars show standard error of the mean (SEM). P value on the graph (**p < 0.0001) was obtained by a paired, two-sided student t-test.**

Figure 5.3a. shows that sputum which was not treated with DNase was unable to support growth of PAO1. As indicated by the profile of the blue line in figure 5.3a., the growth yield doesn't appear to increase after 24 hours compared to the start inoculum at 0 hours. However, when DNase treated, the data in figure 5.3b. reveals an increase in yield of PAO1 after 24 hours when exposed to CF sputum which was previously treated with 60 U/ml DNase (****$p < 0.0001$). Therefore, based on the data, the presence of DNase was shown to stimulate growth whilst there was no growth in the untreated samples.

Because no growth was observed in the untreated sputum when a higher inoculum was administered, gene expression could therefore not be assessed during growth phase. Evidently this was not possible due to limitation of sample. It was therefore decided to expose PAO1 to the samples for 3 hours before spinning down the bacteria and suspending in TRIzol™ reagent for RNA extraction as described in section 5.2.7. The intention was to repeat this at 6, 9, and 12 hours in future experiments.

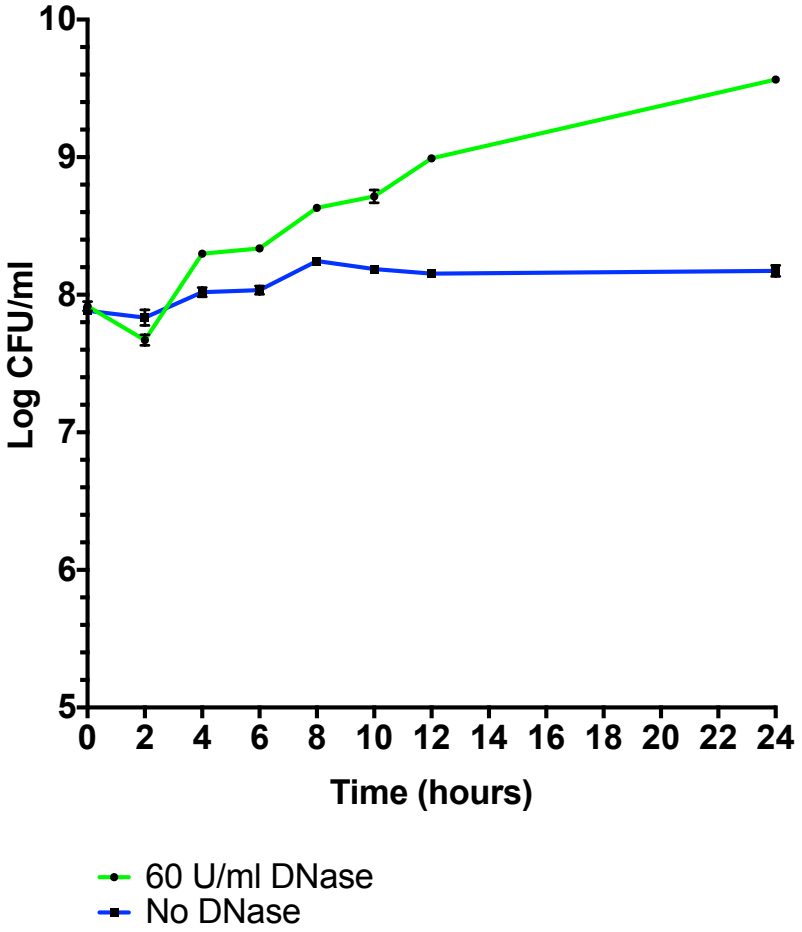

Figure 5.3a.

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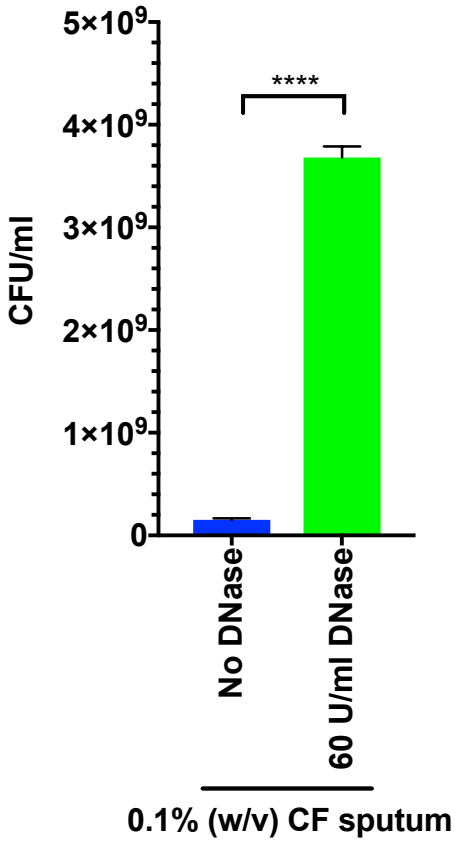

Figure 5.3b.

Figure 5.3: a) Growth of *Pseudomonas aeruginosa* PAO1 over 24 hours in 0.1% (w/v) adult and paediatric CF whole sputum supplemented with M9. Data are represented as the mean log growth (log CFU/ml) at the different time points in CF sputum which was not treated with DNase (blue line, 6 replicates: 3 adult, 3 paediatric), and CF sputum which was treated with 60 U/ml DNase (green line, 6 replicates: 3 adult, 3 paediatric). b) Mean growth yield (CFU/ml) after 24 hours of growth of PAO1 in whole CF sputum which was treated with 60 U/ml DNase (green bar) or was not treated with DNase (blue bar). Error bars show standard error of the mean (SEM). P value on the graph (**p < 0.0001) was obtained by a paired, two-sided student t-test.**

5.3.2. Analysis of RNA samples

5.3.2.1. Analysis of RNA integrity using Bioanalyzer 2100

Total RNA was extracted from PAO1 which was exposed to adult CF sputum which was not DNase treated (aCF 1/2/3, – ve), adult CF sputum which was treated with 60 U/ml DNase (aCF 1/2/3, DNase), paediatric CF sputum which was not DNase treated (pCF 1/2/3, – ve), and paediatric CF sputum which was treated with 60 U/ml DNase

(pCF 1/2/3, DNase). Following extraction, the freshly extracted RNA was assessed via NanoDrop and treated with TURBO DNase. RNA was then immediately assessed for sample integrity as determined by an RNA integrity number (RIN). This was performed using the Agilent RNA 6000 Nano kit with the Bioanalyzer 2100. As a control, RNA ladder was loaded into the designated well as described in section 5.2.8.3.

Figure 5.4a. demonstrates the successful run of the RNA ladder which was run in tangent with the samples. The run could be deemed successful for the following reasons. Firstly, the lower marker was identified at 25 nucleotides. Secondly, the baseline was observed to be flat and the RNA ladder did not appear degraded as indicated by the individual sharp peaks. Finally, as expected for larger ladder fragments found at 2000 and 4000 nucleotides, the peaks were found to be wider. Larger fragments are wider because it becomes increasingly difficult with an increase in ladder size to form individual peaks with greater intensity. For this reason, the signal intensity [FU] also decreases with an increase in ladder size (nucleotides) as shown in figure 5.4a.

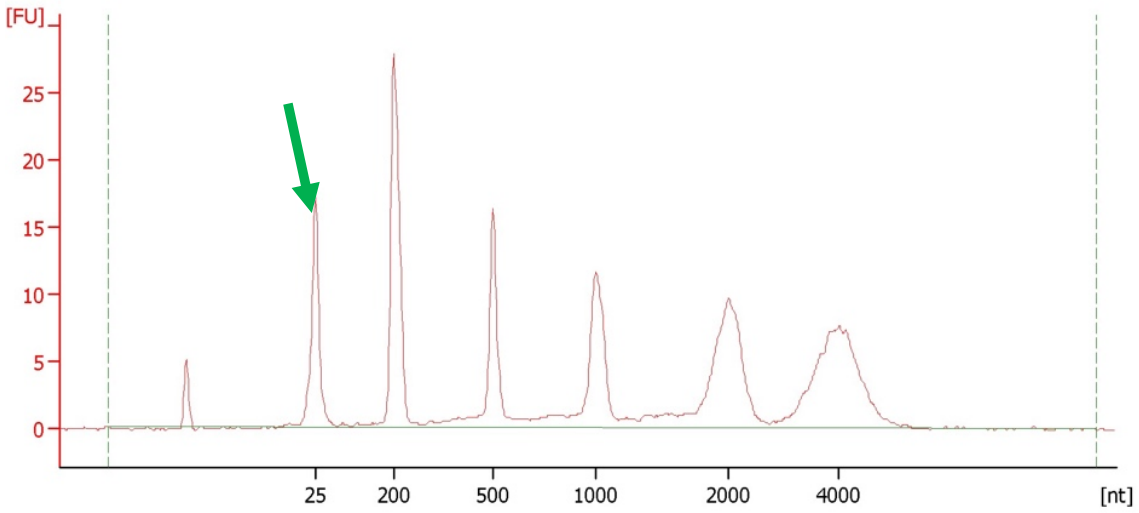

Figure 5.4a.

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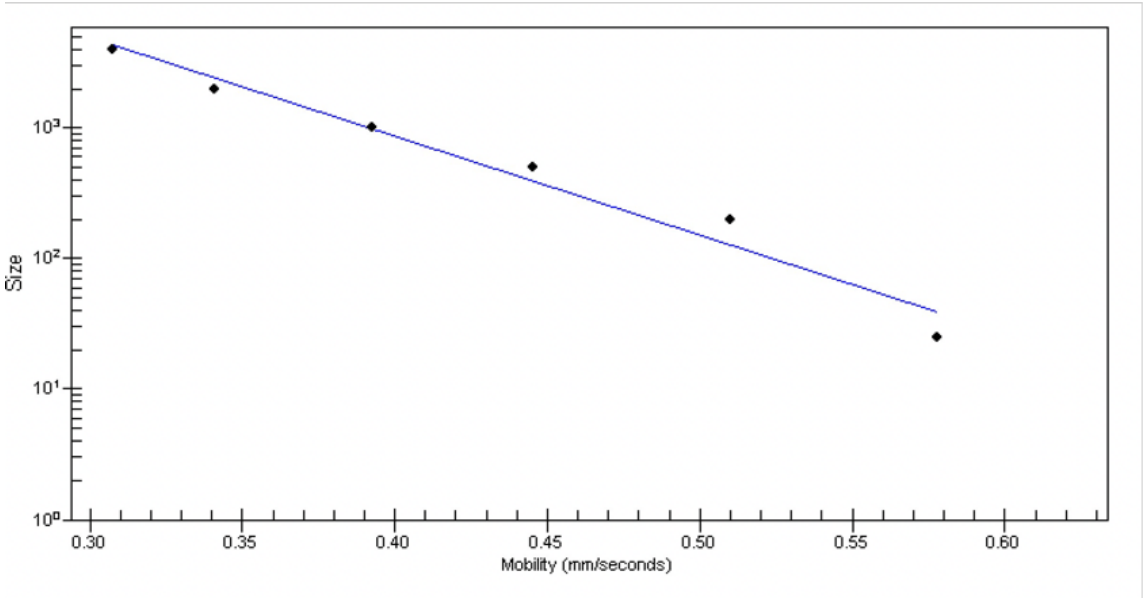

Figure 5.4b.

Figure 5.4: a) Electropherogram of the RNA ladder. Key: 'nt' = nucleotide, 'FU' = fluorescent signal intensity. A DNA fragment of 25 nucleotides was used as the lower marker in the RNA nano assay for alignment as indicated by the green arrow. b) Standard curve generated from the RNA ladder based on the size of the ladder peaks and migration time. This was then used to obtain the size of each sample peak based on the sample mobility.

Figures 5.5, 5.6, 5.7, and 5.8 represent the electropherograms and the individual gel images for each RNA sample. In all twelve electropherograms the 16S (pink arrows) and 23S peaks (green arrows) were clearly identified by the Bioanalyzer. However, additional peaks were observed at 200 nucleotides (blue arrows) and after the 23S RNA peak (red arrows) in all samples. Interestingly, the intensity of this second peak was increased in the DNase treated samples (figures 5.6 and 5.8). The gel images also display these findings. The presence of the extra band just above the 25-nucleotide green marker indicates the presence of small RNA fragments including tRNA and 5S RNA. However, the fluorescent intensity was larger than expected which could indicate degradation of the total RNA.

However, because an exaggerated jagged baseline between the two ribosomal peaks was not observed, re-extraction did not need to be considered. If this was observed, it would represent degradation of the 23S RNA. There would also be a jagged baseline to the left of the 16S RNA peak which would represent degradation of the 16S RNA. Thus, the

RNA integrity number (RIN) generated by the Bioanalyzer 2100 would be reduced. However, the RNA extracted from PAO1 grown in these samples had a mean RIN score of 8.4, with the highest value being 8.9 and the lowest 7.9. This lower value was still considered to be very good for downstream use in qPCR. A review by Fleige and Pfaffl (2006), suggests that a RIN score over 5 is acceptable for use in qPCR whereas a score over 8 is considered ‘perfect’.

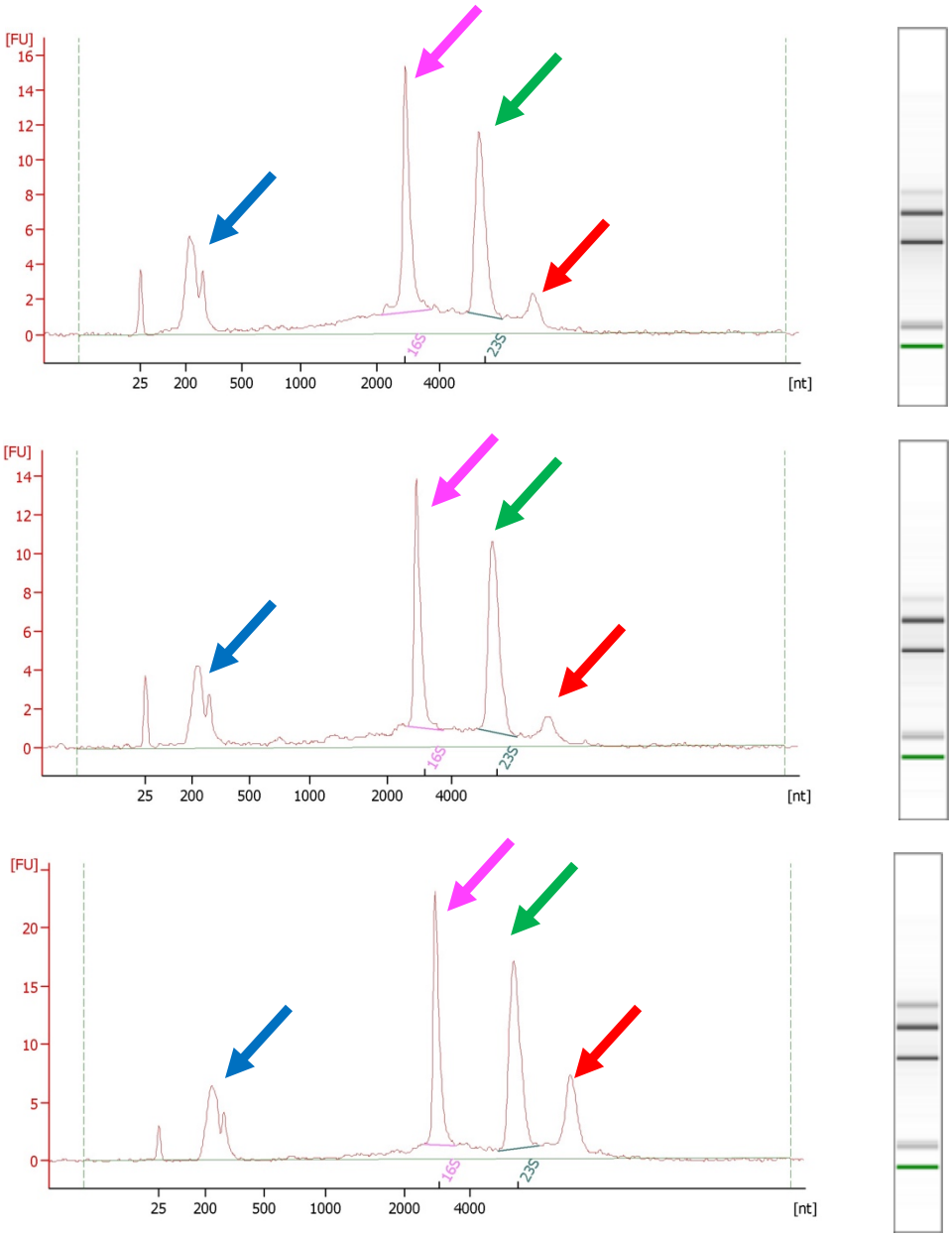

Figure 5.5.

Figure 5.5: Electropherograms of the total RNA extracted from PAO1 following exposure to three adult CF patient sputum samples which were not treated with DNase. The 23S RNA is highlighted on each graph in green font (and green arrows) whilst the 16S RNA is highlighted in pink font (and pink arrows). Blue arrows indicate small RNA fragments. Red arrows indicate additional peak. The figure also shows the nanochip gel images for each sample. Bands indicate RNA in sample. The green band is representative of the 25-nucleotide [nt] marker.

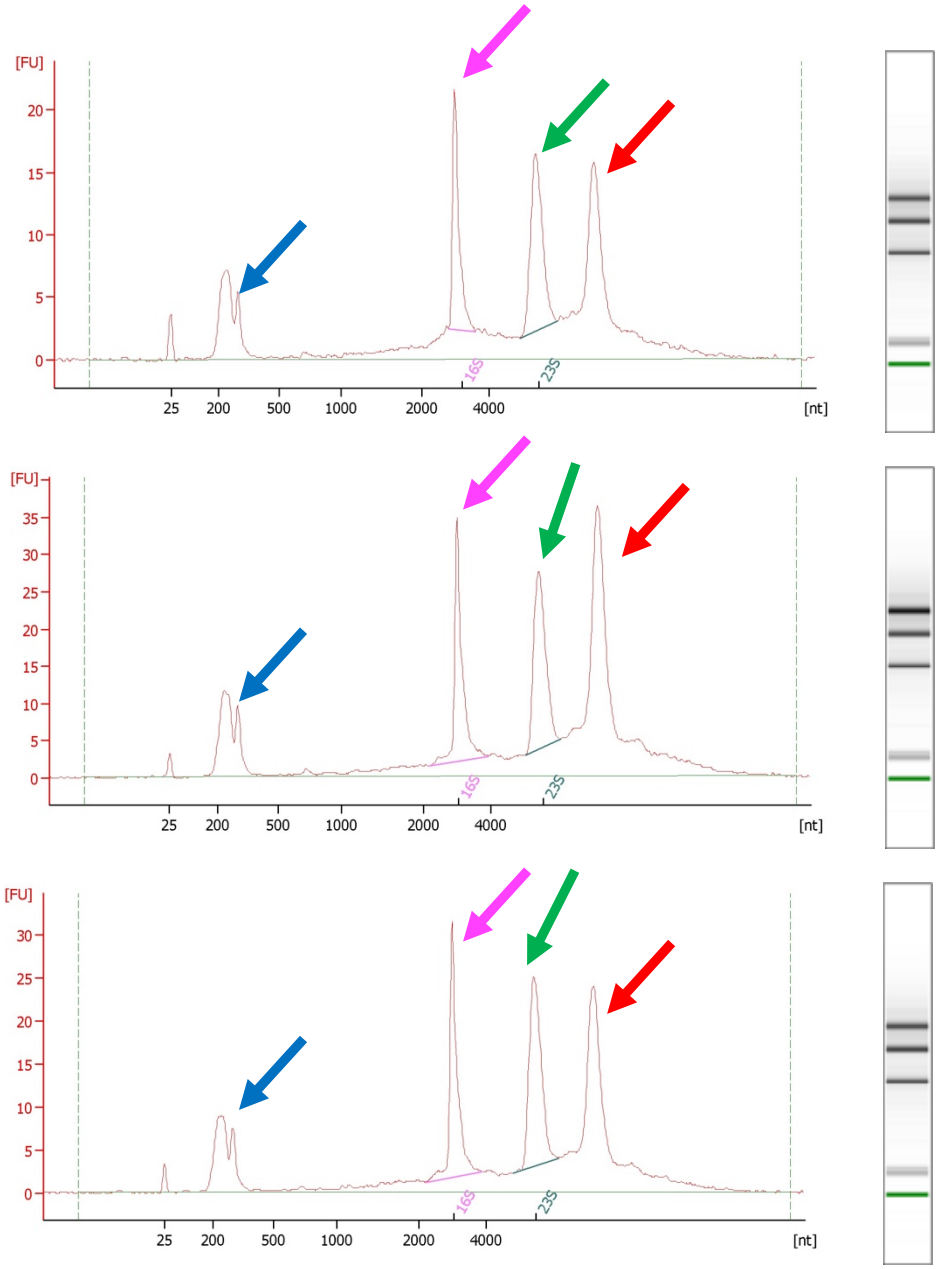

Figure 5.6.

Figure 5.6: Electropherograms of the total RNA extracted from PAO1 following exposure to three adult CF patient sputum samples which were treated with 60 U/ml DNase. The 23S RNA is highlighted on each graph in green font (and green arrows) whilst the 16S RNA is highlighted in pink font (and pink arrows). Blue arrows indicate small RNA fragments. Red arrows indicate additional peak. The figure shows the nanochip gel images for each sample. Bands indicate RNA in sample. The green band is representative of the 25-nucleotide [nt] marker.

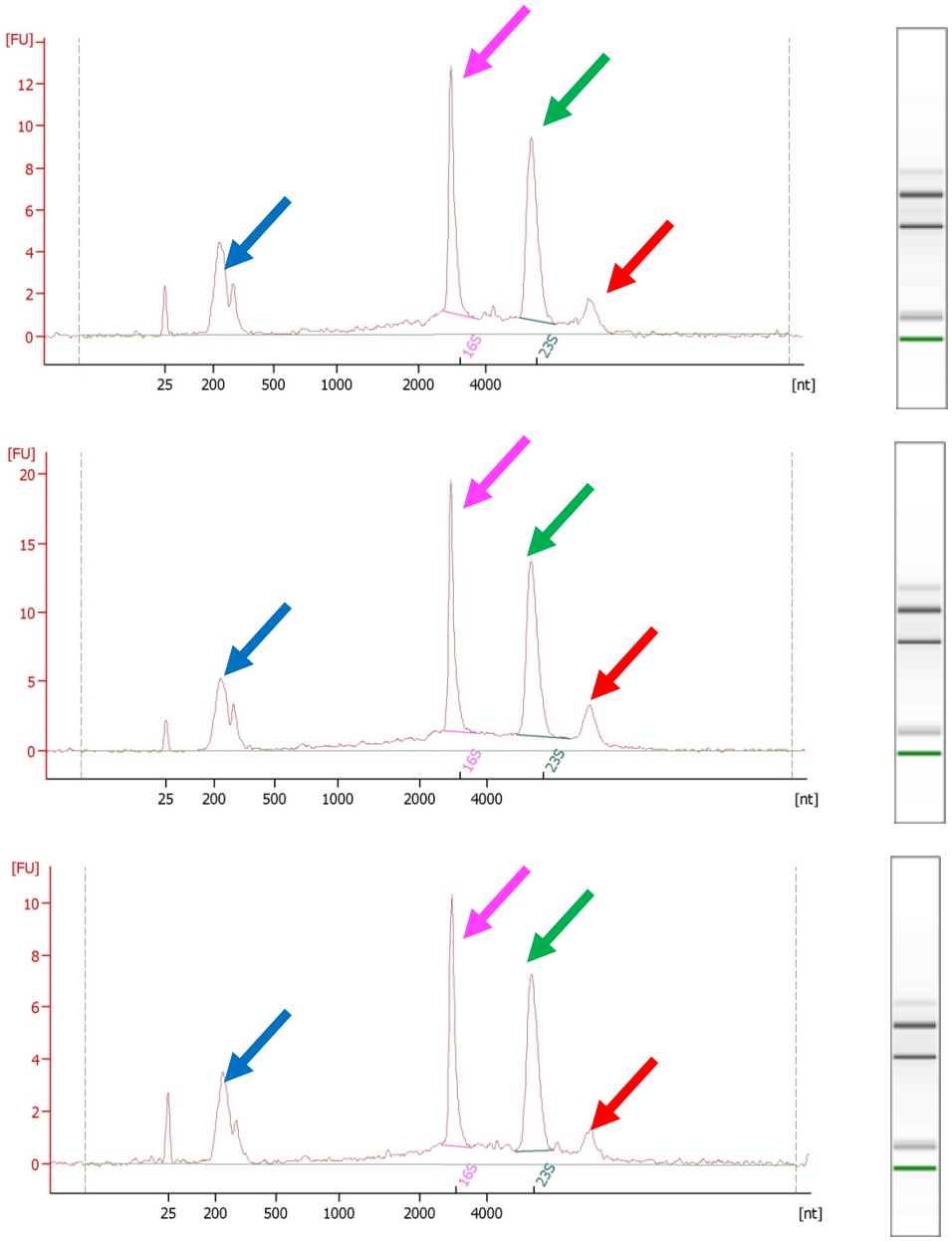

Figure 5.7.

Figure 5.7: Electropherograms of the total RNA extracted from PAO1 following exposure to three paediatric CF patient sputum samples which were not treated with DNase. The 23S RNA is highlighted on each graph in green font (and green arrows) whilst the 16S RNA is highlighted in pink font (and pink arrows). Blue arrows indicate small RNA fragments. Red arrows indicate additional peak. The figure shows the nanochip gel images for each sample. Bands indicate RNA in sample. The green band is representative of the 25-nucleotide [nt] marker.

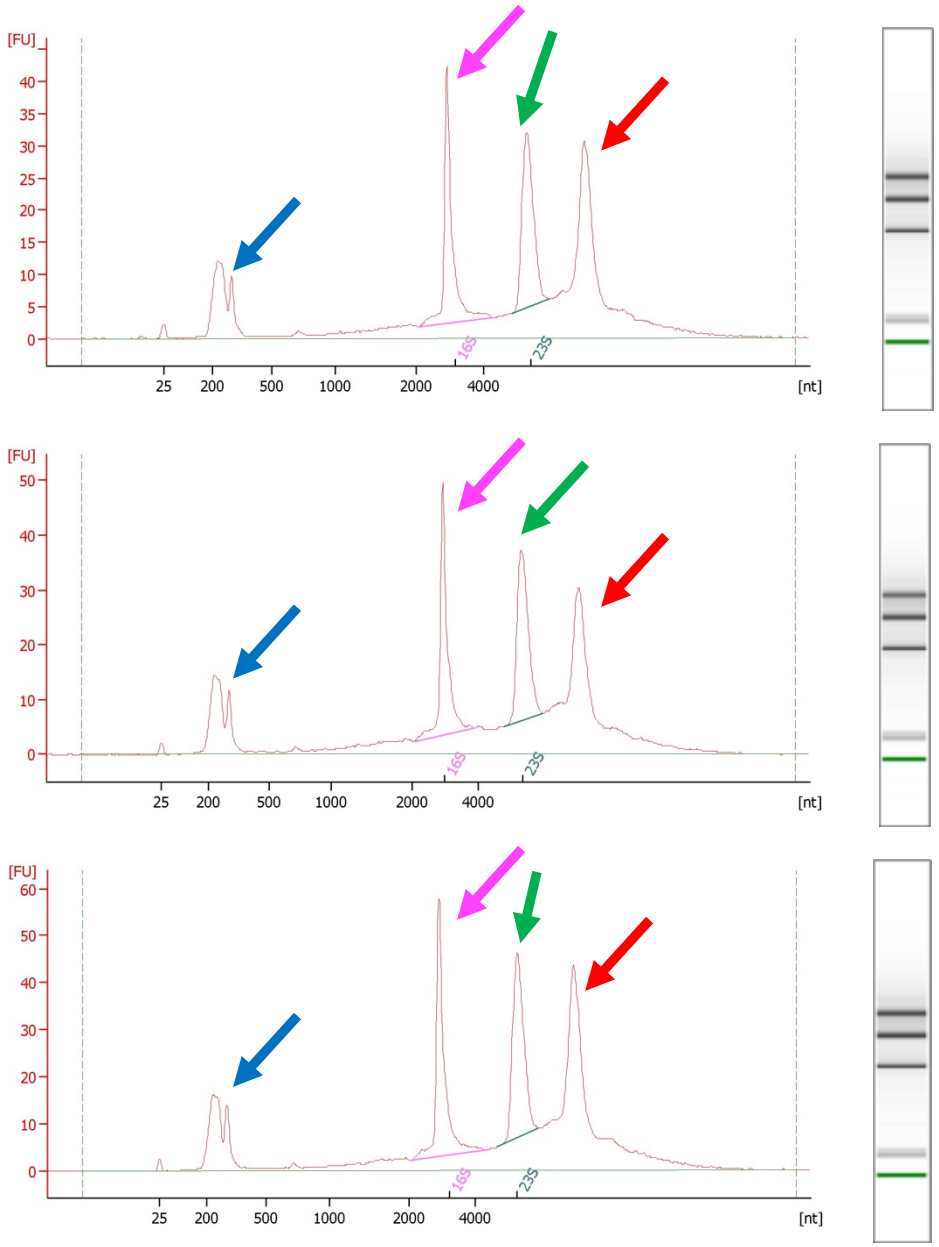

Figure 5.8.

Figure 5.8: Electropherograms of the total RNA extracted from PAO1 following exposure to three paediatric CF patient sputum samples which were treated with 60 U/ml DNase. The 23S RNA is highlighted on each graph in green font (and green arrows) whilst the 16S RNA is highlighted in pink font (and pink arrows). Blue arrows indicate small RNA fragments. Red arrows indicate additional peak. The figure shows the nanochip gel images for each sample. Bands indicate RNA in sample. The green band is representative of the 25-nucleotide [nt] marker.

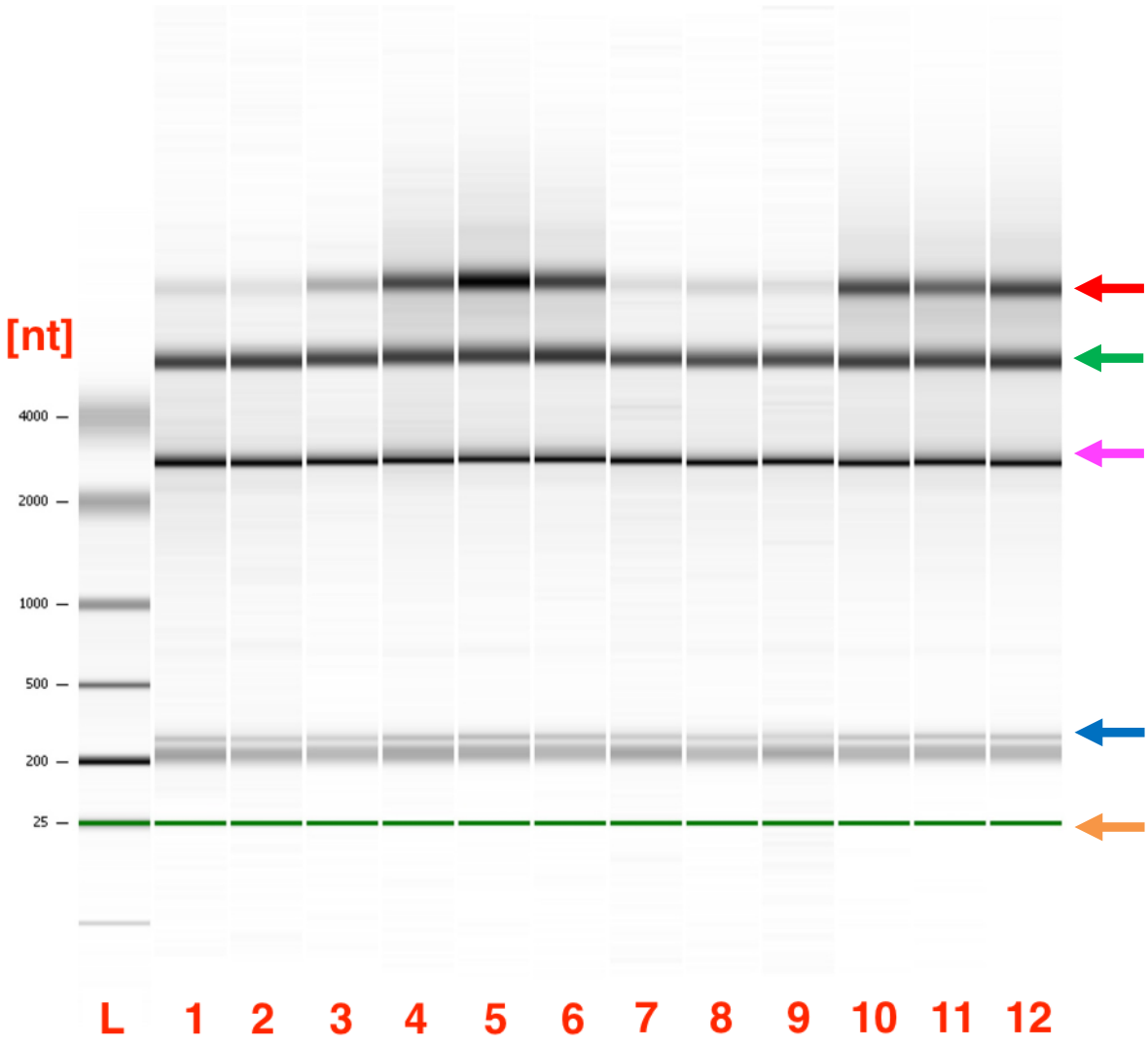

Figure 5.9.

Figure 5.9: Nanochip image shows the RNA ladder in lane L, and RNA extracted from PAO1 which was exposed to CF patient sputum for 3 hours which was either untreated or treated with 60 U/ml DNase. Key: 'a' = adult samples, 'p' = paediatric sample, number indicates different patient samples, '-ve' = samples was not treated with DNase, 'DNase' = samples treated with 60 U/ml DNase. Lane 1 = a1 –ve, lane 2 = a2 –ve, lane 3 = a3 –ve, lane 4 = a1 DNase, lane 5 = a2 DNase, lane 6 = a3 DNase, lane 7 = p1 –ve, lane 8 = p2 –ve, lane 9 = p3 –ve, lane 10 = p1 DNase, lane 11 = p2 DNase, and lane 12 = p3 DNase. Green arrow indicates location of 23S RNA, pink arrow indicates location of 16S RNA, red arrow indicates location of unknown band thought to be RNA which has aggregated prior to sample loading, orange arrow indicates the 25 nucleotide [nt] marker, and blue arrow indicates location of small RNAs.

Figure 5.9 shows the nanogel image for all RNA samples. As previously discussed, an extra peak to the right of the 23S RNA was observed in all electropherogram images. Due to the shape of the peaks it was unlikely that this was due to gDNA. This is because when gDNA is present it usually distorts the 23S RNA peak and this was not evident in the electropherogram images. Additionally, if heavily contaminated then this would be evidenced as a wide peak to the right of the 23S RNA peak. In all instances, the peak to the right appears to be sharp with varying intensities. Finally, prior to analysis on the Bioanalyzer 2100, all samples following NanoDrop One analysis were rigorously treated with TURBO DNase as described in section 5.2.8.2.

5.3.2.2. Analysis of RNA via agarose gel electrophoresis

Aliquots of all samples were then diluted based on the RNA concentration measured for each sample following Bioanalyzer 2100 analysis. These aliquots were diluted to contain approximately 20 ng for agarose gel electrophoresis whilst the main samples remained undiluted and on ice until required for cDNA synthesis. For added reassurance, because the peak to the right of the 23S RNA can sometimes occur due to secondary RNA structures, according to instructions from the Agilent troubleshooting guide, samples were subsequently heat denatured to 72^{o}C for 2 minutes and immediately placed on ice. Denaturation for longer than 2 minutes can cause RNA degradation. These samples were then run on a non-denaturing agarose gel using 1 kbp DNA ladder either side of the samples. Please appreciate that the DNA ladder was used not as a marker for the RNA, but to ensure that the gel had run successfully.

Figure 5.10 shows the ethidium bromide stained RNA agarose gel electrophoresis image. The gel clearly shows that following heat denaturation of the samples that the additional band above the 23S RNA band was no longer present and therefore demonstrates that there was no contaminating gDNA in these samples.

The figure also shows that the RNA extracted from paediatric CF sputum sample 1 which was not treated with DNase (lane 5) was less intense on the agarose gel compared to all the other sample bands. Analysis via NanoDrop of remaining sample from these dilutions revealed that this sample was over diluted to approximately 15 ng/µl whilst the concentration of RNA in the other samples ranged between 18 – 22 ng/µl. However,

although not uniform in intensity, the gel image served to demonstrate the distinct bands of the total RNA. It also demonstrated little smearing between the bands, and finally that no gDNA was present in the samples following rigorous TURBO DNase treatment.

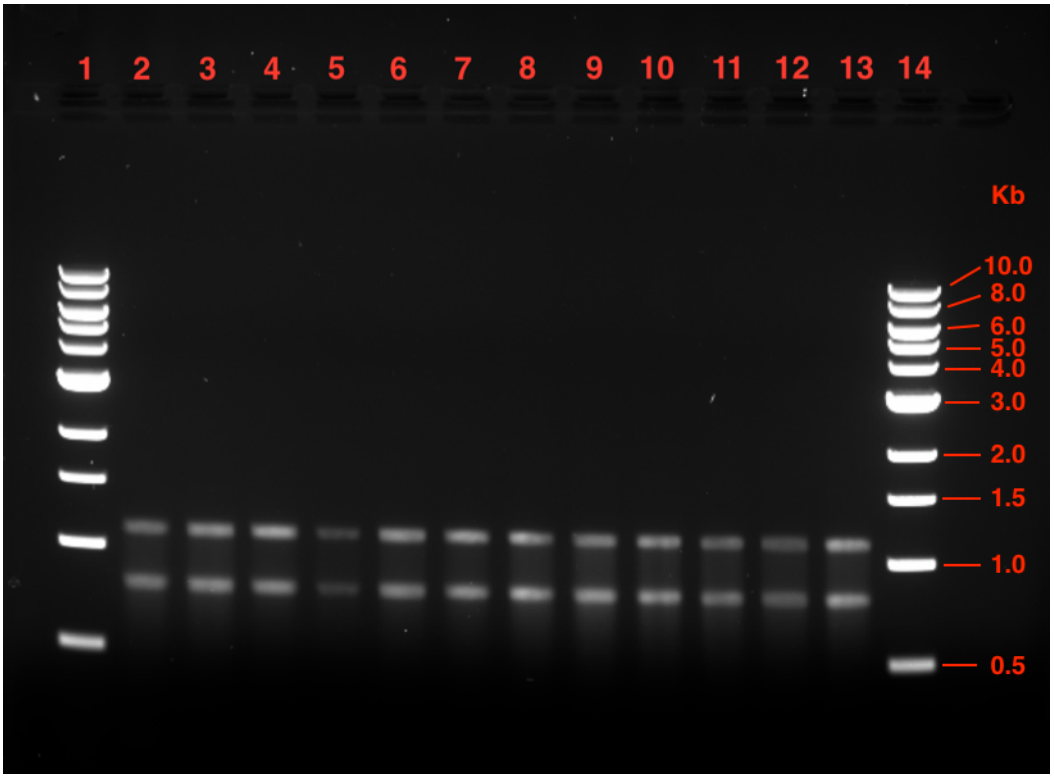

Figure 5.10.

Figure 5.10: Agarose gel electrophoresis image of RNA stained with ethidium bromide following extraction. Lanes 1 and 14 = 1 kbp DNA ladder, Lane 2 = untreated adult CF sputum 1, Lane 3 = untreated adult CF sputum 2, Lane 4 = untreated adult CF sputum 3, lane 5 = untreated paediatric CF sputum 1, lane 6 = untreated paediatric CF sputum 2, lane 7 = untreated paediatric CF sputum 3, lane 8 = DNase treated adult CF sputum 1, lane 9 = DNase treated adult CF sputum 2, lane 10 = DNase treated adult CF sputum 3, lane 11 = DNase treated paediatric CF sputum 1, lane 12 = DNase treated paediatric CF sputum 2, and lane 13 = DNase treated paediatric CF sputum 3.

5.3.2.3. Analysis of RNA via NanoDrop One

As a final measure prior to RT-PCR for the synthesis of first-strand cDNA, RNA was assessed using NanoDrop One. Using the concentrations measured by NanoDrop the samples were then diluted to 30 ng/µl so that when used as a template for reverse transcription, the total amount of RNA in the 5 µl added to the reaction tube would be 150 ng (sections 5.2.8.4 and 5.2.9).

5.3.3. Optimisation

5.3.3.1. Assessment of amplicon product using conventional PCR

Once the first-strand cDNA template for qPCR had been synthesised in RT-PCR, the gDNA was used as a template for end-point PCR, otherwise known as conventional PCR. This was done to check that the correct product size was amplified following use of the designed primers.

As presented in figure 5.11, the agarose gel electrophoresis images show bands between 100 and 200 base pairs, apart from bands shown in lanes 5-9 in figure 5.11d. These primers were taken from another study (Gi et al, 2015).

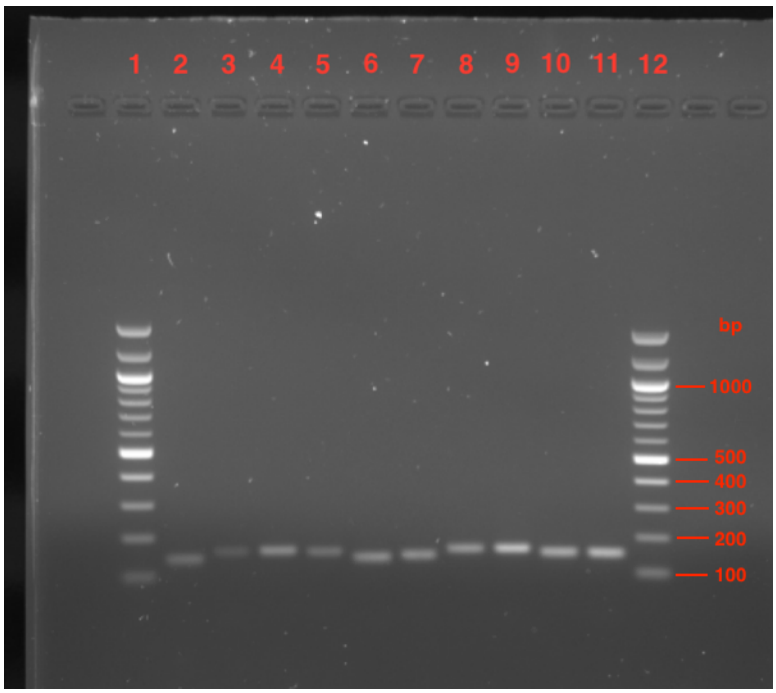

Figure 5.11a.

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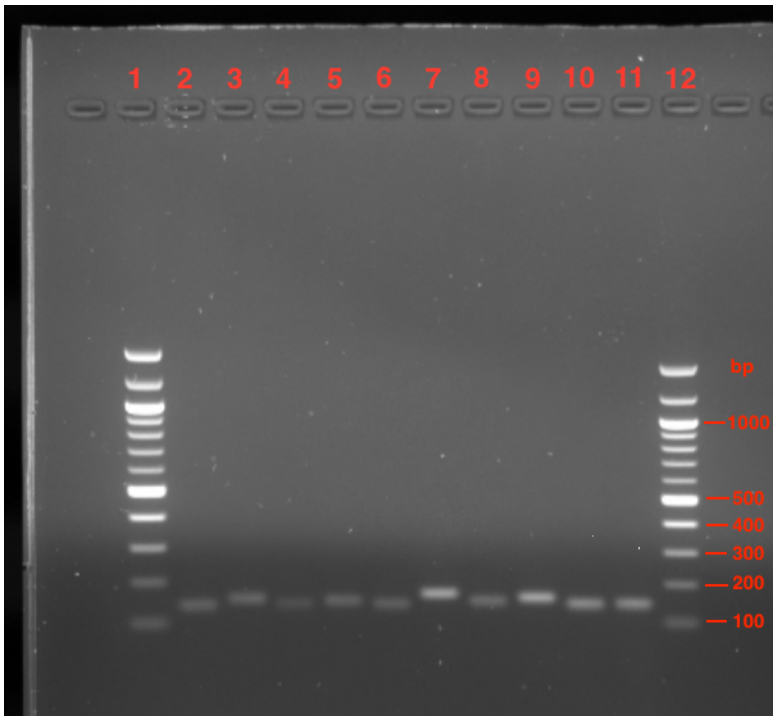

Figure 5.11b.

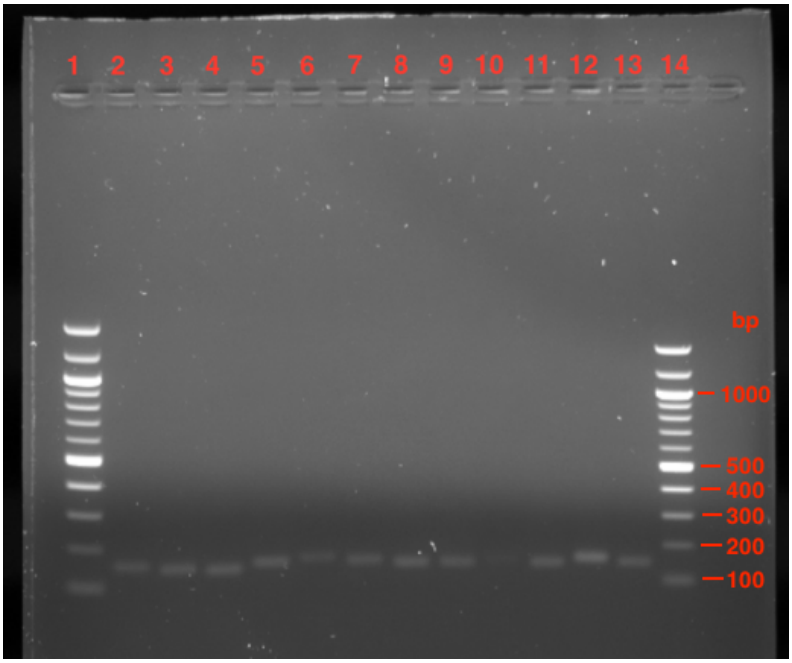

Figure 5.11c.

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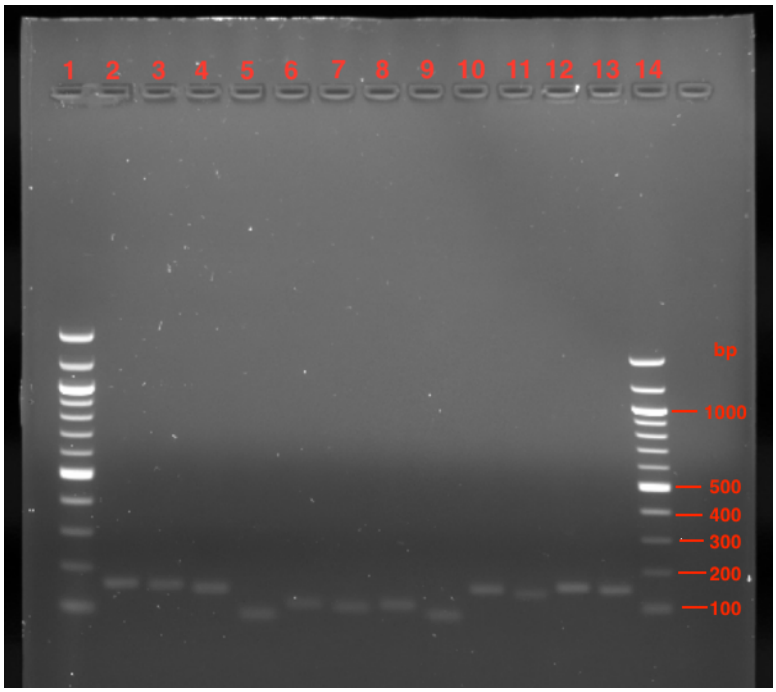

Figure 5.11d.

Figure 5.11: Agarose gel electrophoresis images of end-point PCR product using the designed primers for investigating metabolism in *Pseudomonas aeruginosa* PAO1. Image a), lane 1 = 100 bp DNA ladder, lane 2 = PA5493, lane 3 = PA1124, lane 4 = PA3807, lane 5 = PA0134, lane 6 = PA0143, lane 7 = PA0935, lane 8 = PA1518, lane 9 = PA4868, lane 10 = PA5173, lane 11 = PA1514, lane 12 = 100 bp DNA ladder. Image b), lane 1 = 100 bp DNA ladder, lane 2 = PA0148, lane 3 = PA1523, lane 4 = PA3970, lane 5 = PA3625, lane 6 = PA1155, lane 7 = PA2962, lane 8 = PA0444, lane 9 = PA0437, lane 10 = PA3163, lane 11 = PA0441, lane 12 = 100 bp DNA ladder. Image c), lane 1 = 100 bp DNA ladder, lane 2 = PA0782, lane 3 = PA0865, lane 4 = PA0872, lane 5 = PA2009, lane 6 = PA2249, lane 7 = PA2250, lane 8 = PA5304, lane 9 = PA2322, lane 10 = PA2323, lane 11 = PA3181, lane 12 = PA3186, lane 13 = PA3195, lane 14 = 100 bp DNA ladder. Image d), lane 1 = 100 bp DNA ladder, lane 2 = PA2794, lane 3 = PA3761, lane 4 = PA1432, lane 5 = PA4834, lane 6 = PA4835, lane 7 = PA4836, lane 8 = PA4837, lane 9 = PA0576, lane 10 = PA0668.1, lane 11 = PA2966, lane 12 = PA2232, lane 13 = PA2023, and lane 14 = 100 bp DNA ladder.

5.3.3.2. Standard curve

Once it had been determined that the correct sized product was formed for each set of primers, gDNA which had been extracted from *P. aeruginosa* PAO1 was used to determine the efficiency of the primers. This was done by performing five 10-fold dilutions so that the final concentrations of gDNA template were 20, 2, 0.2, 0.02, 0.002, and 0.0002 ng/µl. Therefore, the final concentrations in each reaction tube were 40, 4, 0.4, 0.04, 0.004, and 0.0004 ng, respectively, because 2 µl of template was added to

each tube. A NTC, composed of RNase/DNase-free dH_2O, was also included in each run, though this was not included in the standard curve generated by the Rotor-Gene software following completion. For each primer pair, the reaction was performed in duplicate.

Initially during optimisation, because it was planned to use the Livak method for analysing gene expression, it was necessary for all primers to have efficiencies ranging between 90 and 100% (Livak and Schmittgen, 2001). Generally, efficiencies above 100% are indicative of primer-dimer formation. This is because instead of the primers binding to the template, they bind to each other and are subsequently amplified rather than the intended gene of interest. The reason why it was not desirable for reaction efficiency to be below 90% is because the expression of the gene of interest is relative to a stably expressed HKG. For example, if gene 'x' has a reaction efficiency of 60% and gene 'y' has an efficiency of 90%, and these are then compared to expression of the HKG with a reaction efficiency of 95%, it could appear like gene 'x' has reduced expression compared to 'y' when it is reduced because of a reduced reaction efficiency. Figure 5.12a. shows an example of a standard curve for a primer pair which was accepted. The reason for this is because the efficiency was 94% and the r^2 value was greater than 0.99.

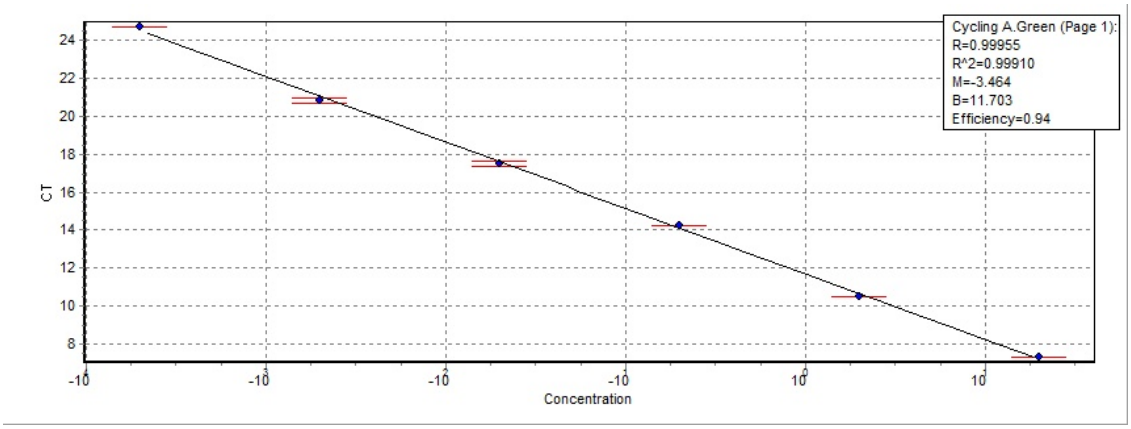

Figure 5.12a.

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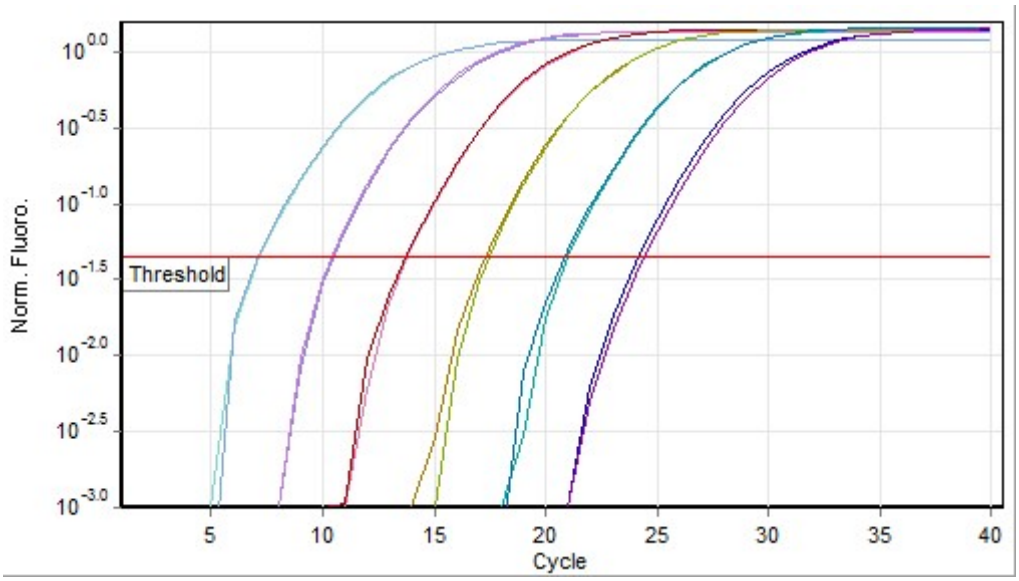

Figure 5.12b.

Figure 5.12: a) standard curve example generated using PA5493 primers. b) quantitation data for each cycle using primers PA5493.

Primers were accepted for further use if their reaction efficiencies were over 90%. However, when it became apparent that there was a problem with amplification in the NTC, primer pairs with lower efficiency were also accepted providing the melt-point analysis checks were satisfied. This was not only due to time and financial constraints at the end of the project, but also following necessary optimisations to reduce amplification in the NTC, the reaction efficiency was unable to be improved further with the designed primers. Thus, the Livak method was no longer suitable and instead the Pfaffl method needed to be employed for analysis of relative gene expression. This was because the latter method considers differences in reaction efficiencies (Pfaffl, 2001; Pfaffl, 2004).

5.3.3.3. Melt analysis

Following completion of each run, melt-point analysis was also performed. This was done by increasing the temperature of the reaction tube by 1^{o}C increments every 5 seconds. Because SYBR dye intercalates with double-stranded DNA, it is for this reason that an increase in fluorescence is measured during qPCR. This dye then remains bound to DNA until DNA becomes denatured due to an increase in heat, which releases the

dye, and thus causes a reduction in fluorescence. The change in fluorescence over time (dF/dT) can then be plotted against the temperature. When there is dissociation of an amplicon product at a specific temperature due to denaturation of the double-stranded DNA, this results in a peak in the melt analysis. Multiple peaks are therefore indicative of non-specific products, although occasionally a double peak can form because more stable regions of the DNA, such as G-C rich regions, require higher temperatures to fully dissociate whilst the rest of the amplicon product may have dissociated at a lower temperature. Primer pairs which demonstrated multiple peaks were not used further. Additionally, primers which had the formation of primer-dimers in the NTC as indicated by melt analysis were only used if the melting point of the non-specific product was different to that of the amplicon product.

In the initial stages of optimisation, primer-dimers formed frequently in the NTC. Initially the problem with the formation of non-specific products occurred between 24 and 30 cycles. This explains why initially the reaction efficiencies for the primers were higher and could have been potentially misleading had melt analysis not been performed. Overall, it appeared that primer-dimers formed when no template was added to the tube, or alternatively when the concentration of template was reduced. In some instances, as indicated by melt analysis, there would be evidence of non-specific product with a lower melting point. With a reduction in template to the lowest concentration of gDNA at 0.0002 ng/µl, occasionally there was evidence of both amplicon product and non-specific products forming. These primers were only used if it was expected that the concentration of cDNA used in the final reaction exceeded this and therefore non-specific product would not be allowed to form.

To try and solve the problem of non-specific products, several attempts were made to eradicate this frequent observation. Steps were followed as suggested by the troubleshooting guide provided with the SensiMix™ SYBR Hi-ROX kit. Steps included using fresh sterile RNase/DNase-free dH_2O, new reagents, fresh tips, new pipettes (only used for PCR), new primers, and finally moving to a lab which had never been used for PCR. Once it was determined that the reason behind amplification in the NTC was not due to reaction set-up, reaction conditions for primers which were demonstrated to have only one peak following melt analysis were further optimised. Firstly, the concentration

of primer was reduced so that 1 µl of 10 µM forward and reverse primer stock was added to each tube. Occasionally excess primer can cause the formation of primer-dimers because more primer is available in the reaction. Secondly, the annealing temperature was adjusted in increments. Initially a temperature of 55^{o}C was used for the annealing temperature of the primers. This was extensively tested at multiple temperatures ranging from 54 to 57^{o}C. Eventually it was determined that the temperatures of 55.5^{o}C and 56 ^{o}C were optimum for the designed primers (data not shown). Next, both annealing and extension times were reduced to 15 seconds. Upon changing the annealing temperatures, it was noticed that for some primer pairs this reduced reaction efficiency, whilst for others it was improved. For this reason, these temperatures were used but all other conditions remained the same. Finally, following extension, an additional fluorescence acquisition step was added to the protocol at an increased temperature of 83^{o}C for all primers except for the HKG, PA0668.1, where acquisition was taken at 80^{o}C. This was added so acquisition could occur at a temperature above the melting point of non-specific products to eliminate errors due to the formation of primer-dimers.

Figure 5.13 shows the melt analysis curves for a selection of primers which were not optimised further. Primers were rejected if there was formation of non-specific products which had melting points at the same temperature as the amplicons (figure 5.13a. and 5.13f.). In figure 5.13a. the pink and blue lines represent the melt analysis for the NTC samples. The figure demonstrates a peak approximately at 73^{o}C and then again at approximately 91^{o}C. Interestingly, there was also a double peak for the sample containing gDNA at 0.0002 ng/µl as indicated by the yellow and red lines in the same figure. Notably, the two peaks occurred at the same temperatures as the NTC samples. If this second peak at 91^{o}C was not present in the NTC, then this primer could have been optimised further but only if a concentration of cDNA above 0.0002 ng/µl was used for the final reaction. Other reasons for primers not being used further were if broad peaks were observed as seen in figure 5.13e., or if there were additional peaks as seen in figures 5.13b., c., and d.

Figure 5.14 shows the melt analysis curves for a selection of primers which were optimised further. In all images the melt peak for the amplicon product (at the highest

melting point) is sharp and defined. The melt peaks of non-specific product due to primer-dimer formation are seen on the furthest left hand side of the images. These primer pairs were accepted for further optimisation because in the NTC, no peak was observed in the same region as the amplicon product.

However, as previously mentioned, when a lower concentration of template is provided, it is not unusual for primer-dimers to form as shown in figures 5.14c., d., f., g., and h. These primers were accepted for further optimisation because at higher concentrations of template these primer-dimers were absent as shown in the figures by the other coloured lines.

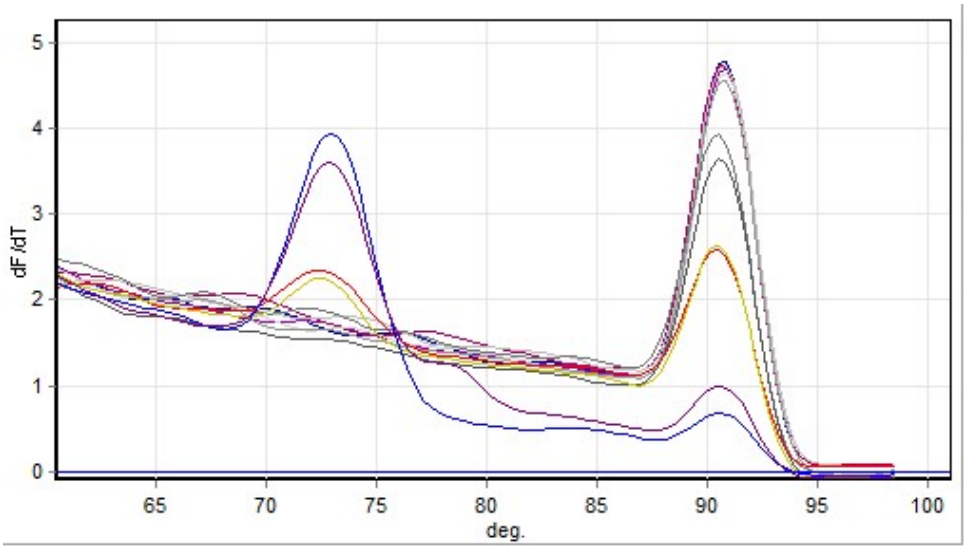

Figure 5.13a.

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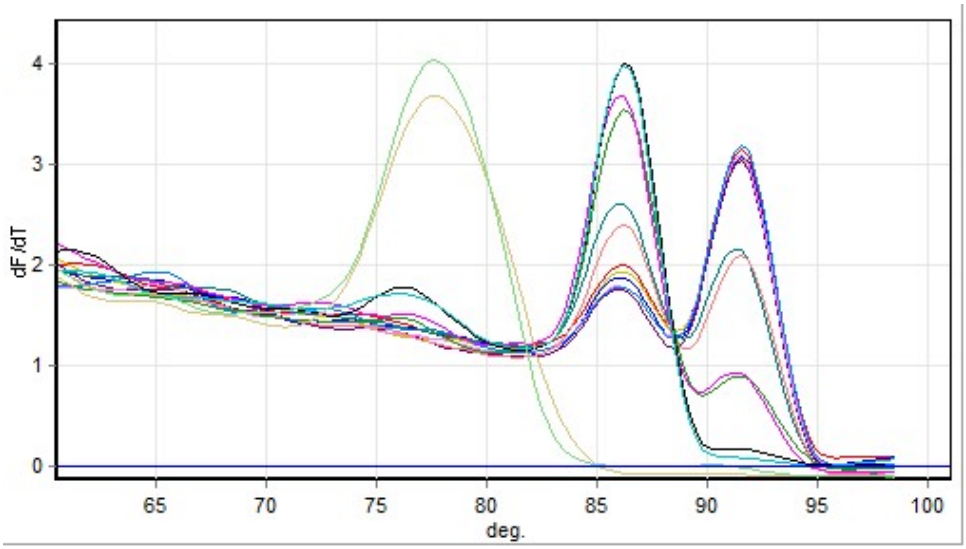

Figure 5.13b.

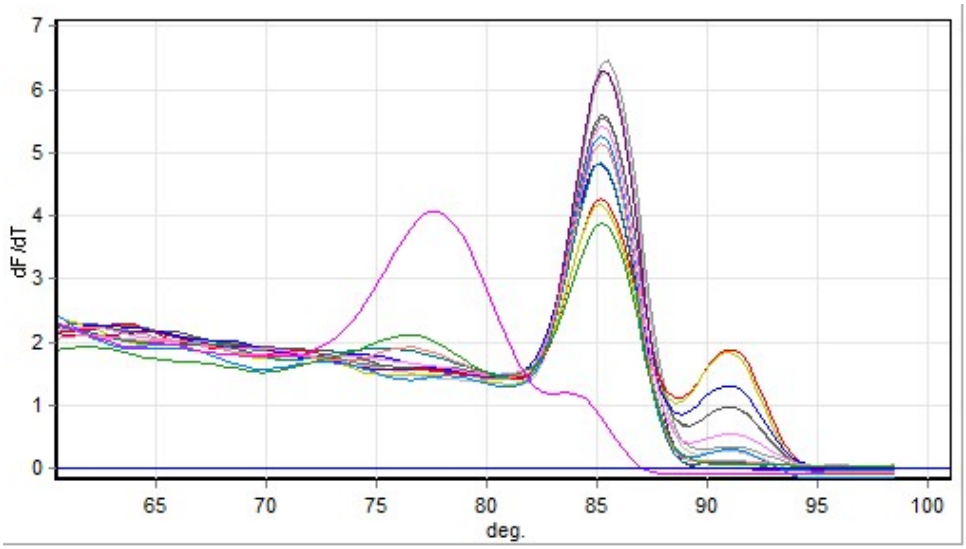

Figure 5.13c.

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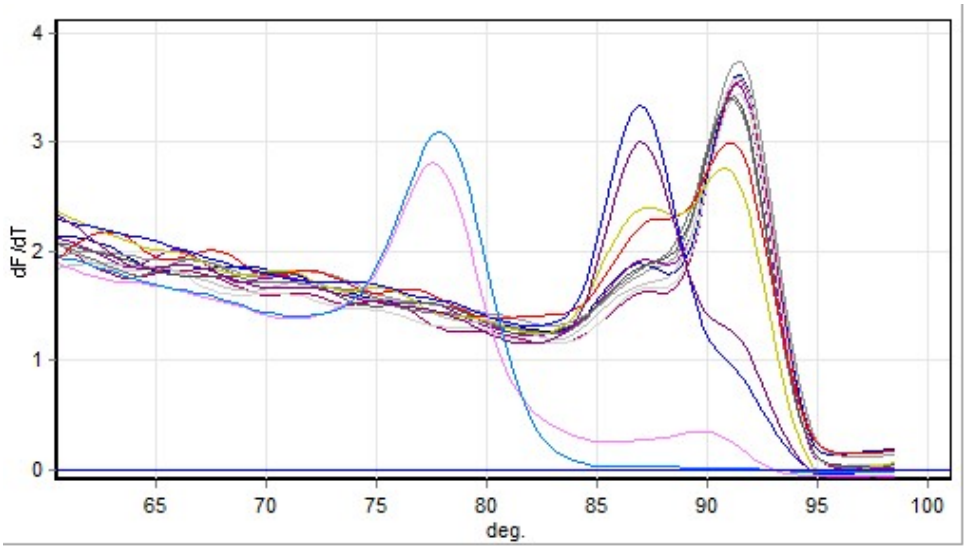

Figure 5.13d.

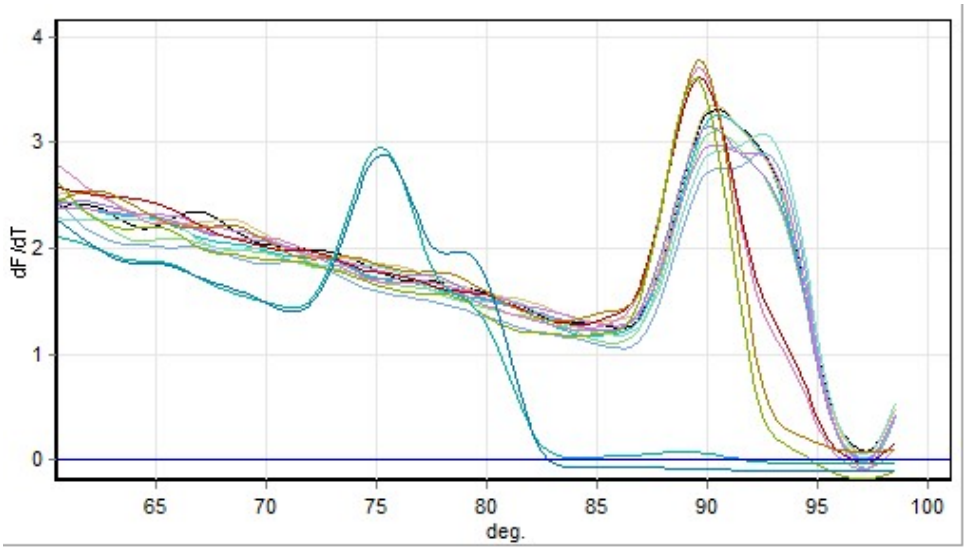

Figure 5.13e.

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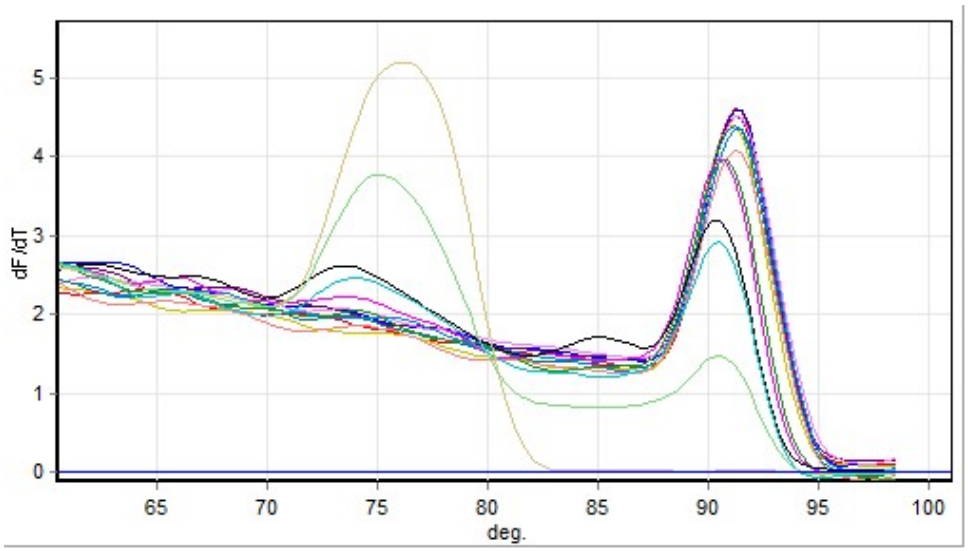

Figure 5.13f.

Figure 5.13: Melt curve analysis graphs show change in fluorescence over time (dF/dT) with an increase in temperature (^{o}C) following qPCR using primers: PA0134 (a), PA1155 (b), PA3163 (c), PA4868 (d), PA3181 (e), and PA2966 (f), which were not used for further optimisation. The different colour lines represent the melt analysis for different sample tubes which contained different starting concentrations ranging from 0.0002 to 20 ng/µl of genomic DNA. In all figures, the NTC samples are the peaks on the furthest left hand side of each graph.

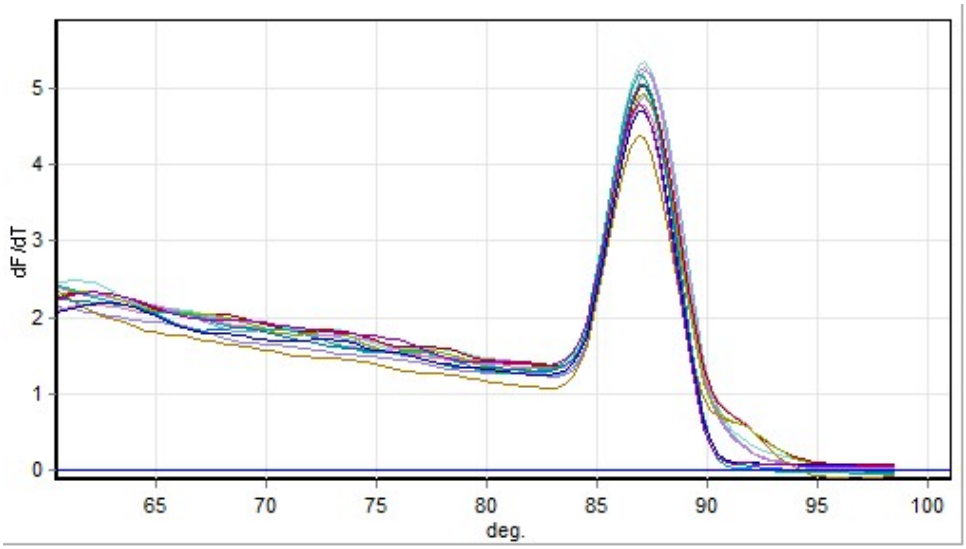

Figure 5.14a.

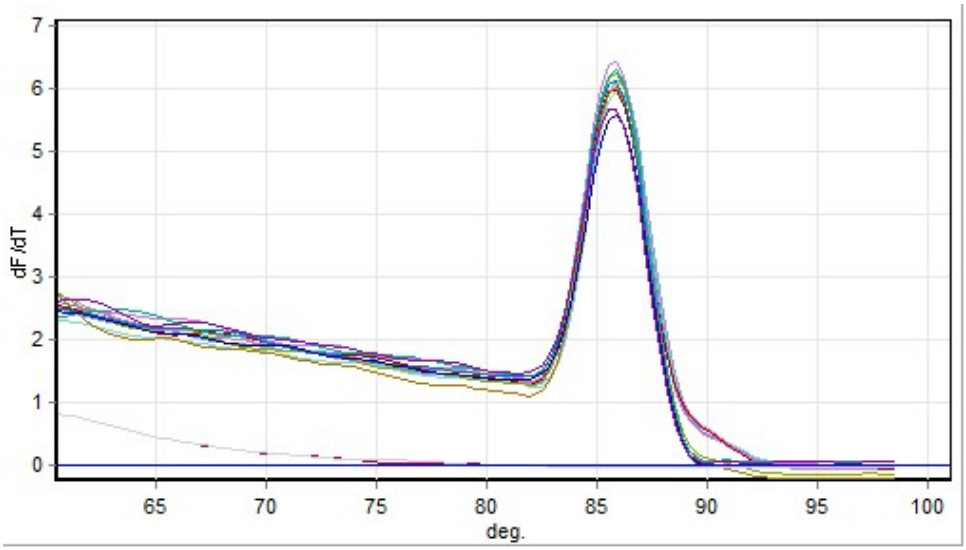

Figure 5.14b.

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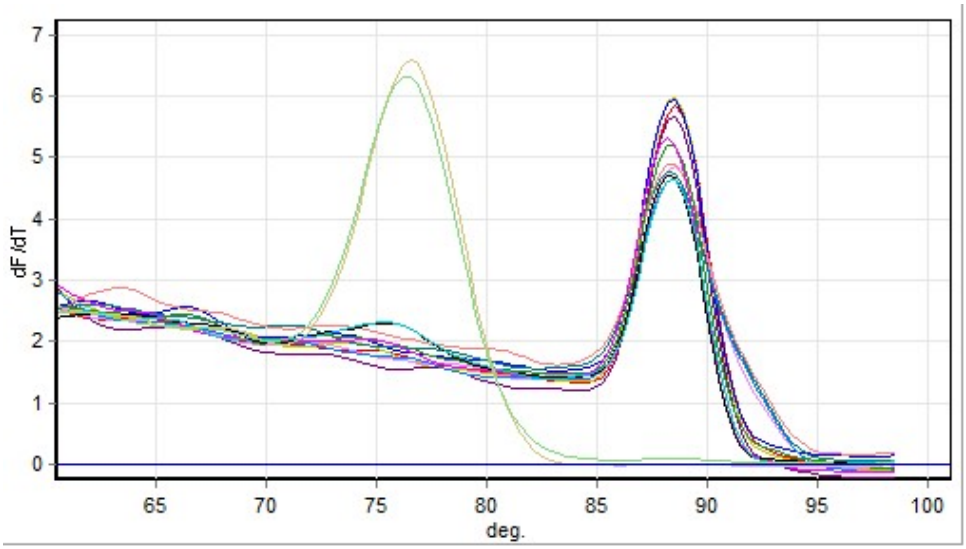

Figure 5.14c.

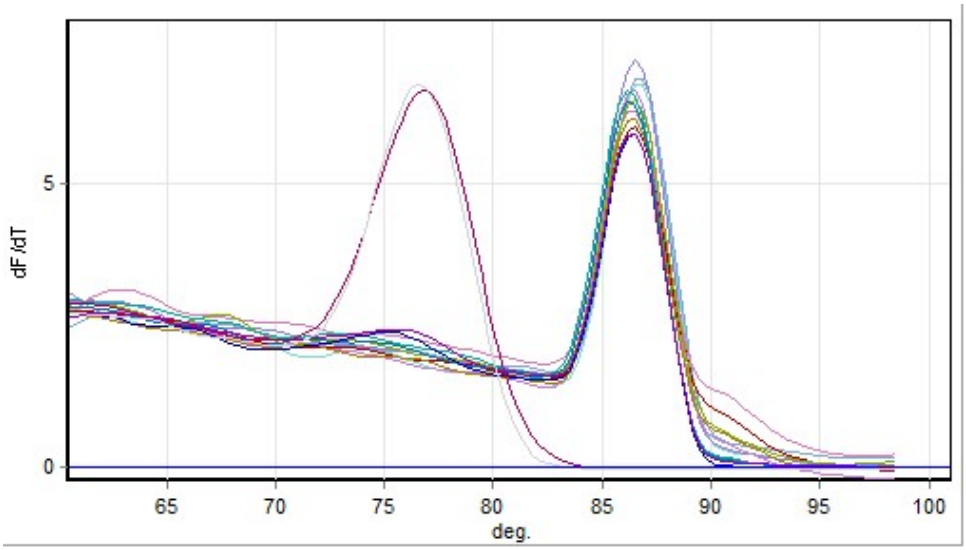

Figure 5.14d.

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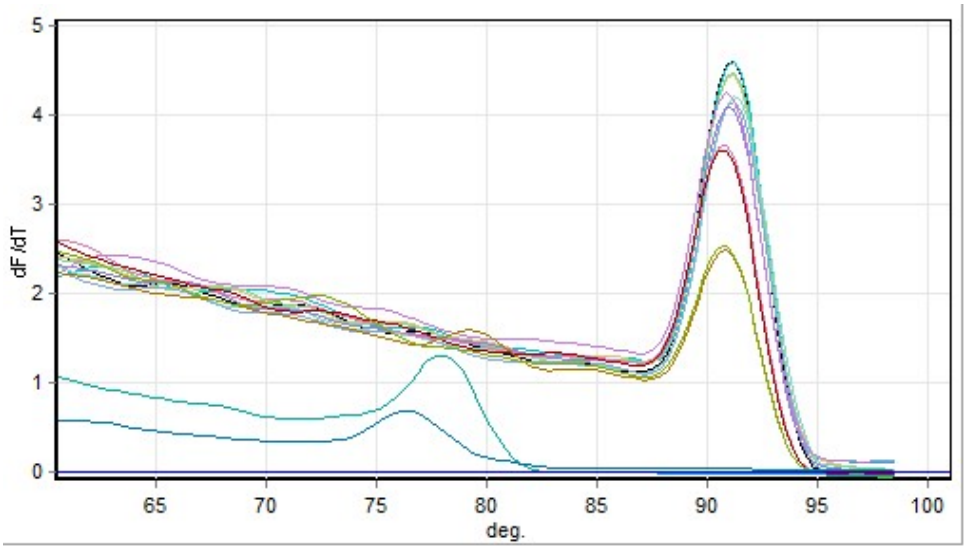

Figure 5.14e.

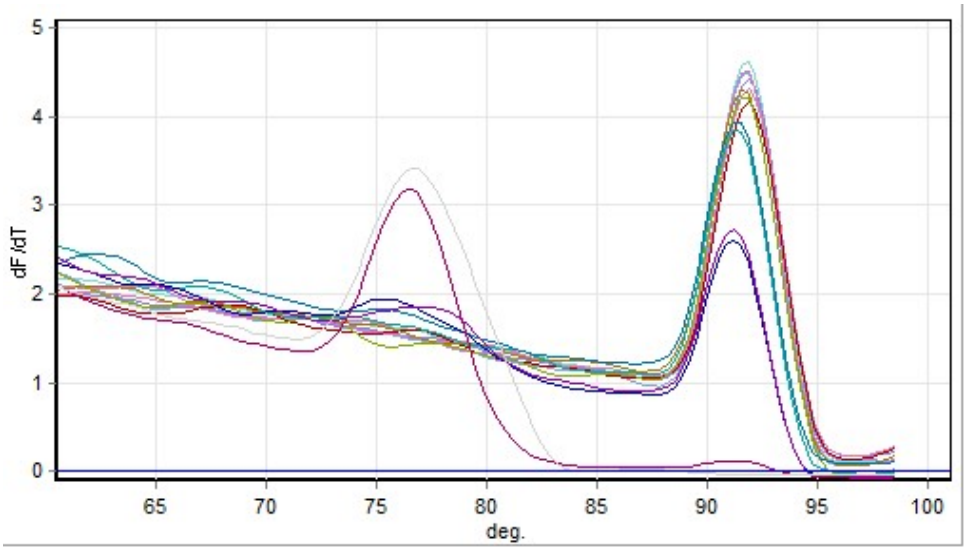

Figure 5.14f.

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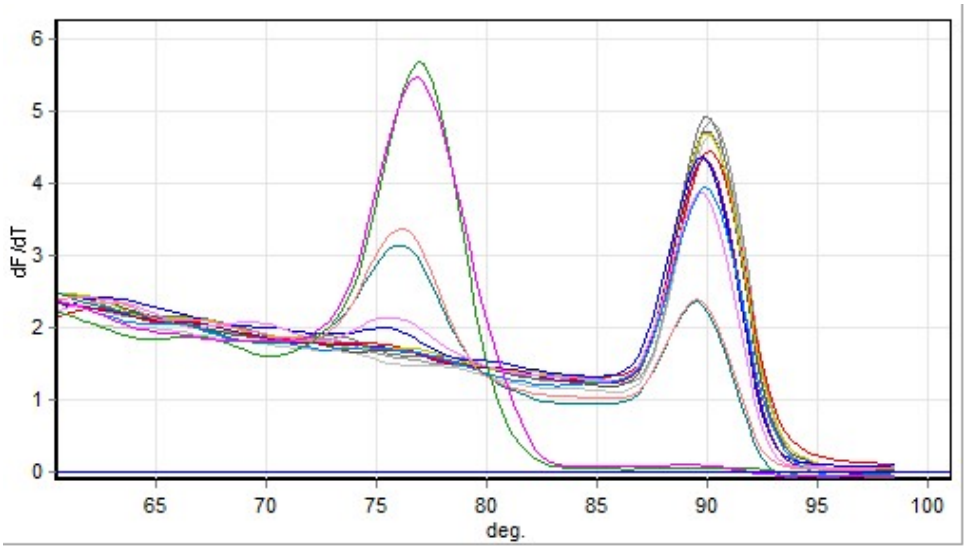

Figure 5.14g.

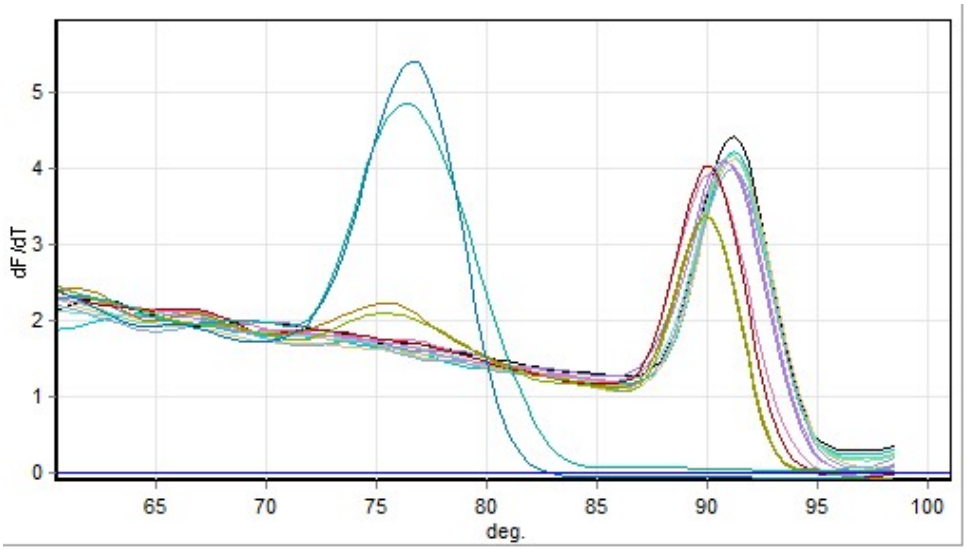

Figure 5.14h.

Figure 5.14: Melt curve analysis graphs show change in fluorescence over time (dF/dT) with an increase in temperature (^{o}C) following qPCR using primers: PA5493 (a), PA0935 (b), PA1514 (c), PA0148 (d), PA3970 (e), PA2962 (f), PA0444 (g), and PA0437 (h) which were used for further optimisation to minimise primer-dimer formation. The different colour lines represent the melt analysis for different sample tubes which contained different starting concentrations ranging from 0.0002 to 20 ng/µl of genomic DNA. In all figures, the NTC samples are the peaks on the furthest left hand side of each graph.

After these adjustments, amplification which previously occurred in the NTC, was overall eliminated. On the occasion in which amplification did occur, this was generally after 35 cycles, and the data for gene expression was only accepted providing there was greater than 10 cycles difference between the CT value for the amplicon product and the NTC samples. Even when amplification occurred after 35 cycles, melt analysis still revealed that primer-dimers were present in the NTC, but were absent in the samples with template. Following adjustments, reaction efficiencies for the primers which were optimised were determined again for each primer pair based on the adjusted qPCR conditions.

5.3.3.4. Selection of a suitable housekeeping gene (HKG)

To assess relative gene expression, a HKG which was stable in all conditions needed to be selected so that gene expression data could be normalised (Pfaffl, 2001). Often a HKG is selected without being checked as to whether it is stably expressed in all tissue types or conditions.

Three primers were initially designed to target the HKGs: *acpP* (*PA2966)*, *rpoD (PA0576),* and *16S rRNA (PA0668.1)*. Unfortunately, *acpP* formed primer-dimers and had low reaction efficiency. The designed primers for *rpoD* were used from a study by Gi and colleagues (2015). Although it was confirmed to be stably expressed in the conditions of this study as shown in in figure 5.15 ($p > 0.05$, one-way ANOVA), the problem with this primer pair was that when the amplicon product was checked via agarose gel electrophoresis, a faint smeared band was detected below the main band (figure 5.16a.). Notably, this main band wasn't distinct either. In every run, an RT control was also included. Therefore, it would be assumed that there would be no cDNA present in this sample since no reverse transcriptase was included in this control (section 5.2.9). Since SYBR dye only intercalates with DNA, it would not be expected to see amplification in these samples containing RNA. Figure 5.16b. demonstrates the faint band at the bottom of the gel in the RT control within the blue dotted line. Melt point analysis revealed the formation of primer-dimers in both the RT control samples, the NTC, and the samples containing cDNA implying that non-specific binding was occurring despite efforts to optimise the conditions. To check that there was not a

problem with the primers themselves, the primers were ordered again, but the same problem occurred. These primers were not considered to be suitable for this study. Instead, *16S rRNA* was used as the HKG. Repeatedly, *16s rRNA* was shown to remain stable in all conditions as shown in figure 5.17.

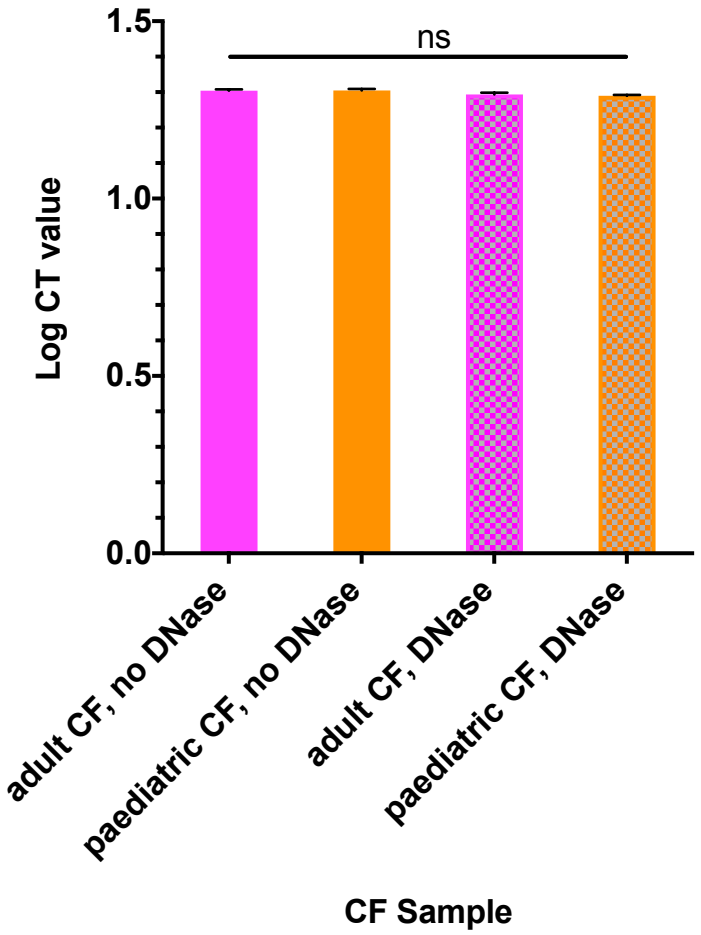

Figure 5.15.

Figure 5.15: Expression (log CT) of HKG *rpoD* (PA0576) in *Pseudomonas aeruginosa* PAO1 exposed to adult CF sputum which was not treated with DNase (pink bar), paediatric CF sputum which was not treated with DNase (orange bar), adult CF sputum treated with DNase (patterned pink bar), and paediatric CF sputum treated with DNase (patterned orange bar). Data represent the log CT value from 6 biological replicates which were treated as described. Error bars represent standard error of the mean (SEM). 'ns' means there is no significant difference as assessed by a one-way ANOVA followed by a Tukey's multiple comparisons test.

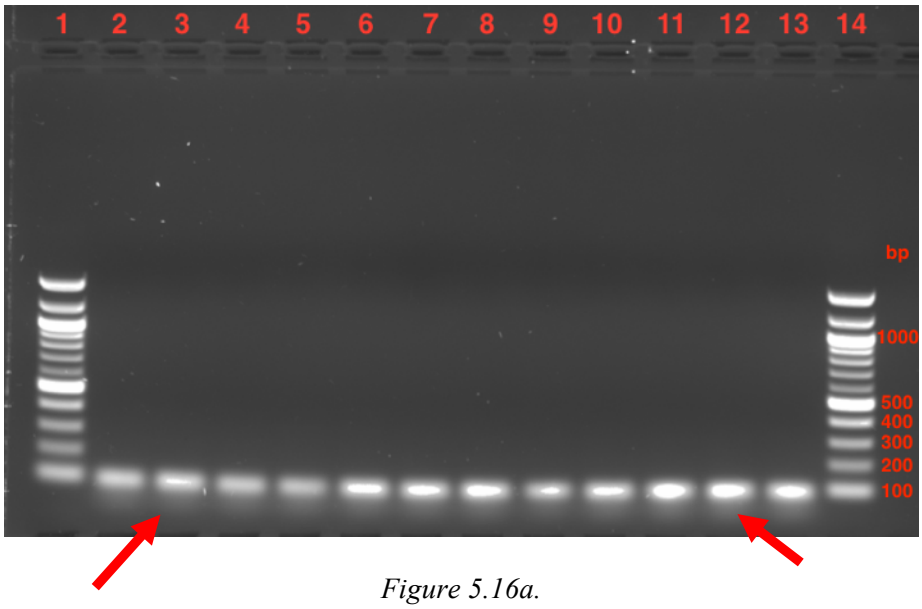

Figure 5.16a.

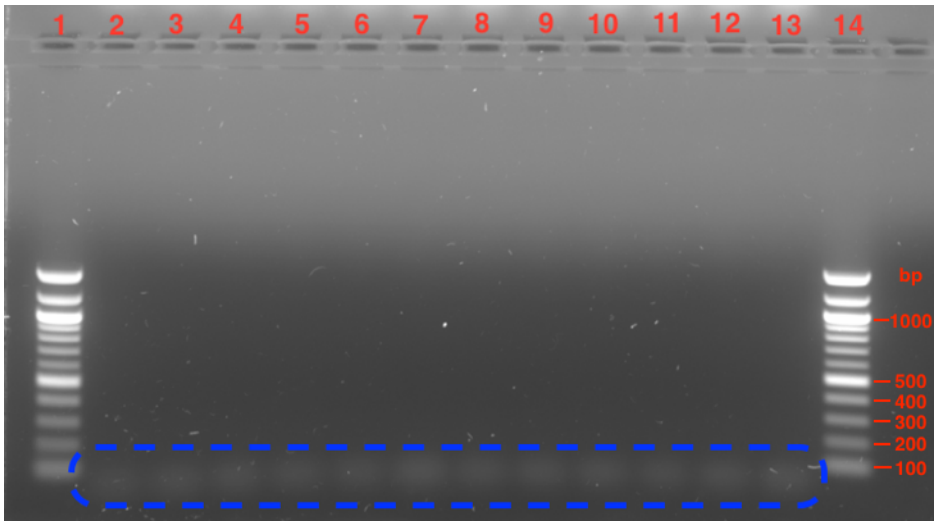

Figure 5.16b.

Figure 5.16: Agarose gel electrophoresis images of the *rpoD* amplicon product following qPCR. a) Image shows agarose gel using cDNA as the template. Red arrows indicate a 'haze' under the main band b) Image shows agarose gel using the RT control (RNA) as the template. Blue dotted line indicates region of faint bands. Lanes 1 and 14 = 100 bp DNA ladder, Lane 2 = untreated adult CF sputum 1, Lane 3 = untreated adult CF sputum 2, Lane 4 = untreated adult CF sputum 3, lane 5 = untreated paediatric CF sputum 1, lane 6 = untreated paediatric CF sputum 2, lane 7 = untreated paediatric CF sputum 3, lane 8 = DNase treated adult CF sputum 1, lane 9 = DNase treated adult CF sputum 2, lane 10 = DNase treated adult CF sputum 3, lane 11 = DNase treated paediatric CF sputum 1, lane 12 = DNase treated paediatric CF sputum 2, and lane 13 = DNase treated paediatric CF sputum 3.

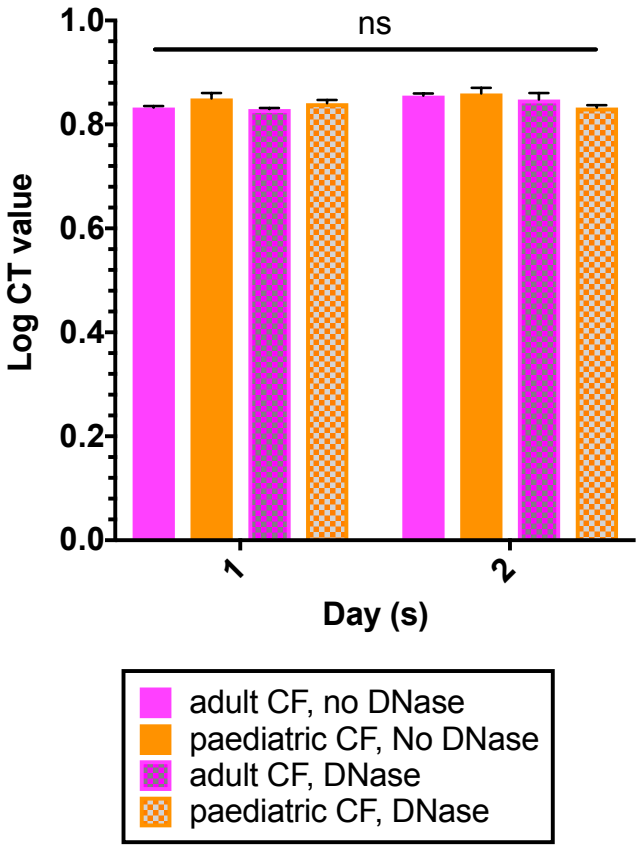

Figure 5.17.

Figure 5.17: Expression (log CT) of HKG 16S rRNA in *Pseudomonas aeruginosa* PAO1 exposed to adult CF sputum which was not treated with DNase (pink bar), paediatric CF sputum which was not treated with DNase (orange bar), adult CF sputum treated with DNase (patterned pink bar), and paediatric CF sputum treated with DNase (patterned orange bar). Data represent the log CT value from 6 biological replicates which were treated as described and the qPCR carried out on 2 separate days. Error bars represent standard error of the mean (SEM). 'ns' means there is no significant difference as assessed by a two-way ANOVA followed by a Sidak's multiple comparisons test.

Before commencing qPCR experiments, dilutions of the cDNA template were used in qPCR experiments using 16S rRNA primers. Finally, a 1 in 100 dilution of template was eventually selected. Amplicon products following qPCR were then assessed via agarose gel electrophoresis. Notably, distinct bands were observed when cDNA was used as a template with the primers for 16S rRNA as shown in figure 5.18.

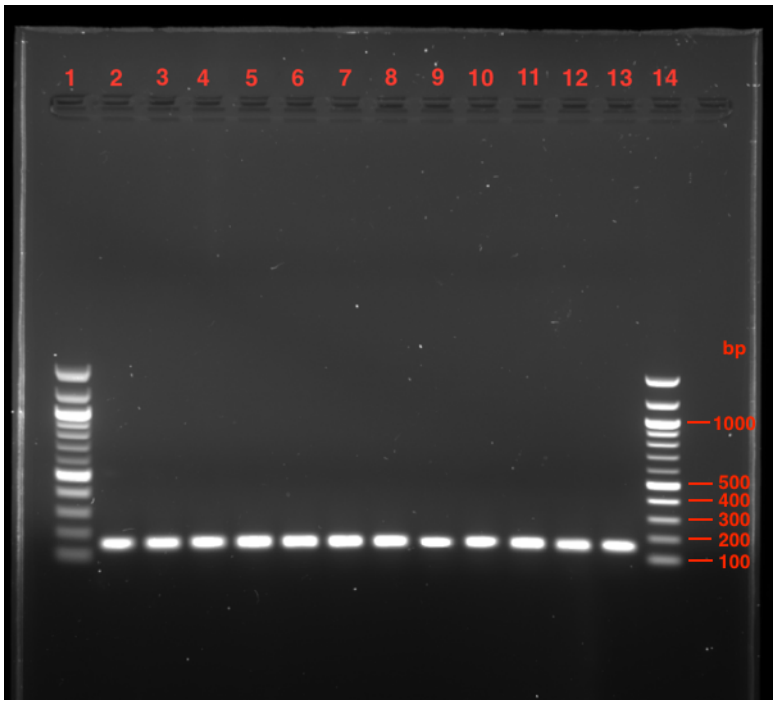

Figure 5.18.

Figure 5.18: Agarose gel electrophoresis images of the 16S rRNA amplicon product following qPCR. Lanes 1 and 14 = 100 bp DNA ladder, lane 2 = untreated adult CF sputum 1, lane 3 = untreated adult CF sputum 2, lane 4 = untreated adult CF sputum 3, Lane 5 = untreated paediatric CF sputum 1, lane 6 = untreated paediatric CF sputum 2, lane 7 = untreated paediatric CF sputum 3, lane 8 = DNase treated adult CF sputum 1, lane 9 = DNase treated adult CF sputum 2, lane 10 = DNase treated adult CF sputum 3, lane 11 = DNase treated paediatric CF sputum 1, lane 12 = DNase treated paediatric CF sputum 2, and lane 13 = DNase treated paediatric CF sputum 3.

Analysis of the melt curves obtained on day 1, where cDNA was used as a template reveals a distinct peak at approximately 84^{o}C as seen in figure 5.19a., whilst the NTC sample (green lines), despite not crossing the threshold (figure 5.19c.), also demonstrates as small peak to the left-hand side and therefore represents the formation of non-specific product at approximately 77^{o}C (figure 5.19a.). Figure 5.19b. represents the melt analysis following qPCR where the RT control (i.e. RNA) was used as the 'template'. The orange lines represent the melt peaks for gDNA, but in the CF samples, these were reduced in height. Figure 5.19c. reveals that amplification of the RT control samples appears at approximately 30 cycles, and therefore greater than 10 CT cycles after amplification when cDNA template was used. These data can therefore be accepted for this reason. Melt analysis of the RT control samples shown in figure 5.19b.

revealed that there was formation of amplicon product at the same melting point that would be expected if cDNA template was provided. Therefore, it appears that non-specific product formed due to trace amounts of gDNA.

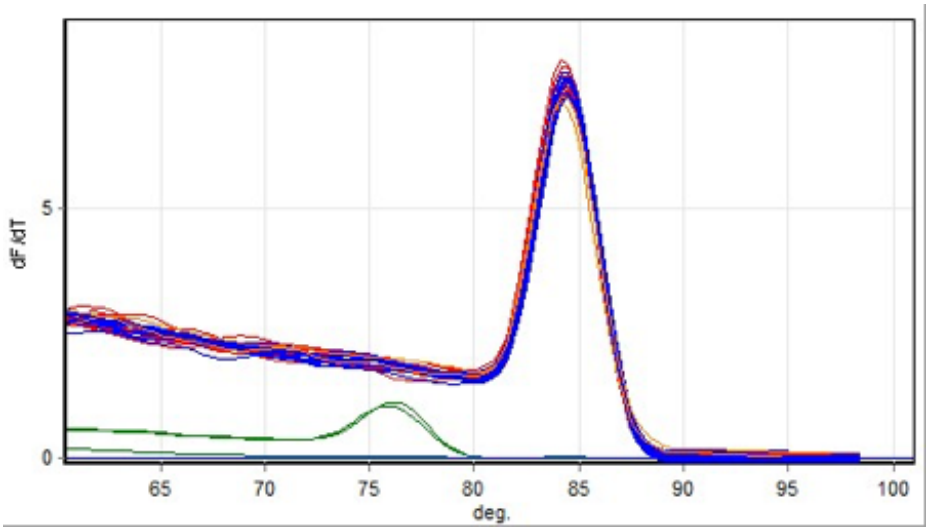

Figure 5.19a.

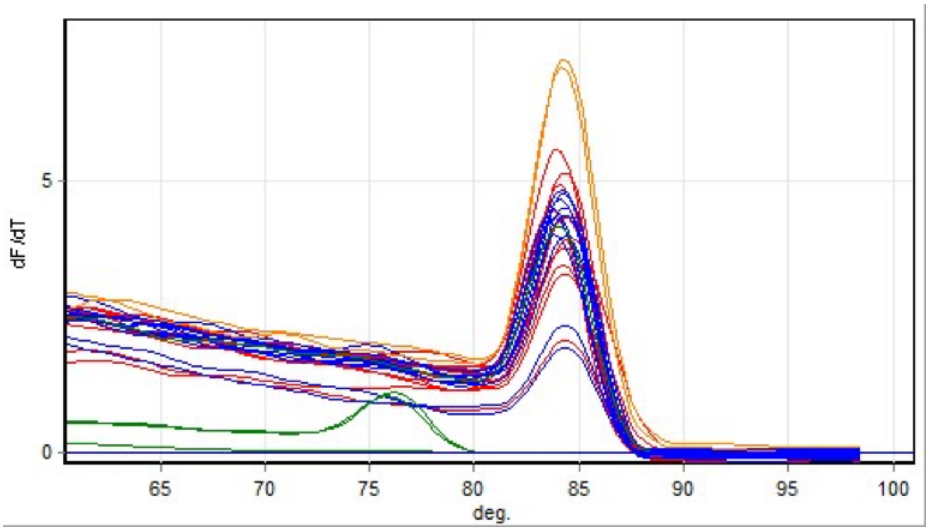

Figure 5.19b.

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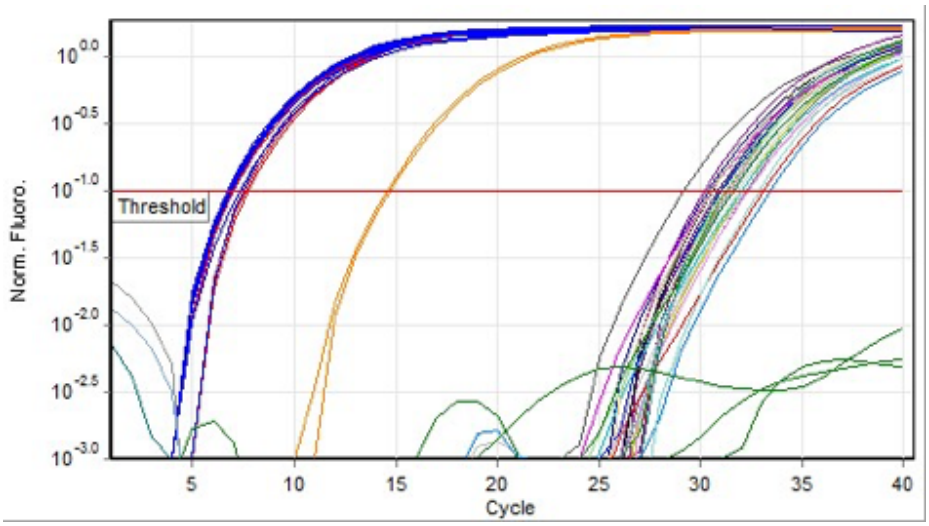

Figure 5.19c.

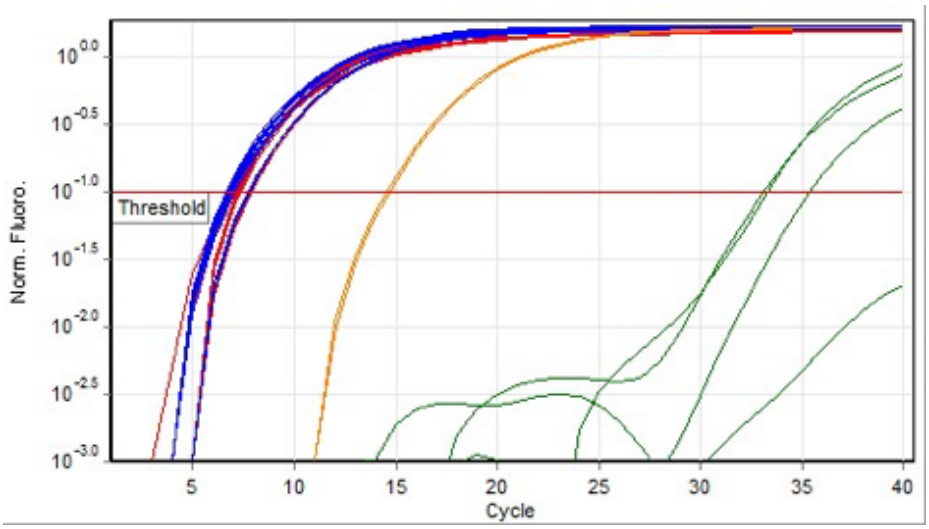

Figure 5.19d.

Figure 5.19: a) Melt analysis of qPCR products using primers for *16S rRNA* HKG using cDNA as a template and in graph b), using RNA as the template (RT control). c) Amplification using 16S rRNA primers over 40 cycles on day 1 and graph d) shows amplification of 16S rRNA gene on day 2. In all graphs, the blue lines represent the untreated CF samples, the red lines represent the DNase treated CF samples, the orange lines represent gDNA template, the green lines represent the NTC, and the multi-coloured lines (c. only) represent amplification in the RT control.

5.3.4. Gene expression of *Pseudomonas aeruginosa* PAO1 in CF sputum in the presence of DNase

Once the conditions of qPCR had been optimised, the final qPCR experiments began. By this point a total of fifteen genes were investigated (excluding the HKG, *PA0668.1*) for their expression in *P. aeruginosa* PAO1 in DNase treated CF sputum vs. non-DNase treated CF sputum. The fifteen selected genes include those involved in carbon, purine, and pyrimidine metabolism. The genes were selected from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways with the purpose of identifying a preferred metabolic pathway involved in nucleotide metabolism in *P. aeruginosa* PAO1. In general, around one to three genes were initially selected to target each pathway, however, as previously described, several genes which were initially selected were found to be unsuitable during optimisation for the final experiments (Appendix 2). Table 5.3 represents the genes found to be significantly upregulated or downregulated, out of these fifteen genes, in CF sputum treated with DNase vs. non-DNase treated sputum relative to the HKG (*PA0661.8, 16S rRNA*).

Table 5.3: Genes found to be significantly upregulated/downregulated in *P. aeruginosa* PAO1 in the presence of DNase treated CF sputum

Gene	Product name and biological function	Upregulated/ Downregulated	Fold expression in presence of DNase treated sputum relative to HKG (PA0668.1, *16S rRNA*)
PA5493 (polA)	DNA polymerase 1: DNA ←→dGTP/dATP dCTP/dTTP → DNA	Upregulated	2.02
PA0935	Nucleoside triphosphate pyrophosphohydrolase: GTP → GMP ATP → AMP UTP → UMP CTP → CMP dTTP → dTMP	Upregulated	2.17
PA3970 (amn)	AMP nucleosidase: AMP → adenine	Upregulated	1.51
PA2962 (tmk)	Thymidylate kinase: dTDP ←→dTMP dUDP ←→dUMP	Upregulated	1.83
PA0437 (codA)	Cytosine deaminase: 5-methylcytosine →	Upregulated	4.63

	thymine cytosine → uracil		
PA3195 (gapA)	Glyceraldehyde 3-phosphate dehydrogenase: glyceraldehyde 3-phosphate → glycerate 1,3-disphosphate	Upregulated	4.43
PA0143 (nuh)	Purine nucleosidase: guanosine →guanine xanthosine → xanthine inosine →hypoxanthine adenosine →adenine	Downregulated	0.49
PA3186 (oprB)	Glucose/carbohydrate outer membrane porin OprB precursor	Downregulated	0.59

As described in section 5.2.10, CT values obtained at the set threshold for all qPCR experiments were then used to calculate ratio gene expression values considering the efficiency for each primer pair. The results of which are presented in figures 5.20. and 5.21. A repeated-measures ANOVA was chosen to analyse the data for two reasons. Firstly, the HKG had been proven to be stable in all twelve samples on repeated days (figure 5.17), and secondly, because the same samples were repeatedly used for analysis of gene expression on different days. In the study design, the primer pairs were defined as the 'treatment' group and the samples were the 'subject' group. Analysis revealed that when a repeated-measures ANOVA was performed that matching was highly effective with a p-value of less than 0.0001. Therefore, an ordinary ANOVA would not have been appropriate for this data. However, analysis also revealed that there were significant differences between the variances, or in other words, sphericity had not been met (****$p < 0.0001$). To correct this, because the epsilon value was less than 0.75, the Geisser and Greenhouse correction was applied. The epsilon value generated reveals the degree of sphericity violation. For example, a value of 1 indicates no violation of sphericity whilst a value closer to the lower bound value dictated by the number of treatments is indicative of violation. The Geisser-Greenhouse correction accounts for this violation by making an adjustment to the degrees of freedom. This in turn results in a higher p-value than if no correction had been applied. The Dunnett's post hoc test was used for comparisons against the HKG, *PA0668.1* (Maxwell and Delaney, 2003).

Figure 5.20 demonstrates six genes which were significantly upregulated, two genes which were downregulated, and seven genes which demonstrated no difference in expression when DNase treated compared to non-DNase treated CF sputum. The genes which were significantly upregulated were *PA5493* (*polA*) with a value of 2.02 ± 0.19 SEM (**p < 0.01), *PA0935* with a value of 2.17 ± 0.18 SEM (***p < 0.001), *PA3970* (*amn*) with a value of 1.51 ± 0.10 SEM (**p < 0.01), *PA2962* (*tmk*) with a value of 1.83 ± 0.21 SEM (*p < 0.05), *PA0437* (*codA*) with a value of 4.63 ± 0.56 SEM (***p < 0.001), and finally *PA3195* (*gapA*) with a value of 4.43 ± 0.51 SEM (***p < 0.001).

The genes which were downregulated as shown in figure 5.20 are *PA0143* (*nuh*) and *PA3186* (*oprB*). These genes were downregulated because the gene expression ratios calculated from normalising the data for the genes against the HKG (as indicated by the black bar) were found to be below one. For example, for *PA0143* (*nuh*) the mean gene expression ratio was reported as 0.49 ± 0.06 SEM (****p < 0.0001) highlighting that it is significantly downregulated whilst for *PA3186* (*oprB*) it is 0.59 ± 0.09 SEM (**p < 0.01). The problem with presenting the data this way is that it is not entirely clear which genes are significantly downregulated.

However, when the data was log transformed, values below one became negative values. This log transformed data are presented in figure 5.21. Analysis revealed another gene which was found to be significantly downregulated. For example, *PA0865* (*hpd*) was found to have a log value of – 0.98 ± 0.25 SEM (*p < 0.05) compared to the HKG, *16S rRNA, PA0668.1* at baseline zero. This value was not found to be highly significant but notably there was a large standard error of the mean which would be affecting this. Transformation of the data was also found to affect the significance of other genes. Where previously the p-value for *PA0143* was less than 0.0001, following transformation it was less than 0.001. This similarly applied to genes which were upregulated. Following transformation, expression of *PA0935, PA0437*, and *PA3195* were found to be highly significant (****p < 0.0001) compared to the HKG baseline at 0.

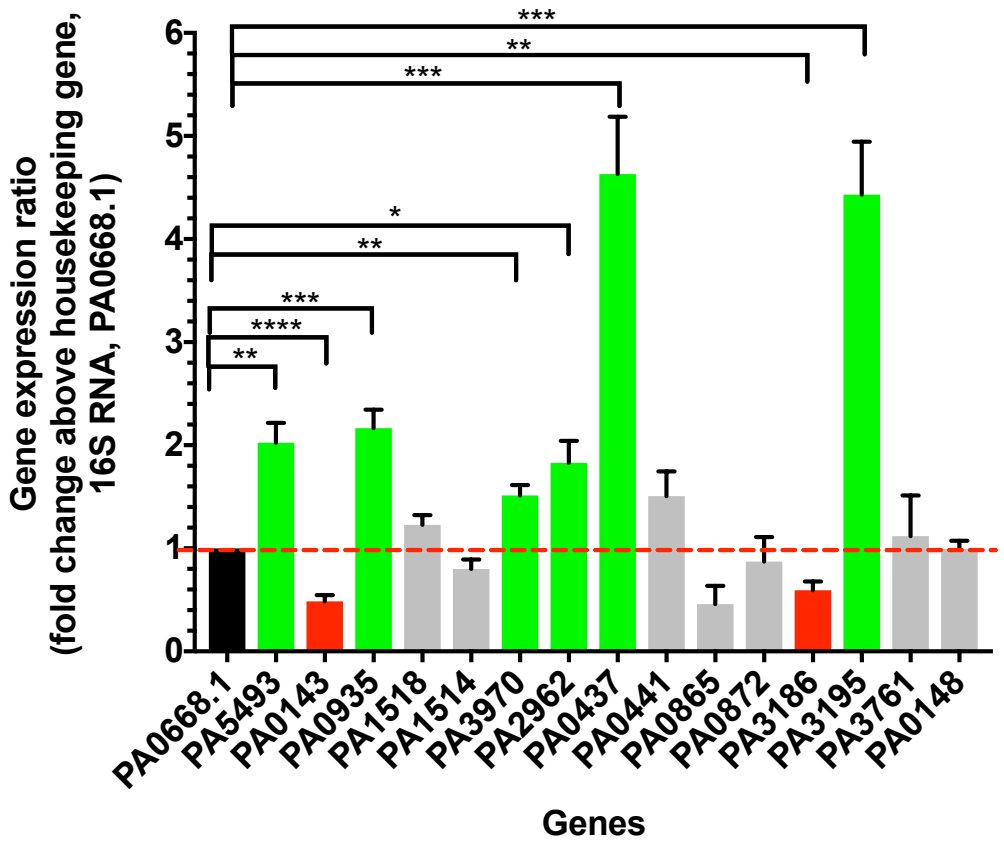

Figure 5.20.

Figure 5.20: Expression of selected genes in *Pseudomonas aeruginosa* PAO1 when exposed to DNase treated and non-DNase treated CF sputum for 3 hours. Expression is DNase treated sputum relative to non-DNase sputum, normalised against expression in the HKG, *16S rRNA (PA0668.1)*. Data represent the mean gene expression ratio from 6 biological replicates (3 adult, 3 paediatric) which were either DNase treated or were untreated. Expression is therefore in 12 samples performed in duplicate. Error bars show standard error of the mean (SEM). The black bar represents the baseline to which expression was compared to, i.e. *16S rRNA (PA0668.1)*. This baseline is represented by the red dotted line. Bars above this line indicate genes which were significantly upregulated (green bars) and bars below this line indicate genes which were significantly downregulated (red bars). Grey bars indicate genes which were neither significantly upregulated or downregulated. P values on the graphs (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test. Sphericity was not assumed in this test. The Greenhouse and Geisser correction was performed.**

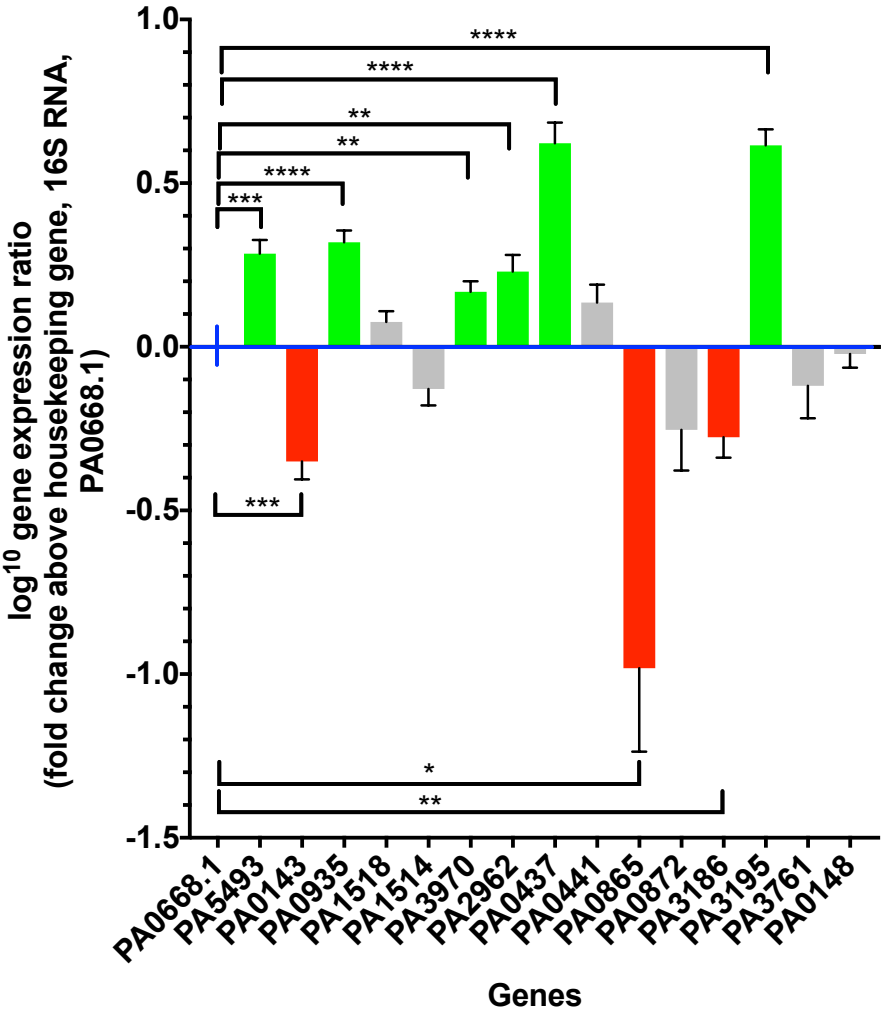

Figure 5.21.

Figure 5.21: Log expression of selected genes in *Pseudomonas aeruginosa* PAO1 when exposed to DNase treated and non-DNase treated CF sputum for 3 hours. Expression is DNase treated sputum relative to non-DNase sputum, normalised against expression in the HKG, *16S rRNA (PA0668.1).* Data represent the mean log gene expression ratio from 6 biological replicates (3 adult, 3 paediatric) which were either DNase treated or were untreated. Expression is therefore in 12 samples performed in duplicate. Error bars show standard error of the mean (SEM). The blue line represents the baseline to which expression was compared to, i.e. the HKG, *16S rRNA, (PA0668.1)* as indicated by the blue cross (+). Bars above this line indicate genes which were significantly upregulated (green bars) and bars below this line indicate genes which were significantly downregulated (red bars). Grey bars indicate genes which were neither significantly upregulated or downregulated. P values on the graphs (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test. Sphericity was not assumed in this test. The Greenhouse and Geisser correction was performed.**

5.3.5. Confirmation of gene product

For qPCR, a few controls were necessary. These included gDNA, NTCs, and RT controls using RNA as the template. This final control was added to be certain that any amplification taking place would be due to the presence of cDNA and not contaminating DNA. It is almost impossible to remove every trace of gDNA. However, previous gel images revealed that gDNA was absent. In addition to this, TURBO DNase was also added at a concentration suitable for contaminating DNA according to the manufacturer's instructions following NanoDrop analysis.

To ensure every qPCR performed was successful, following completion, a melt curve was produced. Production of a single peak, as shown in figure 5.22, confirmed the presence of one product. In all but one example, single peaks were produced at the expected melting temperatures. In the example where there were two peaks, for the primer; PA0444, this data was excluded from the final results. This primer pair had the ability to produce non-specific products when template at a lower concentration was used. The concentration of cDNA template used in this experiment was at a low enough concentration for this to occur and it was for this reason the data for this primer pair was excluded.

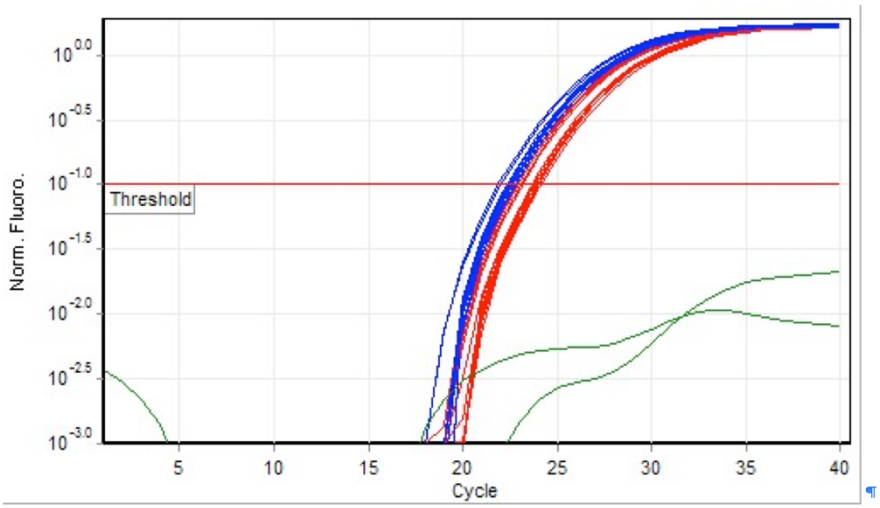

Figure 5.22a.

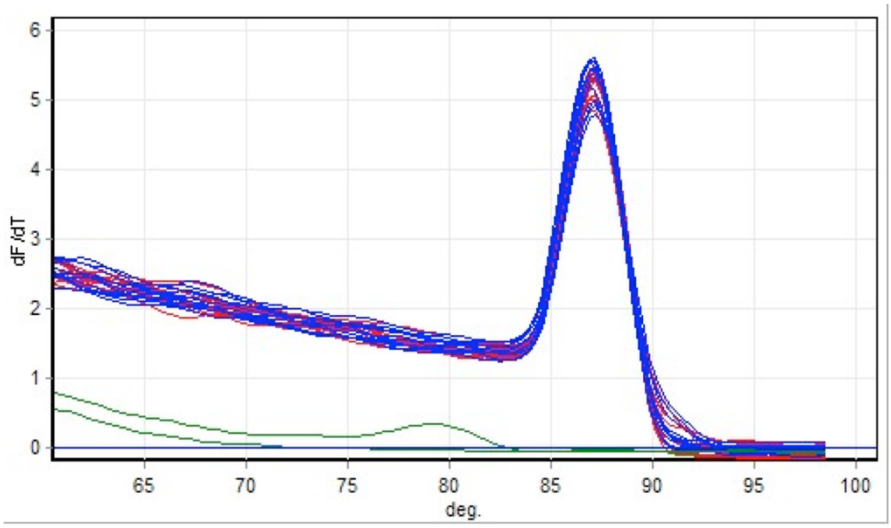

Figure 5.22b.

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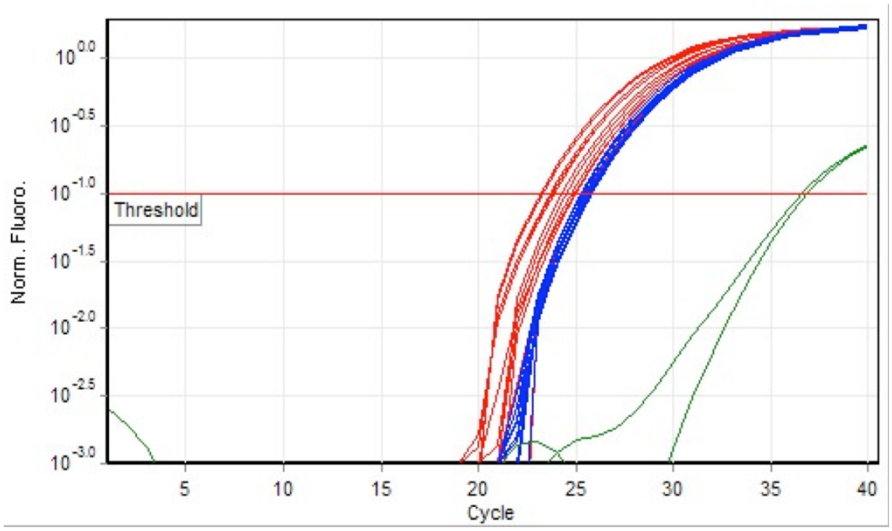

Figure 5.22c.

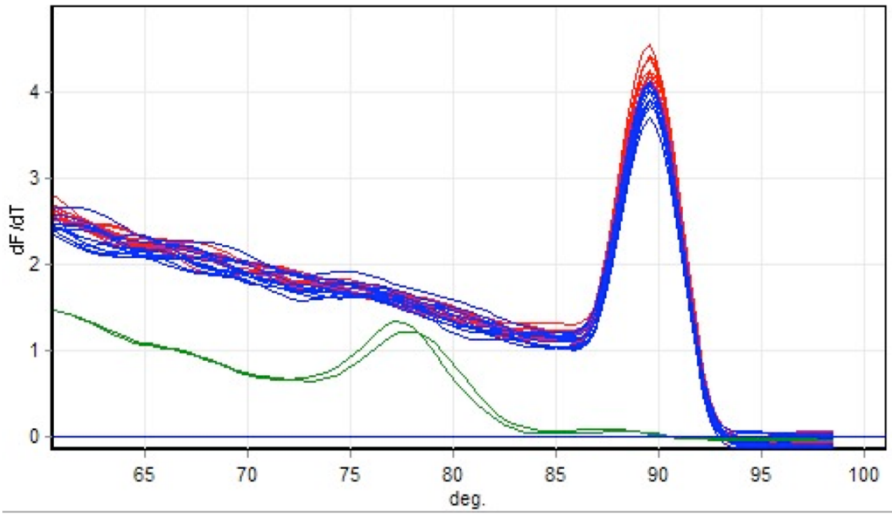

Figure 5.22d.

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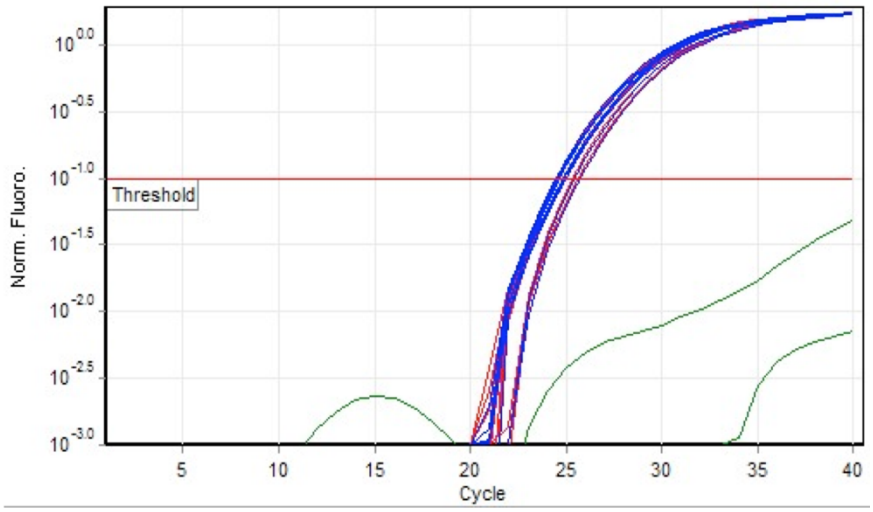

Figure 5.22e.

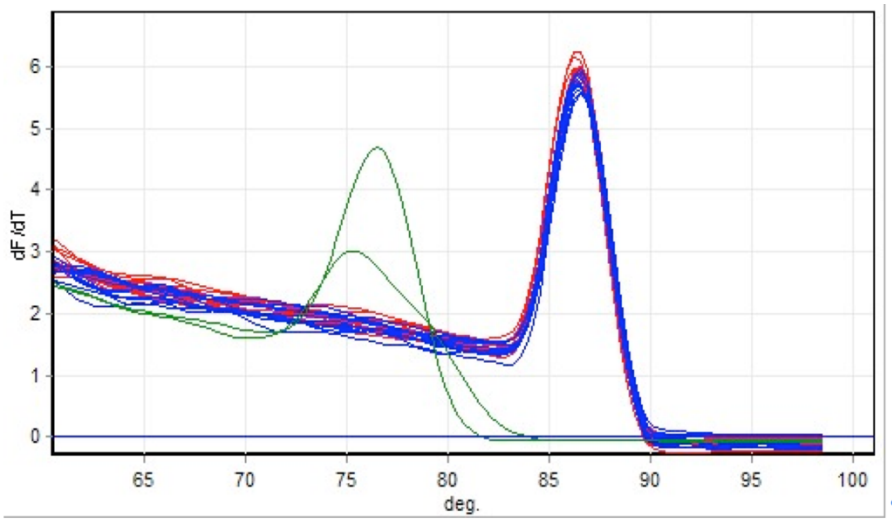

Figure 5.22f.

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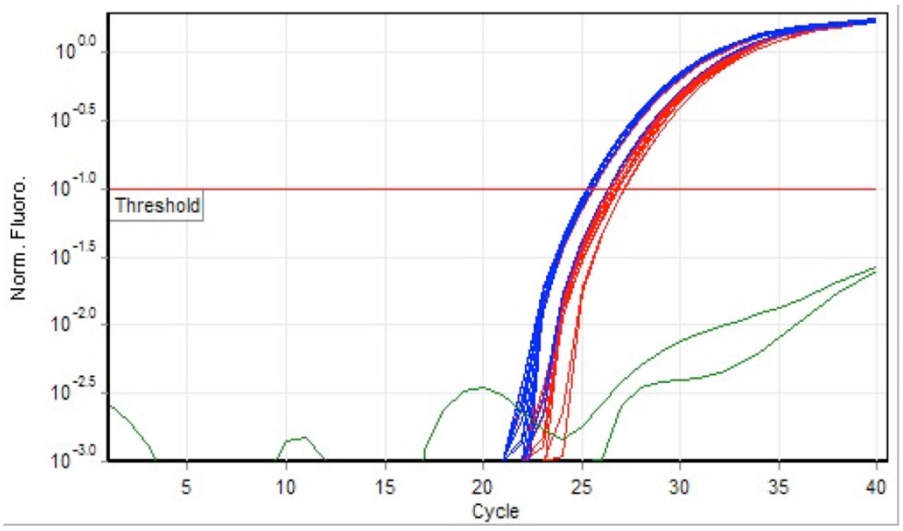

Figure 5.22g.

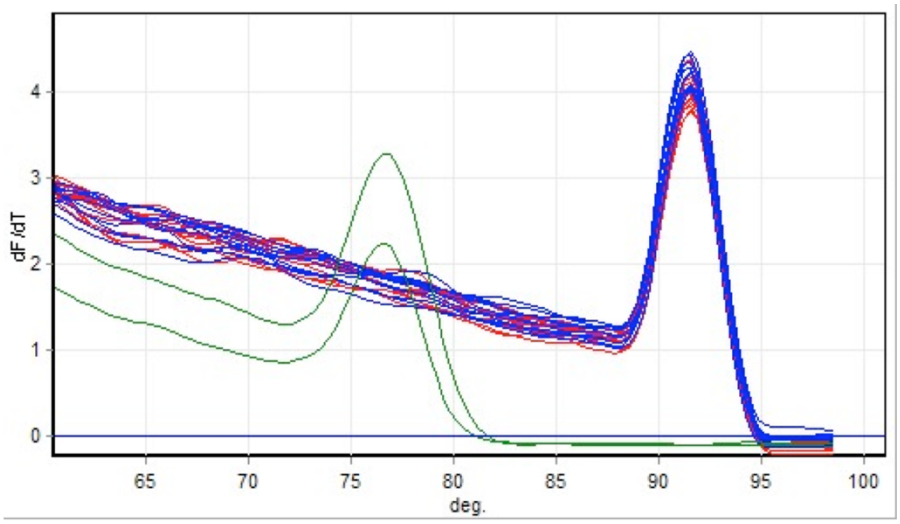

Figure 5.22h.

Figure 5.22: Graphs showing amplification of cDNA over 40 cycles using primers PA5493 (a), PA0143 (c), PA0148 (e), PA2962 (g). Melt curve analysis graphs show change in fluorescence over time (dF/dT) with an increase in temperature (^{o}C) following qPCR using primers: PA5493 (b), PA0143 (d), PA0148 (f), PA2962 (h). The different coloured lines represent the 14 samples including 6 DNase treated (red), 6 non-DNase treated (blue), and 2 NTC samples (green).

Figure 5.22 shows some examples of amplification and melt curve analysis for the amplicon product obtained following qPCR for a selection of primer pairs. Figures 5.22a., e., and g., show no amplification in the NTC whereas in figure 5.22c. the NTC is shown to cross the threshold. However, the data was accepted for this because there was

greater than 10 CT values between the NTC and the sample. Figure 5.22b., d., f., and h., represent the melt curve analysis for each run following qPCR. In many cases, a melt peak was also produced in the NTC samples and the RT control (RNA) samples as shown in figure 5.23. It appears that this was the case due to the absence of template when the primers have no template to anneal to. In most cases, amplification in the NTC and the RT samples overall did not reach the uniformly set threshold. In the scenario where this did occur, it was either after 35 CT values in the RT control and was therefore comparable to values for the NTC or there was greater than 10 CT values between amplification of the RT control/NTC and that of the amplicon product. Notably the data was only accepted if there were greater than 10 CT values between amplification of the amplicon product and the NTC. In two instances, there were differences of 7 CT values between amplification of the amplicon product and the NTC, but because amplification efficiencies of these primers were over 90%, this was accepted.

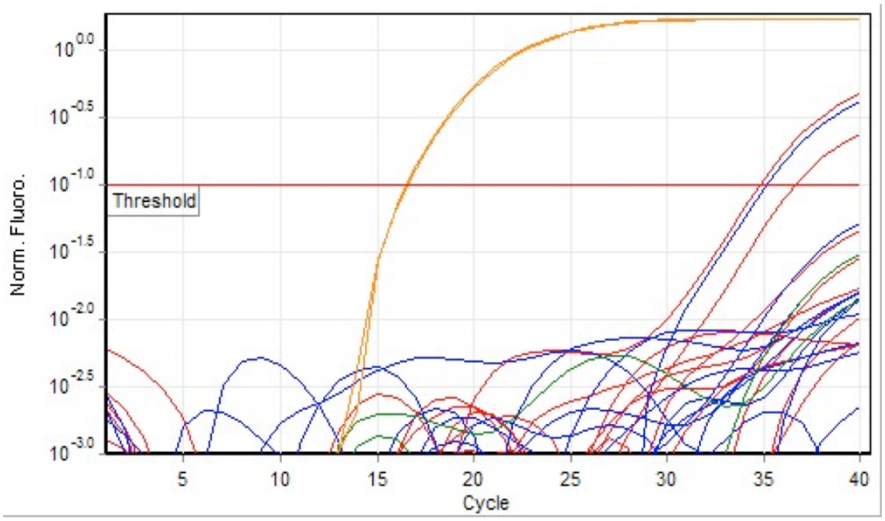

Figure 5.23a.

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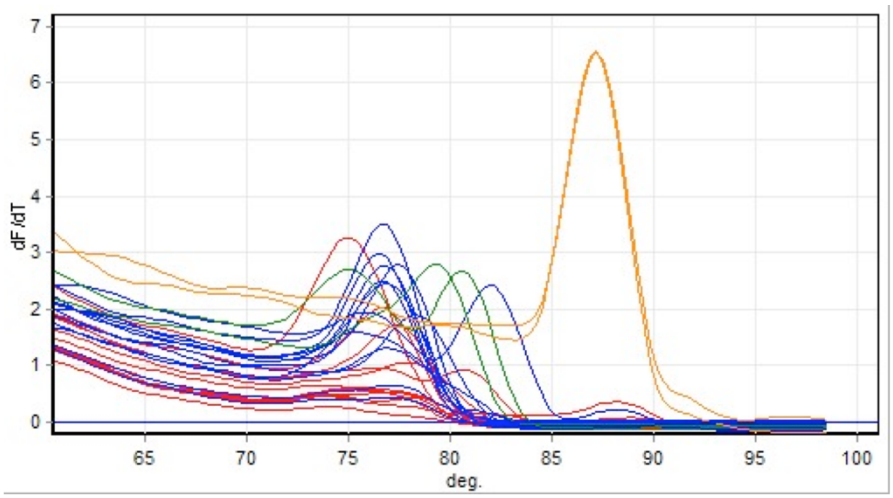

Figure 5.23b.

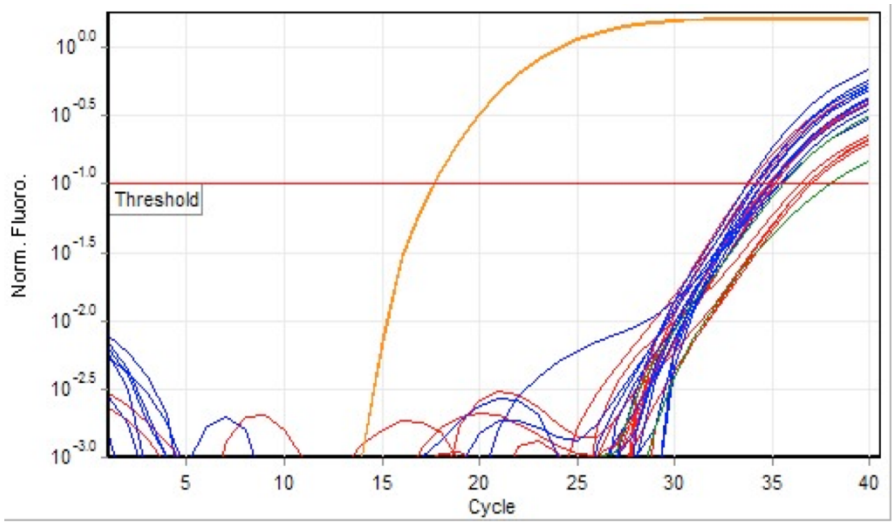

Figure 5.23c.

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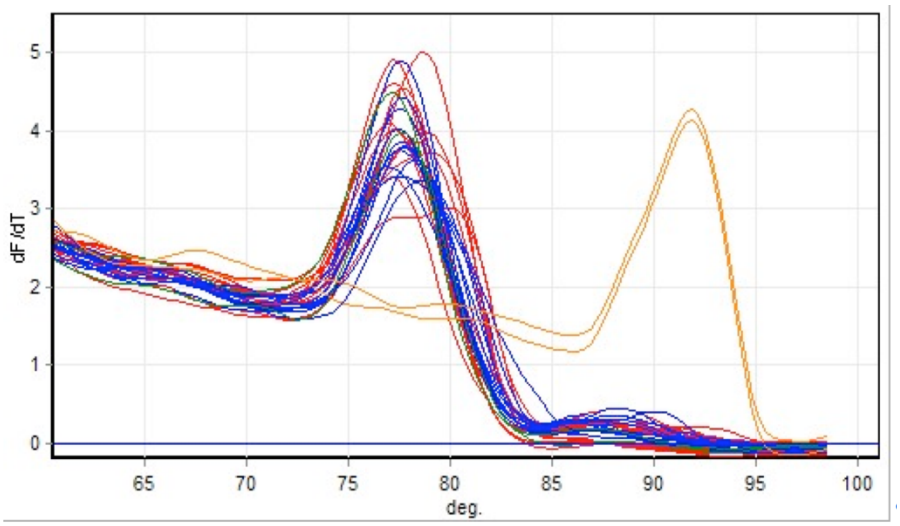

Figure 5.23d.

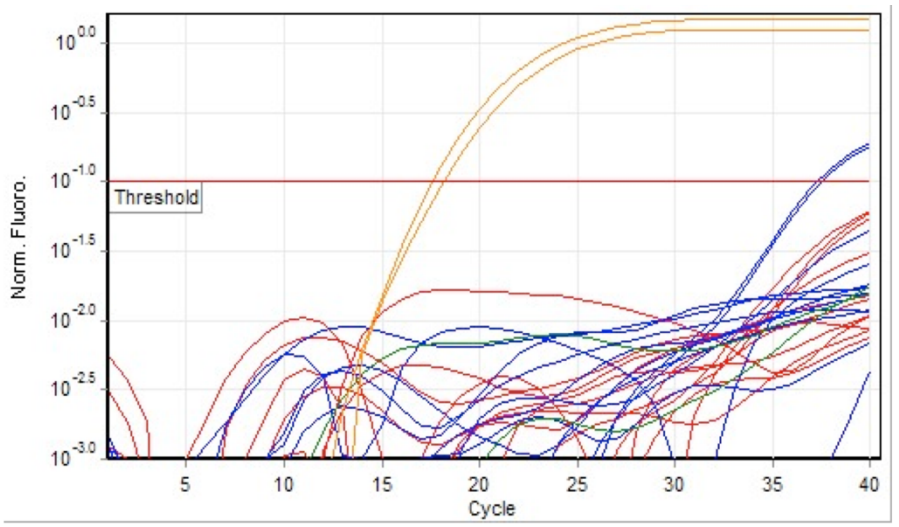

Figure 5.23e.

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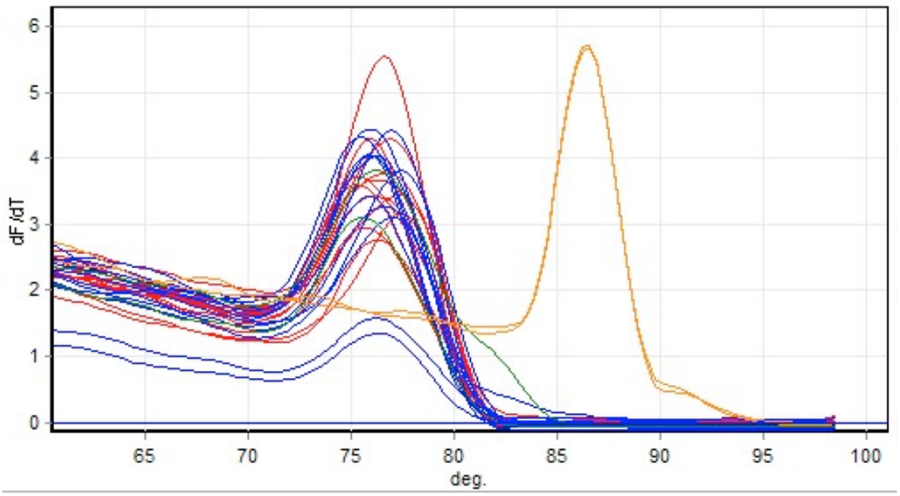

Figure 5.23f.

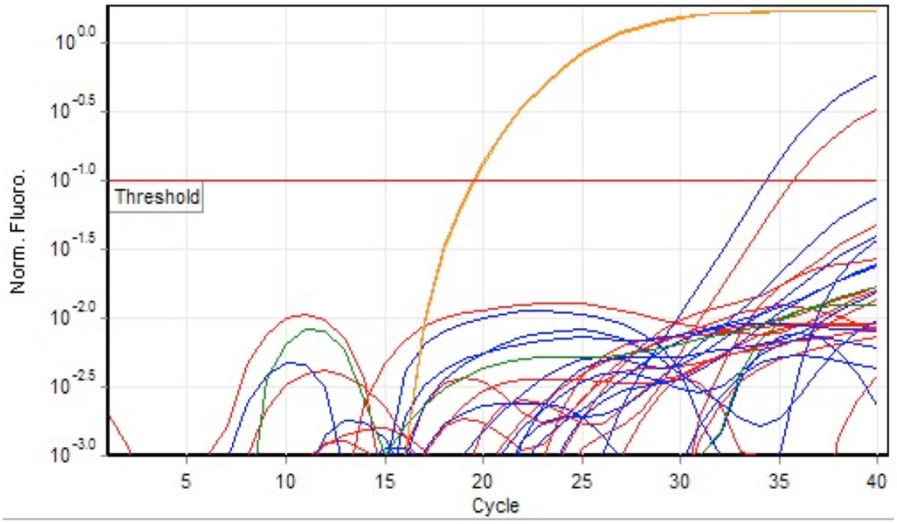

Figure 5.23g.

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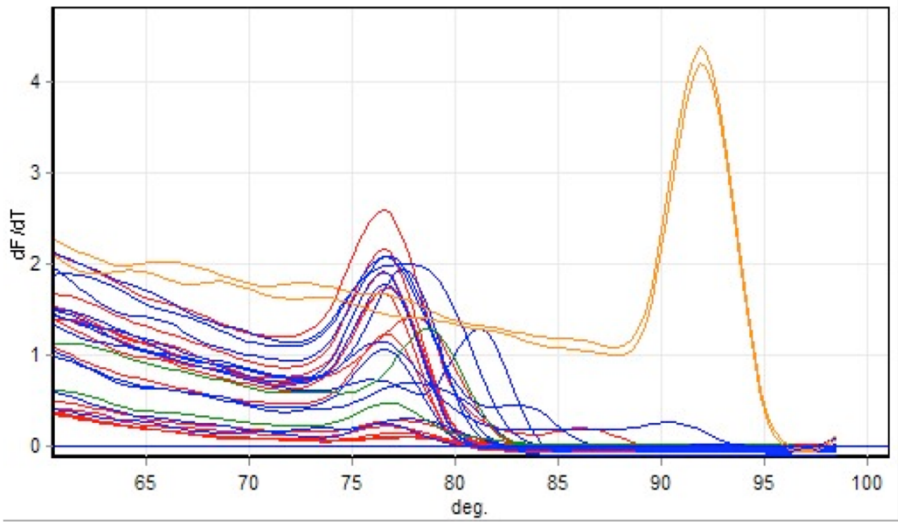


Figure 5.23h.

Figure 5.23: Graphs showing amplification of RT control and genomic DNA over 40 cycles using primers PA5493 (a), PA0143 (c), PA0148 (e), PA2962 (g). Melt curve analysis graphs show change in fluorescence over time (dF/dT) with an increase in temperature (^{o}C) following qPCR using primers: PA5493 (b), PA0143 (d), PA0148 (f), PA2962 (h). The different coloured lines represent the 12 RT control samples, 2 gDNA samples (orange) and 2 NTC samples (green).

5.4. Discussion - Gene expression analysis

Since it had been shown that degraded DNA could promote *P. aeruginosa* PAO1 growth in CF sputum, PGM, and salmon sperm DNA, and because the presence of measured nucleic acid was shown to strongly correlate with the growth rate and yield, it was questioned what components within DNA were serving as a nutrient source. The data in this study (chapter 4) demonstrated that thymine and deoxyribose could promote growth, but growth studies with the other nucleobases presented challenges as previously discussed. Therefore, the aim of this next section of work was to identify mechanisms by which degraded DNA is utilised as a nutrient source.

5.4.1. MIQE guidelines for qRT-PCR and limitations

Gene expression data was obtained by qRT-PCR. Although very sensitive, many problems can arise with this method, especially using the non-specific DNA binding dye SYBR green. Therefore, standardisation and thorough analysis was necessary to ensure that the PCR product amplified during PCR was representative of real gene

expression and not residual contaminating DNA. Therefore, to minimise problems with reproducibility, it was necessary to adopt a methodical approach to ensure production of reliable qPCR data. Details of the 85 steps which should be carried out to obtain more accurate and reliable results have been published as '**M**inimum **I**nformation for publication of **Q**uantitative real-time PCR **E**xperiments (MIQE)' by Bustin (2009).

Therefore, RNA which was extracted upon analysis of integrity using the Agilent Bioanalyzer 2100 and assessment of purity via NanoDrop One, was immediately reverse transcribed to cDNA. This was to avoid freeze-thawing which causes sample degradation in addition to minimise effects of RNase which is ubiquitously present in the environment (Röder et al, 2010; Shao et al, 2012). Therefore, it is worth considering that even if the RNA extracted is of good quality, care must be taken during handling to ensure swift production of cDNA which is more stable than RNA. A study by Vermeulen and colleagues (2011) assessed how RNA quality before reverse transcription affects gene expression results in HKGs and highlights the necessity of highly intact RNA prior to reverse transcription.

In the process of reverse transcription, notably the same amount of total RNA (150 ng) for samples was used as the template for reverse transcription. Following reverse transcription all cDNA was stored at -20^{o}C until required, but in all cases, was used for experiments within 2 weeks of storage. Storage of cDNA in water is generally not advised in the long-term. Although DNA is more stable than RNA, when stored in water, the formation of ice crystals is thought to destroy the nucleic acid strands. Evidence for this theory has been shown in a study by Krajden and colleagues (1999) which demonstrated that the addition of glycerol acts as a cryoprotectant during storage and maintains the DNA integrity (Schaudien et al, 2007). For this reason, although the cDNA was stored in water, as it has been stored in many other studies, the cDNA was frozen only once and used within 2 weeks (Vermeulen et al, 2011). The reason for only allowing one cycle of freeze-thawing is based on evidence showing that ferrous iron exists at high concentrations in nucleic acid samples, which through the Fenton Reaction can then generate damaging ROS in the presence of iron as previously discussed in chapter 1 (Touati, 2000). Therefore, for long term storage it is advised to add a chelating agent to samples such as EDTA. However, EDTA itself can be

problematic for downstream reactions such as qPCR as it is an inhibitor of this reaction even at low concentrations (Huggett et al, 2008). Upon thawing, the cDNA was freshly diluted to 4 ng/µl, or total RNA equivalent, for use as the template in qPCR. Samples were therefore uniformly diluted to the same concentration and handled in the same manner. The purpose of this was to minimise variation of results which could arise due to compromised integrity of the starting sample.

To ensure there was no contamination of the reagents, NTCs in duplicate were included in every PCR run for every primer pair. Amplification in the NTC is indicative of contamination with template/exogenous DNA or primer-dimer formation. Following qPCR, melt curve analysis confirmed specificity of primers due to a single peak at the anticipated temperature required for melting of the PCR product. Overall, it was noticed that primer-dimer formation occurred when no template was present, but this was considered satisfactory since the signal did not cross the threshold, and the melt peak was at a different melt temperature to that of the amplicon. This was accepted based on the advice given in the Thermo Fisher Scientific 'Real-time PCR Handbook' (2014), which states: "There are situations in which primer-dimers are present, but may not affect the overall accuracy of the real-time PCR assay. A common observation is that primer-dimers are present in the no-template control but do not appear in reactions containing template DNA. This is not surprising because in the absence of template, primers are much more likely to interact with each other. When template is present, primer-dimer formation is not favored. As long as the peak seen in the NTC is absent in the plus-template dissociation curve, primer-dimers are not an issue."

Selection of a suitable HKG was also necessary for data normalisation in ensuring reliable data. Generally, it is recommended to use 3-5 reference genes, but in this study, it was not possible due to problems previously described with the HKG primers. Notably, use of *16S rRNA* in qPCR is not always favoured since it doesn't remain stable under all conditions, despite its wide use as a HKG (Rocha et al, 2015; Srinivasan et al, 2015). However, it must be highlighted that although this was the case in other studies, it was confirmed to be stable in the samples used in these experiments.

Finally, for experimental reproducibility it is recommended to minimise biological and technical variability of the data. To minimise biological variability, it is recommended to have at least 3 biological replicates. In this study, there were a total of six biological replicates. To minimise technical variability, as recommended, there were two technical replicates for every biological sample to minimise variability which can arise due to pipetting error or sample differences (Taylor et al, 2010).

5.4.2. Gene expression of *Pseudomonas aeruginosa* PAO1 when exposed to degraded DNA due to DNase treatment of whole patient sputum

Although many previous studies have investigated the expression of *Pseudomonas* in CF sputum, this was the first assessing the effect of DNase treatment on *P. aeruginosa* gene expression in CF sputum (Palmer et al, 2005; Palmer et al, 2007; Fung et al, 2010; Turner et al, 2015). The aim of this section of work was to identify genes involved in KEGG identified pathways which become upregulated or downregulated due to exposure to degraded DNA. Analysis of the gene expression data revealed several genes which were significantly upregulated whilst others were downregulated.

5.4.2.1. Downregulated genes

Firstly, analysis revealed the downregulation of the following genes: *PA0143* (*nuh*) which encodes a purine nucleosidase in purine metabolism, *PA0865* (*hpd*) which encodes 4-hydroxyphenylpyruvate dioxygenase involved in tyrosine and phenylalanine metabolism, and finally *PA3186* (*oprB*) encoding OprB, a glucose/carbohydrate outer membrane porin which allows for uptake of sugars into the *Pseudomonas* (Wylie and Worobec, 1995). Interestingly, because *oprB* was downregulated, this may suggest that *P. aeruginosa* is not utilising the liberated deoxyribose as a nutrient source and instead the nucleobases are preferred. The data reveals that when *P. aeruginosa* PAO1 is exposed for only 3 hours to degraded DNA because of DNase treatment, metabolism of amino acids and uptake of sugar is reduced compared to expression in samples with comparatively intact DNA (no DNase added). Although it must be noted that this is only based on the assessment of genes investigated in this study. Therefore, this is a limitation of this study that only select genes could be investigated due to limitations

with primer optimisation. Global gene expression in the conditions used in this study would be more informative.

As previously mentioned, other studies have determined that amino acids are important nutrients for *P. aeruginosa* in the CF environment. It is therefore interesting that when in the presence of degraded DNA, the mentioned genes involved in metabolism of amino acids and uptake of sugar are downregulated. However, this finding may elucidate why high concentrations of amino acids are measured in CF sputum. This is because if DNase treatment is regularly administered in the form of dornase alfa, then degraded DNA would instead be preferentially available as a nutrient over amino acids. However, when freshly degraded DNA is not present, the data suggest that *Pseudomonas* will favour utilisation of amino acids. This could therefore explain why numerous studies have shown that amino acid degradation is required for high density growth. It could be because degraded DNA has already been used a nutrient source within the airway by the time the sputum is expectorated (Son et al, 2007, Palmer et al, 2005; Barth and Pitt, 1996; Siegel et al, 1977). The data in this study, although interesting, does not fully explain nutritional preferences. It would therefore be interesting in further studies to compared gene expression to that in glucose and amino acids, with the aim of determining whether degraded DNA is preferred over amino acids.

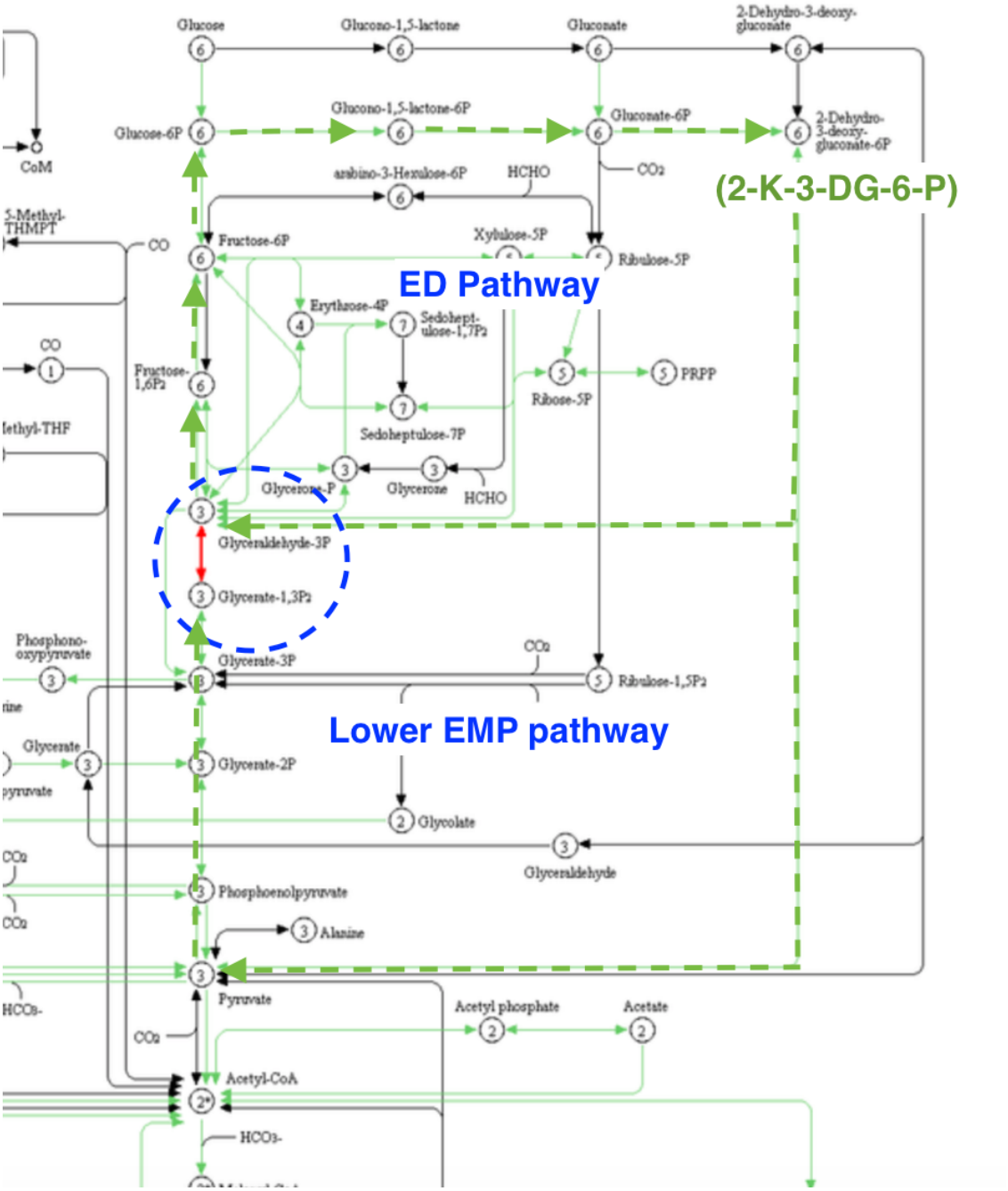
Additionally, downregulation of *PA0143* in purine metabolism implies that purines are not preferred substrates and instead pyrimidines are preferred substrates for *P. aeruginosa*. Alternatively, it could mean that purines are further degraded using other enzymes present in the pathway which have not been investigated. Unfortunately, because not all the primers designed in this study were successful, even though some were designed to be able to distinguish alternate pathways with the aim of identifying preferred pathways, the latter cannot be determined by the data in this study alone. Further work is therefore required to be able to determine whether purines are utilised. It could be that pyrimidines are more easily utilised by *P. aeruginosa* because they contain one less double bond than purines and have a single ring structure (Jordan, 1952).

5.4.2.2. Upregulated genes

Whilst a few genes involved in amino acid metabolism and sugar uptake were downregulated in addition to *PA0143* in purine metabolism, six other genes involved in purine and pyrimidine metabolism were confirmed to be significantly upregulated. This successfully demonstrates via gene expression analysis, in addition to thorough growth assays that *P. aeruginosa* can utilise degraded DNA as a nutrient source for growth. The genes which were significantly upregulated include: *PA5493* (*PolA*) encoding DNA polymerase I in both purine and pyrimidine metabolism, *PA0935* encoding nucleoside triphosphate pyrophosphohydrolase involved also in both purine and pyrimidine metabolism, *PA3970* (*amn*) encoding AMP nucleosidase in purine metabolism, *PA2962* (*tmk*) encoding thymidylate kinase in pyrimidine metabolism, *PA0437* (*codA*) encoding cytosine deaminase in pyrimidine metabolism, and finally *PA3195* (*gapA*) which encodes glyceraldehyde-3-phosphate dehydrogenase involved in carbon metabolism (figure 5.24). Although it appears that more genes involved in pyrimidine metabolism are upregulated, it cannot be conclusively stated that *P. aeruginosa* prefers pyrimidines over purines based on the data obtained.

Carbon metabolism

However, upregulation of *PA3195* (*gapA*) does reveal that carbon metabolism is increased in PAO1 exposed to degraded DNA in DNase treated CF sputum relative to PAO1 exposed to non-DNase treated CF sputum after 3 hours. Also, because growth assays prior to RNA extraction revealed that when a higher inoculum was administered there was no observed growth of PAO1 in non-DNase treated CF sputum, but there was a significant increase in yield after 24 hours when grown in DNase treated CF sputum, the data reveals that degraded DNA can modify gene expression after 3 hours of exposure that allows for growth of *P. aeruginosa*. Since carbon metabolism is activated when sputum was treated with DNase this confirms utilisation of degraded DNA present within CF sputum DNA (figure 5.24.).



http://www.genome.jp/kegg-bin/show_pathway?pae01200+PA3195

Figure 5.24.

Figure 5.24: Carbon metabolism pathways in *P. aeruginosa* PAO1. The red arrow within the blue circle indicates where glyceraldehyde-3-phosphate dehydrogenase (PA3195) acts. The green arrows indicate the ED pathway and lower EMP pathway as previously shown in figure 1.4 (Carbon metabolism in PAO1).

Degraded DNA as a nutrient

As evidenced by gene expression data and growth studies, it appears that degraded DNA is a nutrient for *P. aeruginosa* in the CF airway. The data in this study has revealed that *codA (PA0437)* is upregulated when sputum is DNase treated. The gene *codA* specifically encodes the enzyme cytosine deaminase which catalyses the conversion of cytosine to uracil and 5-methylcytosine to thymine (5-methyluracil) (figure 5.25b.). Notably, this study has demonstrated that thymine can be utilised as a nutrient source for growth as shown in section 4.3.4.2. However, degraded DNA may not only serve as a nutrient, but may also have a role in *Pseudomonas* virulence in the airway because a previous study by Ueda and colleagues (2009) revealed that uracil has a role in the virulence of *Pseudomonas* by influencing quorum sensing and biofilm formation. Therefore, whilst the presence of intact DNA hinders bacterial clearance and aids biofilm formation, it appears that *P. aeruginosa* upregulates genes in the presence of degraded DNA that enable adaptation to the new environment following DNase treatment so that uracil is produced and can promote its survival.

The literature reveals that there are three pathways by which bacteria degrade pyrimidines (Vogels and van der Drift, 1976). The most prevalent of these is the reductive pathway by which thymine and uracil are converted to β-aminoisobutyric acid and β-alanine (Soong et al, 2001). This has been demonstrated in *P. aeruginosa* where specifically it was shown that nitrogen within uracil and thymine could be utilised as a nutrient source (Kim and West, 1991). Evidence for this pathway has not only been observed in *P. aeruginosa* but also other *Pseudomonas* species including *P. fluorescens*, *P. syringae*, and *P. putida* (West, 1988; Gant et al, 2007; West, 2001). Whilst previous studies have demonstrated reductive pathway activation in many *Pseudomonas* species, my data suggests that the reductive pathway may occur in CF patient sputum following treatment with DNase. This is because my data has demonstrated the upregulation of *codA (PA0437)*. It therefore appears that *P. aeruginosa* can utilise degraded DNA within CF patient sputum as a nutrient source via the upregulation of cytosine deaminase to produce uracil or thymine so that it can be degraded via the reductive pathway of pyrimidine metabolism. This could be a mechanism by which *Pseudomonas* thrives in the CF lung environment.

This reductive pathway involves three steps. Initially, uracil and thymine are converted to dihydrouracil and dihydrothymine, respectively, via the enzyme, dihydropyrimidine dehydrogenase. Two further enzymatic reactions then occur which lead to the production of β-aminoisobutyric acid and β-alanine (Vogels and van der Drift, 1976). A study by Kim and West (1991) revealed that maximum dihydropyrimidine dehydrogenase activity in *P. aeruginosa* was observed when NADH acts as the nicotinamide cofactor for reduction of uracil and thymine instead of NADPH, which was preferential in *P. fluorescens* and *P. lemonnieri* (Santiago and West, 1999; Burnette et al, 2006). Interestingly, studies by Kim and West (1991) also revealed that maximum dihydropyrimidine dehydrogenase activity in *P. aeruginosa* was induced by the presence of uracil as a nitrogen source together with glucose as a carbon source, but activity was reduced when grown in glucose alone. Alternatively, thymine was required as a nitrogen source for maximum activity in *P. stutzeri* and succinate was required as a carbon source for maximum activity in *P. fluorescens* (Xu and West, 1992; Santiago and West, 1999). This therefore highlights that different *Pseudomonas* species require altered combinations of nutrients for maximum enzyme activity for pyrimidine metabolism. Based on the literature, it would be interesting in future studies to measure dihydropyrimidine dehydrogenase activity in *P. aeruginosa* clinical isolates to assess whether these pathways are active *in vivo*. It is important to note that dihydropyrimidine dehydrogenase activity was not investigated in this study because it was necessary to first confirm that *Pseudomonas* metabolism pathways were activated in response to DNase treatment.

These previous findings demonstrating that glucose and uracil are needed for maximum dihydropyrimidine dehydrogenase activity in *P. aeruginosa* are interesting because glucose has previously been implicated in driving the growth of respiratory pathogens in CF (Kim and West, 1991; Garnett et al, 2013). Therefore, it could be that the presence of elevated glucose levels together with degraded DNA could be promoting biofilm formation. This is because previous studies by Ueda and colleagues (2009) have demonstrated that uracil is essential for quorum sensing and biofilm formation. Additionally, because previous growth studies in section 4.3.4.2. demonstrated that *P. aeruginosa* could utilise deoxyribose as a nutrient source for growth, it would also be interesting to determine how the combination of deoxyribose within degraded DNA

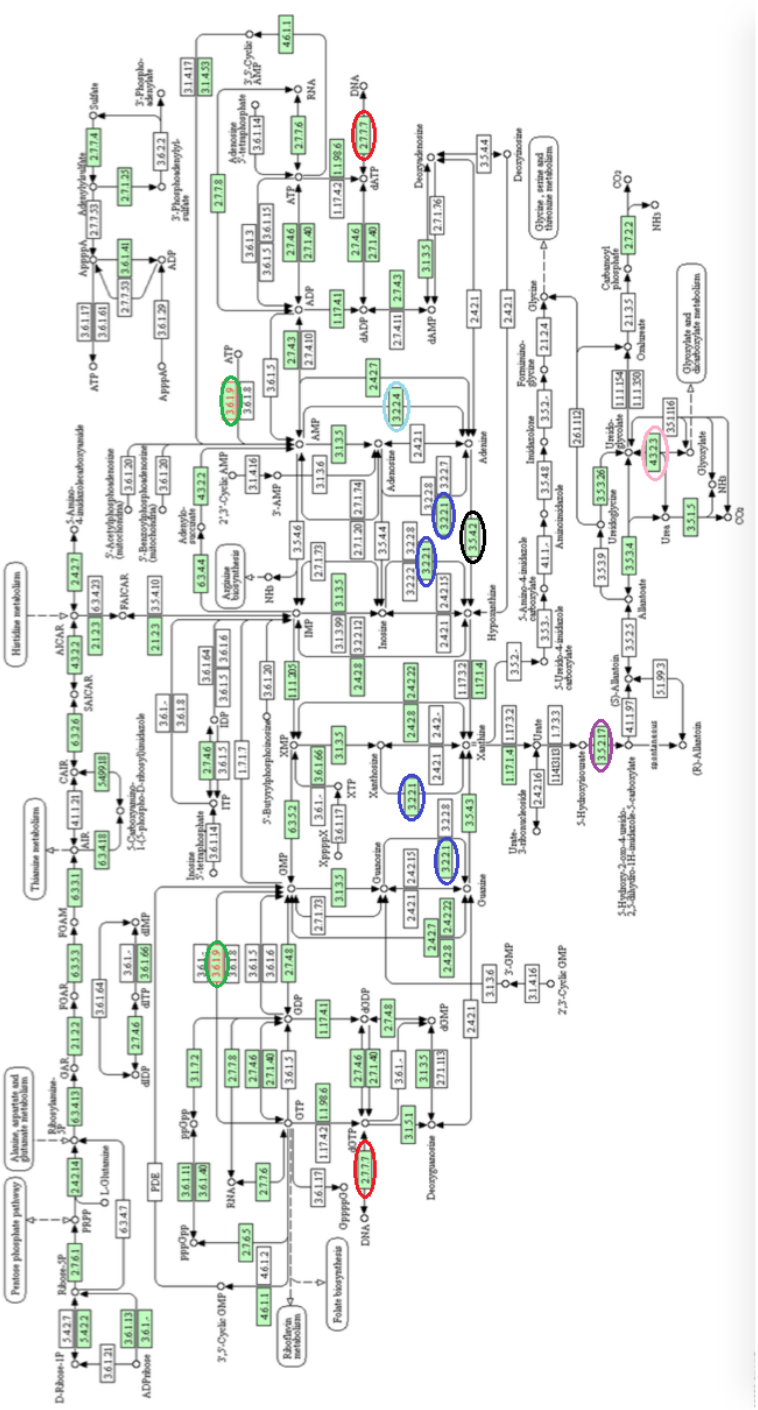
together with uracil or thymine influences dihydropyrimidine dehydrogenase activity. It would then be of further interest to compare the enzyme activities in the presence and absence of glucose vs. deoxyribose together with pyrimidines. This would be important to determine since it would shed light on possible mechanisms by which *Pseudomonas* thrives in the CF airway, since glucose is known to already to be elevated and deoxyribose is also likely to be in abundance because of inhaled dornase alfa (Baker et al, 2007; Brennan et al, 2007; Garnett et al, 2013).

Importantly, it is not just in CF that *Pseudomonas* persists as a problem, but also in CF patients with diabetes, non-CF bronchiectasis, chronic obstructive pulmonary disease, and primary ciliary dyskinesia (Merlo et al, 2007; McShane et al, 2013; Gallego et al, 2014; Alanin et al, 2015). Interestingly in CF patients with diabetes where glucose levels are even more elevated, there is a 6-fold increase in mortality and an increase in pulmonary exacerbations with *P. aeruginosa.* Despite good compliance with inhaled therapies deterioration in lung function still occurs for reasons that are currently unknown (Merlo et al, 2007). Therefore, in view of the data obtained in this project, perhaps this can be explained by the preferential combination of available uracil because of DNA degradation together with higher levels of glucose resulting in maximised activity of the enzyme dihydropyrimidine dehydrogenase (Millia et al, 2000; Kim and West, 1991; Finkelstein et al, 1988). For example, whilst glucose concentrations have been shown to be approximately 0.4 mM in healthy subjects, in CF patients it is approximately 2 mM, and in CF patients with diabetes glucose it is approximately 4 mM (Baker et al, 2007; Garnett et al, 2013). Furthermore, it has been shown in more recent studies that in individuals with diabetes there is increased production of NETs (containing DNA) from neutrophils (Wong et al, 2015). Therefore, the combination of elevated glucose with increased production of uracil in the presence of dornase alfa is likely to contribute to increased *P. aeruginosa* exacerbations in CF patients with diabetes. It therefore may be worth assessing whether dornase alfa is an appropriate treatment for these patients.

Whilst uracil in combination with glucose has been reported to be the preferential combination for maximum dihydropyrimidine dehydrogenase activity, the data in this study revealed that *dht (PA0441)* which encodes the enzyme dihydropyrimidinase

involved in the next stage of the reductive pathway of catabolism of uracil was not upregulated. It could therefore be that uracil is used for biofilm formation whilst thymine is utilised instead as a nutrient source for growth. Alternatively, uracil could be degraded via another pathway which was not investigated using the primers in this study. Further work could therefore focus on determining the nutritional preferences of *P. aeruginosa in vivo* to determine what specifically *P. aeruginosa* is utilising as a nutrient source within degraded DNA.

Finally, although differences in gene expression have been identified, it must be appreciated that gene expression of PAO1 was only investigated after 3 hours of exposure to DNase treated sputum. Further studies could therefore assess whether gene expression is consistently altered following exposure to degraded DNA after 6, 9, and 12 hours in samples. This was therefore a limitation of the study because there was not enough patient sample for further gene expression work. Though, it must be appreciated that the same patient samples were used for mucin purification through to gene expression studies. To eliminate this problem, further gene expression work could use commercially available salmon sperm DNA to elucidate metabolic preferences of *P. aeruginosa*.



http://www.genome.jp/kegg-bin/show_pathway?pae00230

Figure 5.25a.

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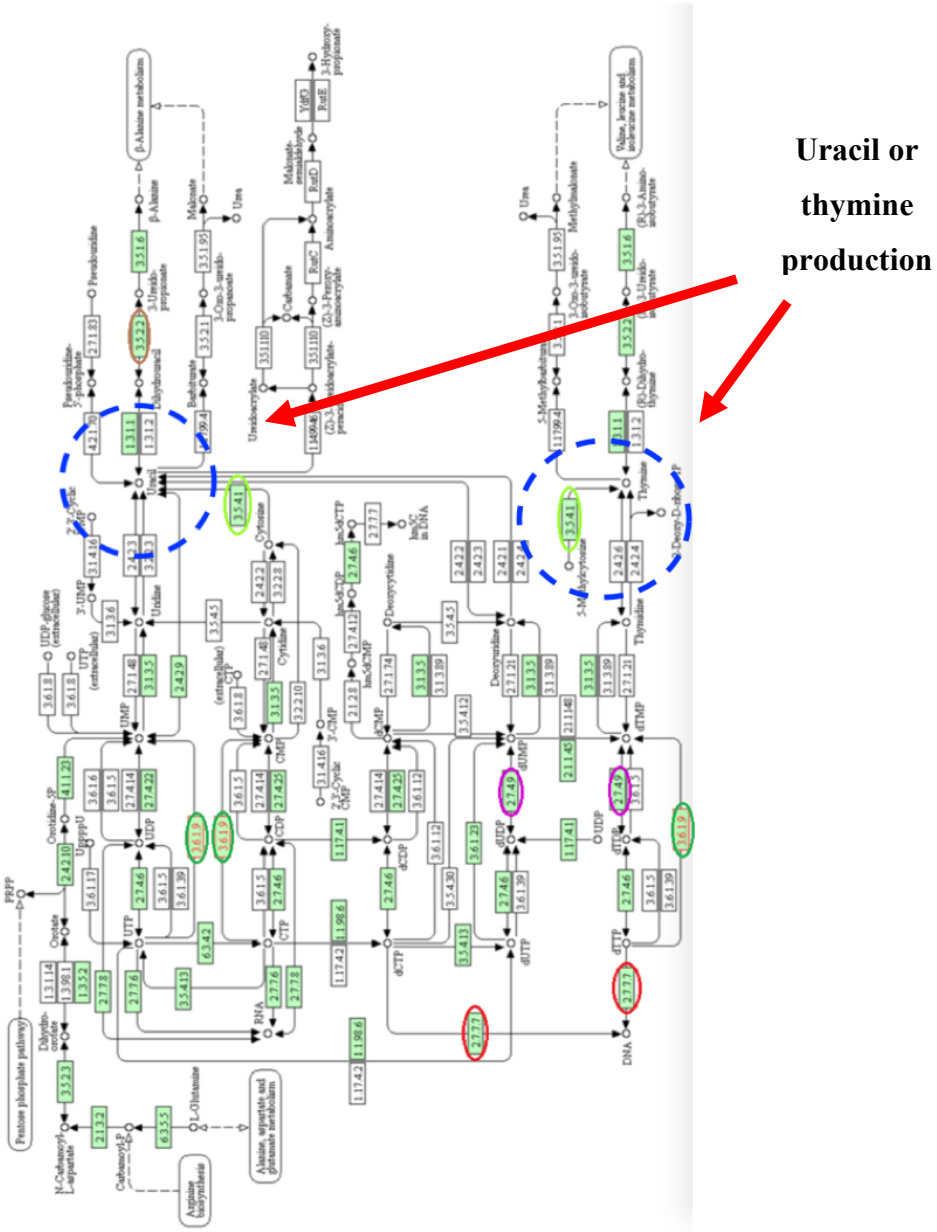

http://www.genome.jp/kegg-bin/show_pathway?pae00240

Figure 5.25b.

Figure 5.25: a) Purine metabolism pathway in *P. aeruginosa* PAO1. Genes investigated are shown in the coloured oval circles: *PA5493* (red), *PA0143* (dark blue), *PA0935* (dark green), *PA1518* (purple), *PA1514* (light pink), *PA3970* (light blue), and *PA0148* (black). b) pyrimidine metabolism pathway in in *P. aeruginosa* PAO1. Genes investigated are shown in the coloured oval circles: *PA5493* (red), *PA0935* (dark green), *PA2962* (magenta), *PA0437* (light green), and *PA0441* (brown).

5.5. Chapter conclusions

The fourth and final aim of this project was as follows:

4) To identify *P. aeruginosa* genes involved in nutrient metabolism which are upregulated or downregulated when exposed to CF sputum.

Based on the data obtained in this chapter, following exposure to degraded DNA in CF patient sputum *P. aeruginosa* has been shown to upregulate several genes involved in the further degradation of DNA. The data has shown pathways involved in carbon metabolism are upregulated in addition to pathways leading to uracil and thymine production. Together with the existing literature, it appears that *P. aeruginosa* is able to grow in degraded DNA due to activation of the reductive pathway which allows for utilisation of the pyrimidines, uracil and thymine, as nutrient sources. The findings of this study are novel because for the first-time degraded DNA has been identified as a viable nutrient source for *P. aeruginosa* in the CF lung environment by using CF patient sputum. This finding is important because DNA is measured at high concentrations in the CF airway and therefore needs to be addressed further to identify the long-term effect of degraded DNA on the CF microbiome both in terms of providing a nutrient source and pathogen virulence.

Chapter 6. Conclusion

6.1. Purpose of the study

Pseudomonas aeruginosa is an important pathogen in CF because up to 80% of adult CF patients become chronically colonised with this organism (McCallum et al, 2001). Once acquired it is very difficult to eradicate. Consequently, *P. aeruginosa* is the major cause of morbidity and mortality due to a severe decline in lung function in these patients (Emerson et al, 2002). Mechanisms by which *P. aeruginosa* thrives in the CF airway currently remain unclear because its nutritional requirements are not fully understood. Because mucus is in abundance in the CF airway this study set out to investigate whether *P. aeruginosa* can utilise airway mucin within mucus as a nutrient source for growth. This was important because identification of nutrients that mediate *Pseudomonas* survival may facilitate the development of novel therapies that could exploit these *Pseudomonas*-nutrient interactions with the aim of reducing the potential of *Pseudomonas* to colonise and thrive in the airway.

6.2. Further work

Based on the data discussed in this thesis, presented in table 6.1 are hypotheses and aims which have stemmed from this project.

6.3. Conclusion

This study set out to answer the question: "Does altered airway mucus composition promote chronic respiratory infections in cystic fibrosis by providing a nutrient source for pathogenic bacteria?" Although the hypothesis was disproved because it was found that *P. aeruginosa* was unable to utilise mucin originating from CF patient sputum as an immediate nutrient source for growth, instead, following DNase treatment it was demonstrated that growth of *P. aeruginosa* was promoted due to utilisation of available degraded DNA within CF patient sputum as a nutrient source. Therefore, it is likely in the CF airway, following dornase alfa treatment, that when degraded DNA is liberated it alters the airway mucus composition in a manner that promotes *Pseudomonas* growth.

This ability of *Pseudomonas* to utilise degraded DNA from CF patient sputum naturally presents a conundrum in the treatment of CF patients. The benefit of dornase alfa in improving lung function cannot be denied (Zahm et al, 1995), but the data suggests a highly novel role of degraded DNA in CF disease and the pathogenicity of *P. aeruginosa.* It is also important to acknowledge that *Pseudomonas* infection does not solely occur in CF, but there are increased pulmonary exacerbations in patients with diabetes (Merlo et al, 2007), non-CF bronchiectasis (McShane et al, 2013), chronic obstructive pulmonary disease (Gallego et al, 2014), and primary ciliary dyskinesia (Alanin et al, 2015). Therefore, further work in this area could make an important impact by identification of future therapeutic targets that could lead to the development of a novel drug class which could reduce pseudomonad growth. Hence, this would not be solely limited to its use in CF, but could be used to treat other respiratory diseases where pseudomonads colonise the airway.

Table 6.1: Summary of hypotheses and aims arising from this study

Hypotheses	Aims
- *P. aeruginosa* mutants lacking genes which are normally upregulated in the presence of degraded DNA are unable to grow in the presence of degraded DNA. - Other respiratory pathogens can utilise degraded DNA as a nutrient source for survival. - *P. aeruginosa* strains lacking genes required for metabolism of degraded DNA are unable to form biofilms and establish respiratory infection in healthy animal models. - *P. aeruginosa* strains lacking genes required for metabolism of degraded DNA have reduced biofilm formation and delayed colonisation in CF animal models. - Dihydropyrimidine dehydrogenase is activated in *P. aeruginosa* CF clinical isolates. - Dihydropyrimidine dehydrogenase is activated in *P. aeruginosa* PAO1 when grown in CF sputum treated with DNase - Dihydropyrimidine dehydrogenase is activated in *P. aeruginosa* PAO1 when grown in degraded salmon sperm DNA - Degraded DNA is a preferred nutrient for *P. aeruginosa* over amino acids in the CF lung. - Degraded DNA in combination with glucose causes increased activation of dihydropyrimidine dehydrogenase involved in pyrimidine metabolism. - *P. aeruginosa* prefers pyrimidines over purines as a nutrient source for growth.	- Identify other genes involved in DNA degradation which are upregulated/downregulated in *P. aeruginosa* - Assess *P. aeruginosa* gene expression at 6, 9, and 12 hours following exposure to untreated and DNase treated CF sputum - Compare gene expression in DNase and non-DNase treated sputum to gene expression when exposed to glucose and salmon sperm DNA (degraded and non-degraded). - Identify whether the presence of genes involved in DNA degradation are required for growth of *P. aeruginosa* in CF patient sputum. - Identify whether other respiratory pathogens can use degraded DNA as a nutrient source - Establish mechanisms involved in the degradation and metabolism of degraded DNA - Identify preferred substrates within DNA, i.e. purines or pyrimidines - Establish in an *in vivo* CF model whether time to chronic infection with *P. aeruginosa* can be delayed in animals inoculated with *P. aeruginosa* mutants (degraded DNA gene mutants). - Establish in an *in vivo* CF model whether biofilm formation can be reduced when inoculated with *P. aeruginosa* mutants. - Establish whether biofilms are formed in the airways of healthy animal models when inoculated with *P. aeruginosa* mutants. - Establish whether infection is prevented in healthy animal models when inoculated with *P. aeruginosa* mutants. - Identify mechanisms by which growth in degraded DNA can be reduced. e.g. competitive inhibition using molecules which mimic degraded DNA but cannot be metabolised. - Identify potential molecules which can bind degraded DNA present in airway so it can still be cleared efficiently, but cannot be broken down and used as a nutrient source. - Assess production levels of the enzyme, dihydropyrimidine dehydrogenase, in *P. aeruginosa* CF clinical isolates, in *P. aeruginosa* PAO1 when grown in CF sputum treated with DNase, and in *P. aeruginosa* PAO1 when grown degraded salmon sperm DNA, in the presence and absence of glucose and amino acids.

Chapter 7. Appendices

7.1. Appendix 1 – Preliminary experiments

7.1.1. Growth of *Pseudomonas aeruginosa* reference strains PAO1 and PA14 in cystic fibrosis patient sputum supplemented with Sicard's

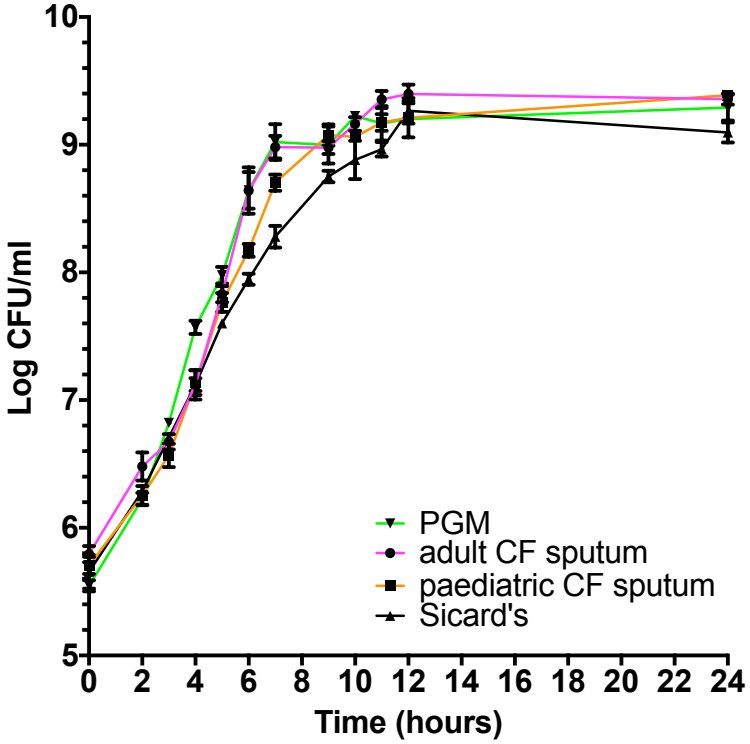

Figure 7.1a.

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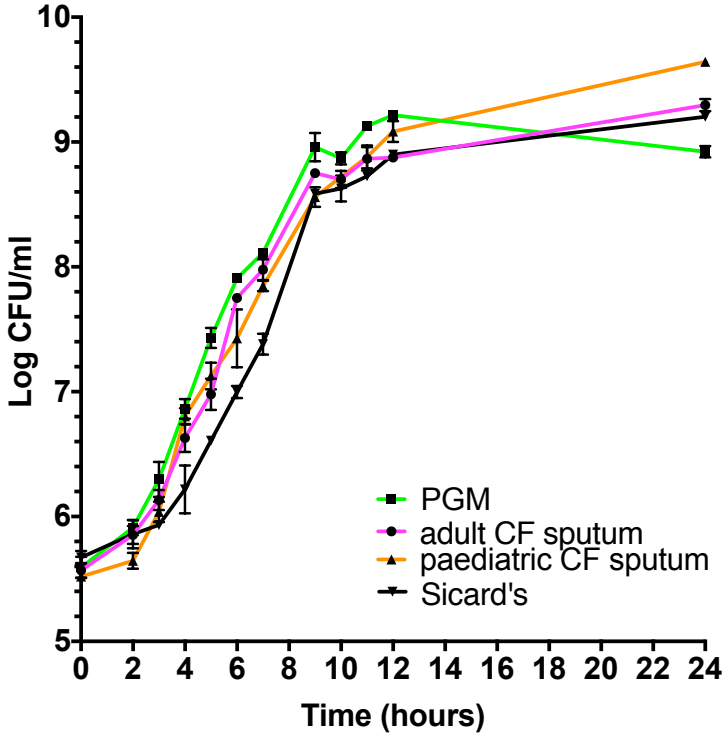

Figure 7.1b.

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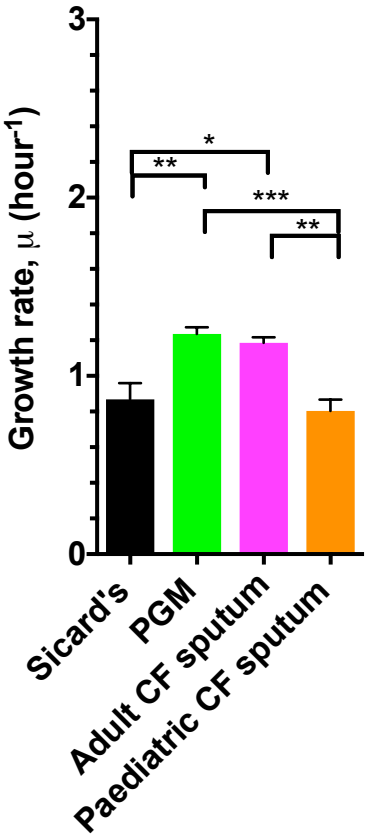

Figure 7.1c.

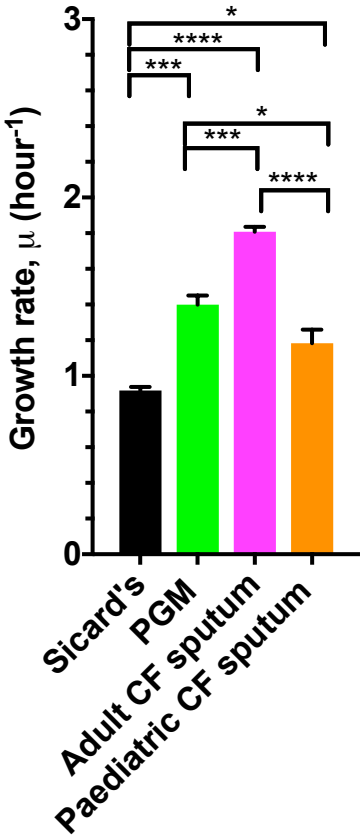

Figure 7.1d.

Figure 7.1: a) Growth of *Pseudomonas aeruginosa* PAO1 growth (log CFU/ml) over 24 hours in 0.4% (w/v) PGM, adult CF sputum, and paediatric CF sputum supplemented with Sicard's. The pink (adult CF sputum), orange (paediatric CF sputum), and green (PGM) lines indicate the mean log CFU/ml of 4, 5, and 4 biological replicates respectively at each time point. b) Growth of *Pseudomonas aeruginosa* PA14 growth (log CFU/ml) over 24 hours in 0.4% (w/v) PGM, adult CF sputum, and paediatric CF sputum supplemented with Sicard's. The pink (adult CF sputum), orange (paediatric CF sputum), and green (PGM) lines indicate the mean log CFU/ml of 5 biological replicates at each time point. c) Mean specific growth rate, μ (hour^{-1}), of PAO1. d) Mean specific growth rate, μ (hour^{-1}), of PA14. Error bars show standard error of the mean (SEM). P values on the graphs (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

7.1.2. Growth of *Pseudomonas* clinical isolates in Sicard's

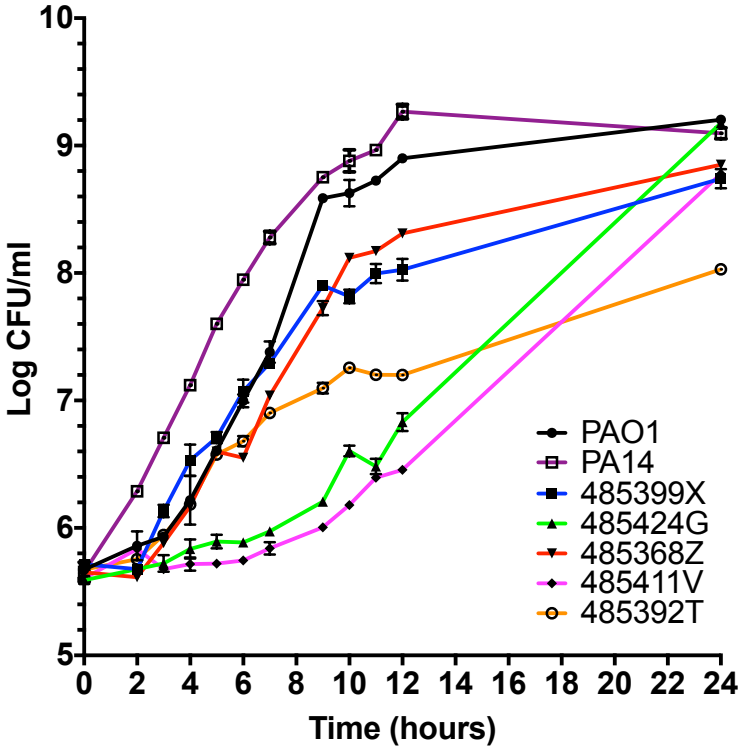

Figure 7.2a.

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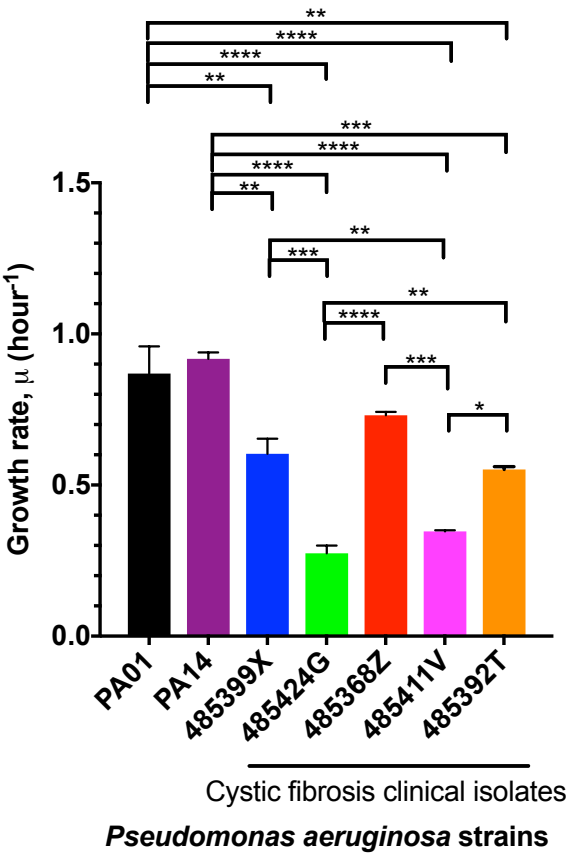

Figure 7.2b.

Figure 7.2: a) Growth (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 (black line/bar), PA14 (purple line/bar), and clinical isolates: 485399X (blue line/bar), 485424G (green line/bar), 485368Z (red line/bar), 485411V (pink line/bar), and 485392T (orange line/bar) growth (log CFU/ml) over 24 hours in Sicard's. b) Mean specific growth rate, μ (hour^{-1}). Data represent the mean from 3 biological replicates. Error bars show standard error of the mean (SEM). P values on the graph (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

7.1.3. Growth of *Pseudomonas* clinical isolates in PGM supplemented with Sicard's

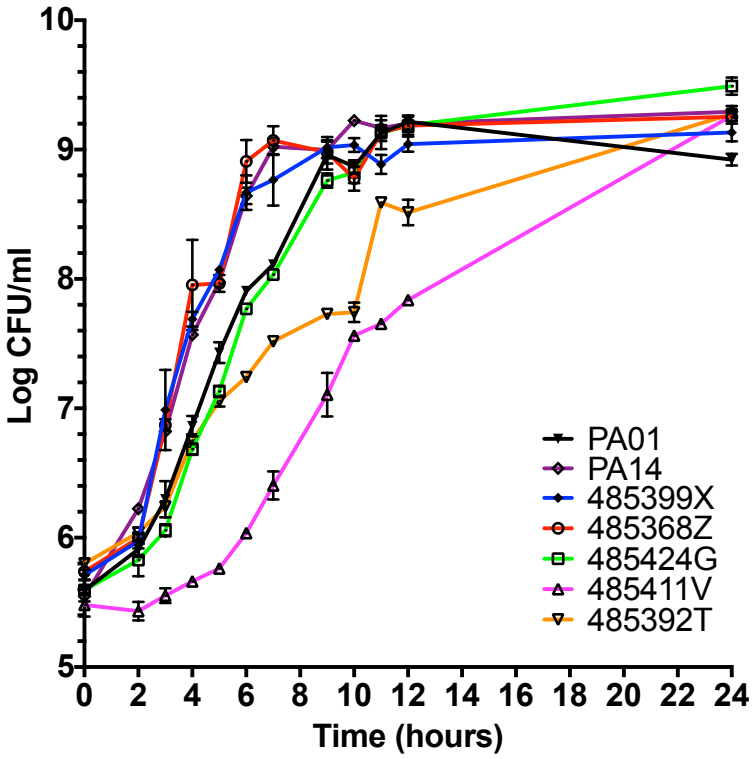

Figure 7.3a.

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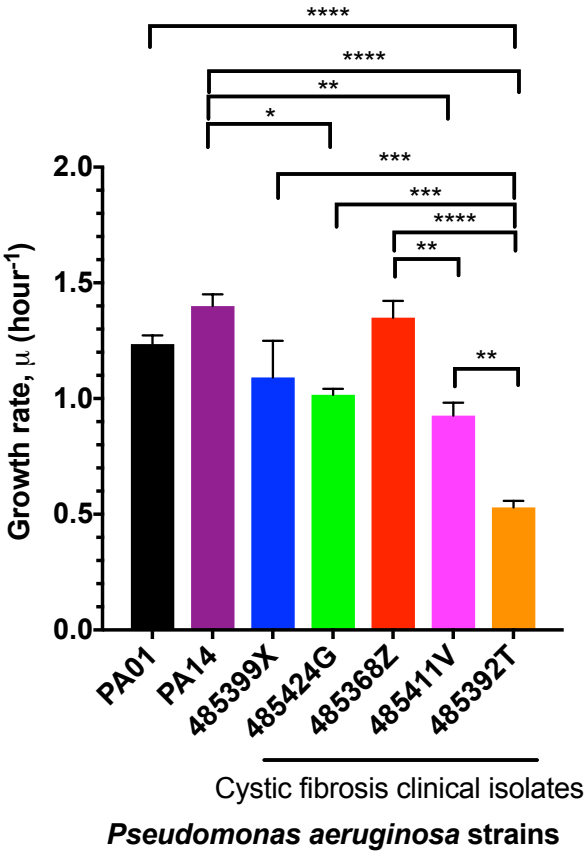

Figure 7.3b.

Figure 7.3: a) Growth (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 (black line/bar), PA14 (purple line/bar), and clinical isolates: 485399X (blue line/bar), 485424G (green line/bar), 485368Z (red line/bar), 485411V (pink line/bar), and 485392T (orange line/bar) growth (log CFU/ml) over 24 hours in 0.4% (w/v) PGM supplemented with Sicard's. b) Mean specific growth rate, μ (hour^{-1}). Data represent the mean of 3 biological replicates. Error bars show standard error of the mean (SEM). P values on the graph (**p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

7.1.4. Growth of *Pseudomonas* clinical isolates in adult and paediatric CF sputum supplemented with Sicard's

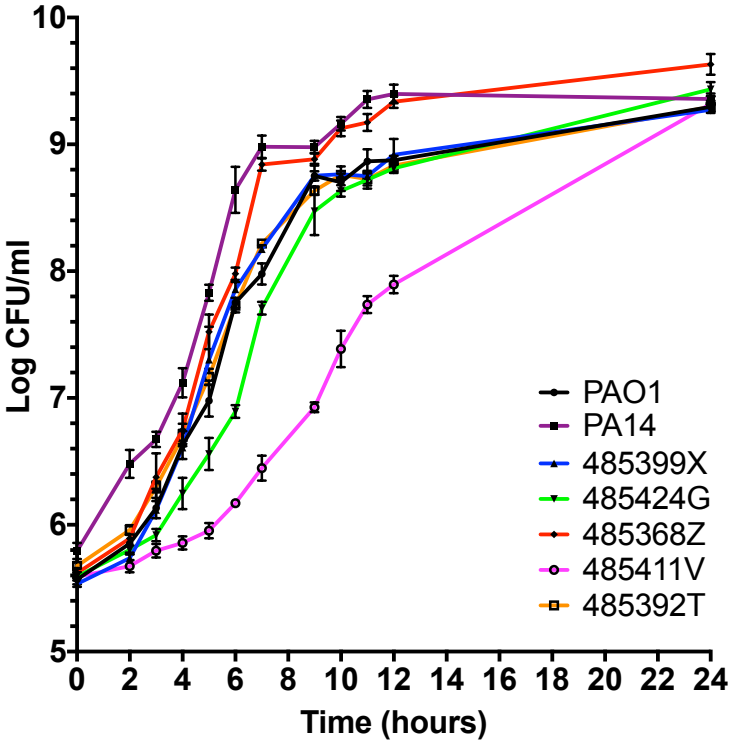

Figure 7.4a.

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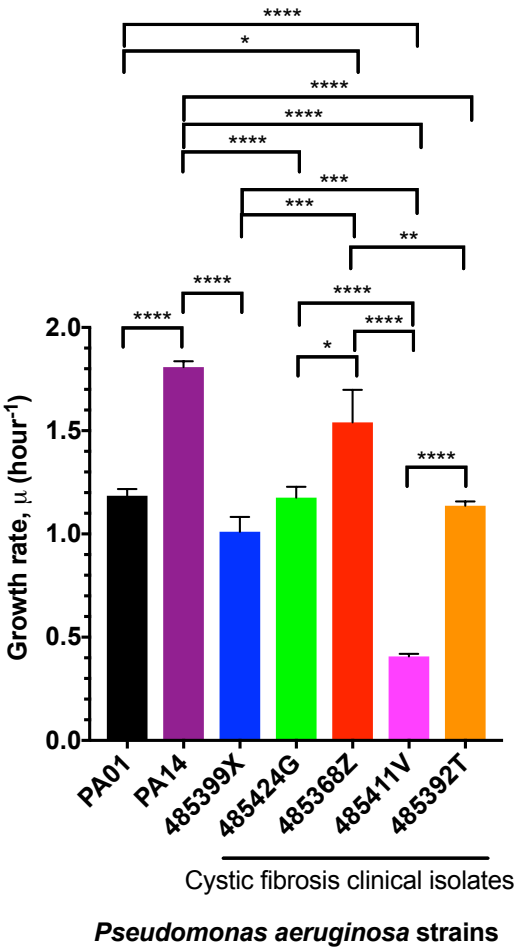

Figure 7.4b.

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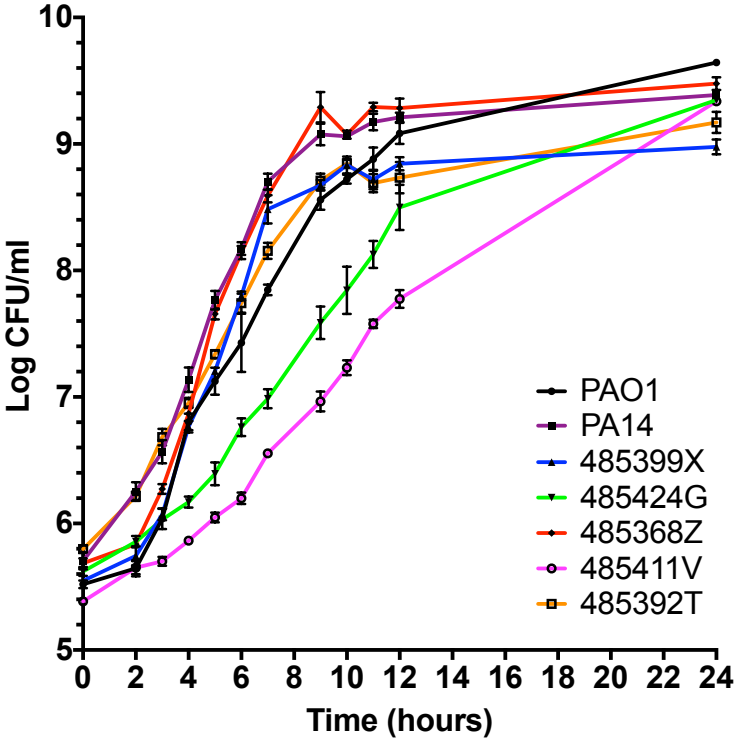

Figure 7.4c.

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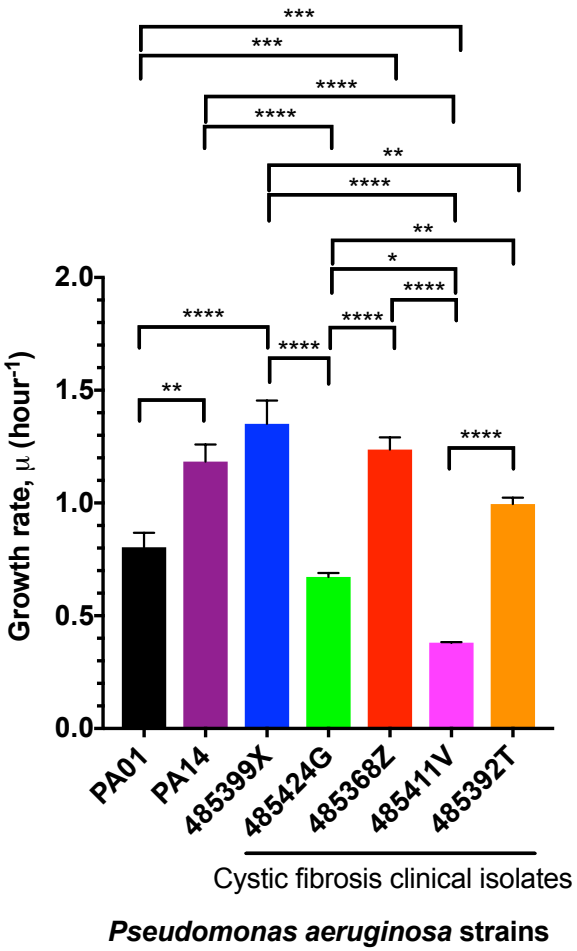

Figure 7.4d.

Figure 7.4: Growth (log CFU/ml) of *Pseudomonas aeruginosa* PAO1 (black line/bar), PA14 (purple line/bar), and clinical isolates; 485399X (blue line/bar), 485424G (green line/bar), 485368Z (red line/bar), 485411V (pink line/bar), and 485392T (orange line/bar) growth (log CFU/ml) over 24 hours in 0.4% (w/v) adult CF sputum (a.) and paediatric CF sputum (c.) supplemented with Sicard's. b) Mean specific growth rates, μ (hour^{-1}), in adult CF sputum. d) Mean specific growth rates, μ (hour^{-1}), in paediatric CF sputum. Data represent the mean from 4 biological replicates. Error bars show standard error of the mean (SEM). P values on the graphs (** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) were obtained by a one-way ANOVA followed by Tukey's multiple comparisons test.**

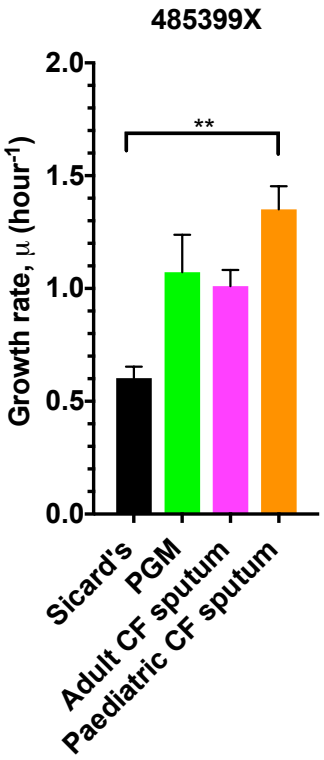

Figure 7.5a.

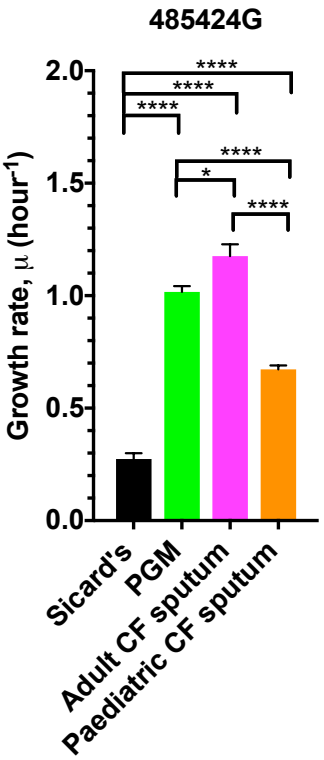

Figure 7.5b.

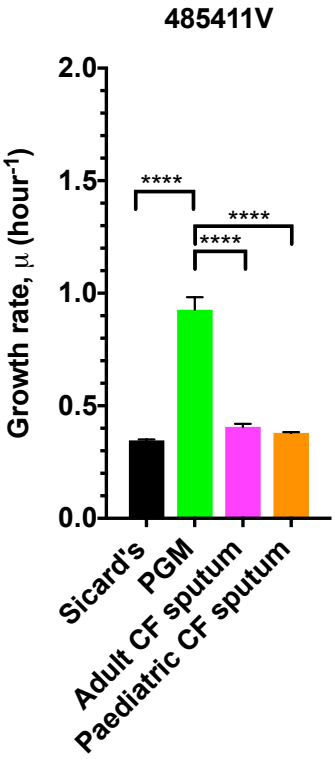

Figure 7.5c.

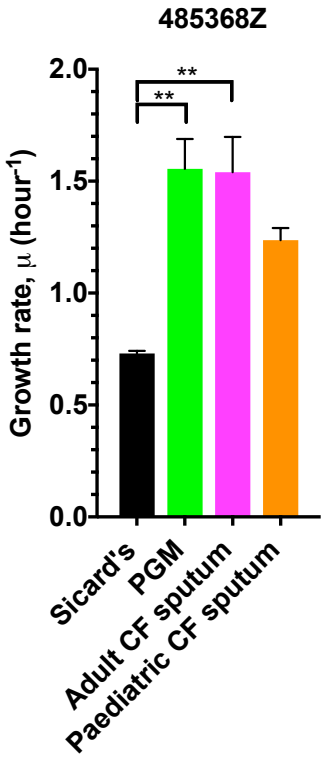

Figure 7.5d.

See legend on the next page

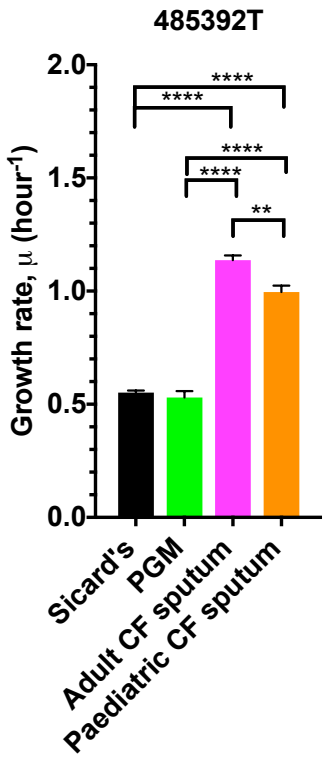

Figure 7.5e.

Figure 7.5: Mean specific growth rates, μ (hour^{-1}) of *Pseudomonas* clinical isolates: a) 485399X, b) 485424G, c) 485411V, d) 485368Z, and e) 485392T when grown in 0.4% (w/v) PGM (green bar), adult (pink bar) and paediatric (orange bar) CF sputum supplemented with Sicard's (black bar). Data represent the mean from 4 biological replicates. Error bars show standard error of the mean (SEM). P values on the graphs (*p < 0.05, **p < 0.01, ****p < 0.0001) were obtained by a one-way ANOVA followed by a Tukey's multiple comparisons test.

7.2. Appendix 2 - Primers for qPCR

Table 7.1: Primers highlighted in green text were used for final qPCR experiments. Genes in black text are those which were not suitable for further study.

Gene name	Primer	Sequence 5'-3'	Primer Reference	Product Name
putA	PA0782 – F	AAGCTGATGGGCAAGCAGT	This study	proline dehydrogenase
	PA0782 – R	AGTCGGCCATGTATTTCTCG		
hpd	**PA0865 – F**	**CGAGATTCGCTACTTCGACA**	**This study**	**4-hydroxyphenyl pyruvatedioxygenase**
	PA0865 – R	**CATGGTATTCGCGGATGAAT**		
phhA	**PA0872 – F**	**CCAGGTCTGGAATACCCTGA**	**This study**	**phenylalanine-4-hydroxylase**
	PA0872 – R	**GGAGAACCCTGTTGATCTCG**		
hmgA	PA2009 – F	GCTATATCGCCGAGAACCAC	This study	homogentisate 1,2-dioxygenase
	PA2009 – R	CCGAGGTATTTCTGCACCAG		
bkdB	PA2249 – F	ATGCCTACCTGACCCAGGAT	This study	branched-chain alpha-keto acid dehydrogenase (lipoamide component)
	PA2249 – R	TAGCTGAAATGCGGGATGC		
lpdV	PA2250 – F	GAAGAAACACGGGGTCGAT	This study	lipoamide dehydrogenase-Val
	PA2250 – R	ATCGGTAGCTCGACGCTCT		
dadA	PA5304 – F	ACCATCCTCGACGAGACCTA	This study	D-amino acid dehydrogenase, small subunit
	PA5304 – R	AGGTCGGTGGTGATCATTTC		
	PA2322 – F	GTGTTCCTGATGCTGCTGAA	This study	Gluconate permease
	PA2322 – R	GGCGTAGCCGAAGGTATAGA		
	PA2323 – F	ATACCATCGAAGCGCTGAAG	This study	probable glyceraldehyde-3-phosphate dehydrogenase
	PA2323 – R	AGGGTGGTGAAGGTTTCGTT		
	PA3181 – F	ATCCACACCGAGCAGTTTCT	This study	2-keto-3-deoxy-6-phosphogluconate aldolase
	PA3181 – R	ACACTTCCGAAGGCGTCAT		
oprB	**PA3186 – F**	**AGTTCCAGTTCACCGTCACC**	**This study**	**Glucose/carbohydrate outer membrane porin OprB precursor**
	PA3186 – R	**CGTCGAAGTACTGCTGCTTG**		
gapA	**PA3195 – F**	**CCATCGGATCGTCTCGAA**	**This study**	**glyceraldehyde 3-phosphate dehydrogenase**
	PA3195 – R	**TGGTACACGTCGGAGAGGTT**		
panA	PA2794 – F	GGCACGCTATGTGATTCCTC	This study	pseudaminidase
	PA2794 – R	CCATCTCCCACAGGAAGGTA		
nagE	**PA3761 – F**	**TGCACCACATCCTCAACAAC**	**This study**	**N-Acetyl-D-Glucosamine phosphotransferase system transporter**
	PA3761 – R	**ACATCATCACCGGGAACATC**		
	PA1432 – F	GGCTGGGACGTTAGTGTCAT	This study	autoinducer synthesis protein LasI
	PA1432 – R	CATGTAGGGGCCAGTGGTAT		
	PA4834 – F	GACCTGCTGAAGAGCGGGGT	Gi et al, 2015	putative nicotianamine synthase
	PA4834 – R	GCTTCCTTGACCAGGGCGTT		

	PA4835 – F	TACGTGCACAGTCCGTTCTT	Gi et al, 2015	hypothetical protein
	PA4835 – R	GATAGGTCCCTCCGGGTAGA		
	PA4836 – F	CTGTTCAACTATCCGGTGGAG	Gi et al, 2015	hypothetical protein
	PA4836 – R	TTCTCCAGGATCAGGGTGTC		
	PA4837 – F	ACCTTGACAGCGCTCGTATC	Gi et al, 2015	probable outer membrane protein precursor
	PA4837 – R	AAGCCACGGACGTTGTACTC		
rpoD	PA0576 – F	AAGGCCCTGAAGAAGCACGG	Gi et al, 2015	sigma factor RpoD
	PA0576 – R	GATCGGCATGAACAGCTCGG		
16S rRNA	**PA0668.1-F**	**TGTAGCGGTGAAATGCGTAG**	**This study**	**16S ribosomal RNA**
	PA0668.1-R	**ACGGCTAGTCGACATCGTTT**		
acpP	PA2966 – F	CCATCGAAGAACGCGTTAAG	This study	acyl carrier protein
	PA2966 – R	TCCTCTTCCAGAGCCATCAC		
pslB	PA2232 – F	GCGAGTTTCTCCTCAACACC	This study	PslB
	PA2232 – R	CGACCGTAGATGTCGTTGAA		
galU	PA2023 – F	GAGATCCAGATCACCGATGC	This study	UTP--glucose-1-phosphate uridylyltransferase
	PA2023 – R	TGCCGGTCTTGTAGAGGTTT		
polA	**PA5493 – F**	**AGGCGTTCCTGTCCTATCAA**	**This study**	**DNA polymerase 1**
	PA5493 – R	**CAGCCAGTTCTTGAACTCCA**		
dgt	PA1124 – F	CAGGGGTTGCTGGATTTCTA	This study	deoxyguanosinetriphosphate triphosphohydrolasE
	PA1124 – R	TTTCATGGCCTCCAGGTAAG		
ndk	PA3807 – F	AGAACGTGATCGGCGAAAT	This study	nucleoside diphosphate kinase
	PA3807 – R	CCGGAAGTCATGAAGGAAAC		
gda2	PA0134 – F	CCCAGTTCCAACCTGTTCAT	This study	probable guanine deaminase
	PA0134 – R	CGCAATTGCTGGATCTTGTA		
nuh	**PA0143 – F**	**TGCAGCCTTCTGCTAGGACT**	**This study**	**purine nucleosidase Nuh**
	PA0143 – R	**AACAGCAGGGCGATCACAT**		
	PA0935 – F	**TGCTGTTCCAGGTGGTCTATT**	**This study**	**nucleoside triphosphate pyrophosphohydrolase**
	PA0935 – R	**ATCGGGCTTGCCATAGAGAT**		
	PA1518 – F	**CGGGGTCTATCAACTGGTGT**	**This study**	**conserved hypothetical protein**
	PA1518 – R	**TAGCTGTAGGGCGAGATCAG**		
ureC	PA4868 – F	CACCATCCACACCTACCACA	This study	urease alpha subunit
	PA4868 – R	CAGACCATCAGCATGTCCAG		
arcC	PA5173 – F	CGGTCTACTCCAGGGAAGAA	This study	carbamate kinase
	PA5173 – R	CTTTCTCCAGCAGCCACTTC		
alc	**PA1514 – F**	**GCGATCATCAGCATCTTCAG**	**This study**	**ureidoglycolate hydrolaseYbbT**
	PA1514 – R	**TACAGGTACATCGCCAAGTG**		
add	**PA0148 – F**	**GCTGTCCAACACCAAGCTCT**	**This study**	**adenine deaminase**
	PA0148 – R	**CATGGAAGTTCTCGGTGACA**		
xdhB	PA1523 – F	ATGGAGGAACTGGTCTGGAA	This study	xanthine dehydrogenase
	PA1523 – R	CTTGGAATGGAACACGGTCT		
amn	**PA3970 – F**	**AGGAAGCCCACCATGAGTAT**	**This study**	**AMP nucleosidase**
	PA3970 – R	**TCCTTGATGTAGCGCTTGAG**		
surE	PA3625 – F	ACGGATTCATCAGCCTCAAC	This study	survival protein SurE
	PA3625 – R	CACGGTACCGGAATAGAGGA		
nrdB	PA1155 – F	TGCTGGAAGGCATCTTCTTC	This study	NrdB, tyrosyl radical-harboring component of class Ia ribonucleotide reductase
	PA1155 – R	CACGTCGATACCGAAGTTCA		

tmk	**PA2962 – F**	**AAGAGCACCAACCGCGACTA**	**This study**	**thymidylate kinase**
	PA2962 – R	**CATCAGCAACAGCTCGGTAT**		
hyuC	PA0444 – F	AATCCACCCAGCGACACAT	This study	N-carbamoyl-beta-alanine amidohydrolase
	PA0444 – R	ACCATTGCACGAAGAGATCG		
codA	**PA0437 – F**	**CACATCCATCTCGACACCAC**	**This study**	**cytosine deaminase**
	PA0437 – R	**GATCTGCCATTTCAGGGTCT**		
cmk	PA3163 – F	GTCAACCATGGTGTCGACCT	This study	cytidylate kinase
	PA3163 – R	ACGGATCACATCGGTCACTT		
dht	**PA0441 – F**	**CAGCTTCAAGCACTTCATGG**	**This study**	**dihydropyrimidinase**
	PA0441 – R	**CTGCAGGTGGAAGACCAGTT**		

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