An investigation of the polymicrobial nature of lower respiratory tract infections in cystic fibrosis patients

Andrew Nelson

PhD

2011

An investigation of the polymicrobial nature of lower respiratory tract infections in cystic fibrosis patients

Andrew Nelson

A thesis submitted in partial fulfilment of the requirements of the University of Northumbria at Newcastle for the degree of Doctor of Philosophy

Research undertaken in the School of Life Sciences and in collaboration with the Freeman Hospital, Newcastle upon Tyne.

April 2011

Abstract

Cystic fibrosis (CF) is a genetically inherited condition most prevalent amongst Caucasians. In previous studies, it has been demonstrated that bacterial, fungal and viral pathogens cause lung function decline and ultimately result in death due to respiratory failure.

Patients with CF produce sputum daily, which makes it an ideal infection to study in terms of access to samples. However, it is unknown how transport of the samples from the patient to the laboratory will affect the results of molecular microbiological analysis. We found that the bacterial community profiles were significantly different in samples stored at room temperature from those which were refrigerated. Furthermore, a significant increase in bacterial load and numbers of Pseudomonas spp. and a significant decrease in number of *H. influenzae* were seen in the samples stored at room temperature.

In this study we also aimed to characterise the factors which have an effect on the bacterial and fungal communities present in the CF lung in patients who possessed the F508del CFTR allele. We found that gender was a significant factor in the assembly of bacterial communities, due to a reduction in bacterial diversity and community evenness. Furthermore, we identified that *P. aeruginosa* colonisation affected bacterial community composition. We have also identified that bacterial community assembly in the CF lung appears to be stochastic. However, our data also shows that gender and *P. aeruginosa* colonisation affect assembly suggesting that, in some respects, a deterministic community assembly is also being observed. Our data also suggests that fungal communities are more diverse than is currently recognised. Additionally, we have found that patients who are homozygous for the F508del

CFTR mutation harbour more rich fungal communities than patients who are heterozygous.

A further objective was to follow these patients longitudinally to determine the stability of the CF lung microbiota, to determine the effects of antibiotic therapy, and to assess if any changes occurred in the CF lung during times of pulmonary exacerbation which could be identified as the causative agent. We did not find a significant relationship between exacerbations and the bacterial communities present in CF. However, in one patient we found that a particular bacterial taxa was present when the patient presented with an exacerbation but was absent when the patient was stable, suggesting that acquisition of a new bacterial taxa can potentially cause an exacerbation. We also found that an increase in bacterial load was not the cause of exacerbations in our cohort. Furthermore, the presence and abundance of fungal species was found not to be the cause of exacerbations.

Table of Contents

Table of Figures

Table of Tables

Acknowledgements

First and foremost, I would like to thank Prof. Steve Cummings for all of the help and encouragement he has given me with the practical work, and for the advice he has given me during the preparation of my thesis.

I would also like to thank Prof. John Perry giving me the opportunity to experience scientific research during my undergraduate degree, without which, I may not have embarked on a career in research. I would also like to thank him for his support during the project, especially regarding the clinical aspects of the microbiology.

I would like to show my gratitude to Audrey Nicholson for helping to collect the samples and for staying late at the Freeman hospital so that I could carry out my DNA extractions. I am grateful to Dr. Anthony De Soyza for his knowledge and advice regarding the clinical aspects of the project and Dr. Steve Bourke for selecting the patients and collating their clinical information.

I am indebted to many of my friends and colleagues from lab A307/321 both past and present. A special mention must go to Dr. Caroline Orr, who has been a great lab mate and has become a very good friend during my time at Northumbria University.

This thesis would not have been possible without the help and support from my family and friends. I am indebted to my mam; the support she has given me throughout my PhD has been invaluable, thank you.

Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. The work was carried out in collaboration with the Microbiology department, Freeman hospital, Newcastle upon Tyne.

Name:

Signature

Date

Abbreviations

ASL	Airway surface liquid
APS	Ammonium persulphate
AZI	Azithromycin
BALF	Bronchoalveolar lavage fluid
Bcc	*Burkholderia cepacia* complex
BMI	Body mass index
bp	Base pair
BSA	Bovine serum albumin
BV	Bacterial vaginosis
CCA	Canonical correspondence analysis
cDNA	Complementary DNA
CEF	Ceftazidime
CF	Cystic fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
CIP	Ciprofloxacin
Cl^-	Chloride ions
COL	Colomycin
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
eDNA	Extracellular DNA

DOX	Doxycycline
ENaC	Epithelial sodium channel
FEV_1	Forced expiratory volume in one second
FLU	Flucloxacillin
g	Gram(s)
x g	Gravity
gDNA	Genomic DNA
GEN	Gentamicin
G.I.	Gastrointestinal
ITR	Itraconazole
I.V.	Intravenous
Kb	Kilobase
LB	Luria-Bertani medium
lasB	*Pseudomonas aeruginosa* elastase
LMG	Laboratory of Microbiology, University of Ghent
LRT	Lower respiratory tract
m	Metre (s)
M	Molar
mA	Milliamps
MCC	Mucociliary clearance
MER	Meropenem
$MgCl_2$	Magnesium chloride
MIN	Minocycline

mRNA	Messenger RNA
MSD	Membrane spanning domain
Na^+	Sodium ions
NBD	Nucleotide binding domain
NCTC	National collection of typed cultures
OF	Oropharyngeal flora
OTC	Oxytetracycline
PCL	Periciliary layer
PMA	Propidium monoazide
PCR	Polymerase chain reaction
R	Regulatory domain
RDA	Redundancy analysis
Rf	Relative front
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RSV	Respiratory syncytial virus
S	Svedberg unit
SCFM	Synthetic Cystic Fibrosis Sputum Medium
SIBO	Small intestine bacterial overgrowth
SMG	*Streptococcus milleri* group
Spp.	Species
TAZ	Tazocin
TAE	Tris-acetate-EDTA

TEMED	N,N,N',N'-Tetramethylethylenediamine
TET	Tetracycline
TMD	Transmembrane domain
TOB	Tobramycin
U	Units
UC	Ulcerative colitis
U.V.	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume

1. Introduction

Cystic fibrosis (CF) is the most common lifespan reducing autosomal recessive condition in the U.K. Most of the morbidity and mortality in CF can be attributed to the chronic lung pathology caused by microbial infection and the subsequent inflammatory immune response. Traditionally, *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex have been seen as the key pathogens in CF due to their link with lung function decline (Cystic Fibrosis Foundation 2009). However, recent analyses using molecular techniques and more investigative culture techniques has demonstrated a wealth of microorganisms that were previously unidentified as colonisers of the CF lung, including many strictly anaerobic bacteria that had previously been regarded as contaminating commensal bacteria from the oral cavity (Rogers et al. 2003; Tunney et al. 2008). These studies have highlighted the drawbacks of microbial culture by revealing that the true microbial diversity of the CF lung may be one or possibly even two orders of magnitude greater than previously recognised and suggest that we need to reassess our understanding of CF microbiology (Rogers et al. 2003; Klepac-Ceraj et al. 2010). One approach would be to adopt a community based perspective to studies of CF microbiology that recognises the polymicrobial nature of this disease and acknowledges the potential contribution of the entire microbial community. This may lead to a more thorough understanding of chronic pulmonary infections based on the complex community involved, as well as demonstrating the role specific organisms, that are frequently found in CF infections, play in the pathogenesis of the disease.

Recent studies on the CF lung have utilised techniques that were traditionally applied to environmental microbiology to examine the relationship between the CF lung microbiology and patient specific factors (Klepac-Ceraj *et al.* 2010; van der Gast *et al.* 2010). These studies have found that bacterial colonisation of the lower respiratory tract of children with CF is influenced by CFTR genotype, the use of inhaled antibiotics and colonisation by *P. aeruginosa* (Klepac-Ceraj et al. 2010). Furthermore, a recent study has identified that bacterial diversity and community richness decreases with patients' age. Similarly, CF patients with a homo or heterozygous F508del mutation are more likely to acquire the emerging fungal pathogen *Geosmithia argillcaea* (Giraud et al. 2010). Further studies applying this knowledge may demonstrate linkages between chronic respiratory infections in CF to patient, environmental factors and the microbial ecology of the lungs which will lead to more effective treatment strategies to improve patient quality of life.

1.1 CFTR structure and function

Cystic fibrosis is a monogenic disease caused by mutations in a gene that codes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, the gene is approximately 250 kb in length and is located on the long arm of chromosome 7. This sequence encodes an mRNA molecule of 6.5 kb which is translated into a protein that is 1,480 amino acids long (Kerem *et al.* 1989; Riordan *et al.* 1989). The CFTR protein is a transporter for chloride ions (Cl^-) and a regulator of other ion channels which is located on the apical membrane of epithelial cells. At the apical membrane the CFTR protein forms a Cl^- channel that is responsible for the transport

of salts between the luminal space and the cytoplasm of the cell. The CFTR protein is made up of two hydrophobic membrane spanning domains (MSD) that each consists of six helical transmembrane domains (TMD): two hydrophilic nucleotide binding domains (NBD) associated with the TMD and a regulatory domain (R) (Riordan et al. 1989).

There are over fifteen hundred mutations identified in the CFTR gene to date that can cause CF (CF mutation database). Most CFTR mutations are due to missense (40.85%), frameshift (16.23%) or splice-site (12.39%) mutations of the CFTR gene (Dorfman et al. 2008). The genetic defects of CFTR have been grouped into five classes which are associated with the synthesis and maturation of CFTR protein (Welsh & Smith 1993). In class I, mutations result in no CFTR at the apical membrane due to transcription of a severely truncated mRNA molecule that is not translated into protein. Class II mutations also result in no CFTR expressed at the apical membrane but are caused by degradation of mis-folded proteins by the proteosome within the endoplasmic reticulum. Class III mutations result in some CFTR expression at the apical membrane but cannot be activated due to missense mutations. Class IV mutations result in expression of activated CFTR at the apical membrane but with limited functionality. Class V mutations result in expression of activated CFTR at the apical membrane but in lower numbers than in healthy individuals. Restoration of CFTR protein activity has been a major focus of CF research for over a decade with gene therapy being seen as the most viable treatment strategy. There have been two major vectors investigated to restore CFTR protein to the respiratory epithelium; viral vectors and liposomes (Hyde et al. 2000; Flotte & Carter 1997). However, no major clinical trials have been conducted to date due to

caveats in the systems leading to immune response to the vector or insufficient uptake by the respiratory epithelium (Atkinson 2008).

1.2 Epidemiology of CF

Epidemiology, in a clinical sense, is the study of the prevalence and distribution of a particular disease on a global scale in order to monitor any temporal changes. Many problems are encountered when studying the epidemiology of CF globally. In countries where CF is less prevalent, it is not screened for at birth, resulting in lower detected incidence rates when actually the incidence rate may be much greater (Raskin et al. 2008) . Even in countries where CF is screened for at birth, accurate identification is problematic due to non CF specific clinical manifestations and different genotypes resulting in different phenotypes which have differing clinical consequences that are difficult to detect (Farrell et al. 2008). The prevalence of a mutated CFTR gene in the U.K. is thought to be in the region of 1 mutated CFTR per 25 individuals of Caucasian decent (World Health Organisation 2002). Furthermore, the prevalence of CF is approximately 1 in every 2,600 live births in the U.K. but can be as low as 1 in up to 350,000 live births in Japan (World Health Organisation 2002). In the general U.K. population, CF is only responsible for approximately 0.17 deaths per 100,000. However, CF reduces lifespan and causes of 1-2% of all deaths in the U.K. in patients aged 5 – 24 (Walters & Mehta 2007). Nevertheless, survival of young patients with CF is improving which will hopefully see all CF patients surviving in to adulthood (Kulich et al. 2003).

The most common CFTR mutation is F508del which accounts for 66% of global mutations with the next most prevalent being G542X which is the cause of 2.4% of CF globally (Dorfman et al. 2008). However, F508del incidence varies greatly when observed on a global scale with incidence ranging from 90% in Danish populations to 20% in Turkish populations where other mutations are most prevalent. Many geographical relationships such as this are observed in CFTR prevalence which makes the origin or cause of the disease difficult to pinpoint (Walters & Mehta 2007).

There has been much speculation surrounding the cause of the high incidence levels of CFTR mutation and why different populations have markedly different distributions of each genotype. The first of these hypotheses was that CFTR heterozygotes had a fertility advantage over their peers (Jorde & Lathrop 1988). However, this theory was discredited in 1988 when a study on the Mormon population revealed that CFTR heterozygotes had no reproductive advantage over the control group (Jorde & Lathrop 1988). A second hypothesis was that there was a selective advantage of carriers against infectious diseases. For example, some evidence links the spread of F508del mutation across Europe with the decreased risk of intestinal infectious disease arising from cattle pastoralism (Alfonso-Sánchez et al. 2010). Most compellingly is the observed resistance to *Salmonella typhi* infection, the causative agent of typhoid fever, being significantly reduced in heterozygote and homozygote F508del population of mice compared to those expressing wild type CFTR (Pier et al. 1998). This study demonstrates that translocation of *S. typhi* into the intestinal submucosa of F508del heterozygote 'carrier' mice was 86% less than those expressing wild type CFTR and almost zero in the homozygote F508del population. There is also a correlation between CFTR

genotype and prognostic factors such as lung function, nutritional status, pancreatic insufficiency and *P. aeruginosa* colonisation (McKone et al. 2006). McKone *et al.* (2006) also demonstrated that high risk CF patients had a mean mortality of 24.2 years and low risk patients had a mean survival age of 37.6 years, highlighting the fact that more research needs to be carried out to improve both the length and quality of patients.

1.3 Systemic effects of CFTR mutation

As CFTR is found on the surface of epithelial cells the disease manifestations are present in many organs such as pancreas, gastrointestinal tract and lungs. Much of the problems in CF are due to impaired activity of CFTR that causes reduced chloride ion transport into the lumen which results in osmotic potential that drives water out of the lumen and in to the epithelium (Quinton 1990). Likewise, unregulated influx of sodium ions (Na^+) through the epithelial sodium channel (ENaC) exacerbates the movement of water out of the airway lumen resulting in dehydration of mucosal membranes (Stutts et al. 1995). This results in mucus plaque formation at the epithelial surface which can impair the function of the effected organs. In CF sufferers factors that affect nutrition can cause severe problems. In neonates meconium ileus can be the first clinical symptom of CF and effects 10-15% of CF sufferers, although it is not specific to CF (Ziegler 1994). Meconium is the first stool passed by a child and is made up of undigested products from its time in the uterus. Meconium ileus is the mechanical blockage of the ileum, due to viscous mucus, by the meconium in CF. However, G.I. tract complications do not end in

infancy. The small intestine is known to be colonised more readily by Gram-negative bacteria in CF sufferers than healthy individuals (Littlewood 1992; Lewindon *et al.* 1998; Fridge *et al.* 2007). Small intestine bacterial overgrowth (SIBO) may be due to several factors including; abnormal accumulation of mucus; suppression of gastric acid to maintain lipase activity and slowed intestinal transit (Borowitz et al. 2005). SIBO may be present in up to 50% of CF patients studied and it is thought that SIBO may contribute to malnutrition often observed in CF patients because of bacterial competition for nutrients and inflammation caused by bacterial enterotoxic metabolites (Borowitz et al. 2005). This hypothesis has been tested in a murine model where CF mice and wild type were compared (Norkina et al. 2004). The results of this study show that CF mice treated with antibiotics were only 10% smaller than the wild type mice, whereas the untreated CF mice were 30% smaller. Furthermore, 16S rRNA analysis of the small intestine showed significantly reduced bacterial species diversity with ~90% of the community belonging to the Enterobacteriaceae family (Norkina et al. 2004). After a link between SIBO and reduced ability to thrive in CF had been established, De Lisle (2007a) took this line of investigation further and examined gastric emptying and intestinal transit in CF mice. De Lisle (2007b) confirmed one hypothesis put forward by (Borowitz et al. 2005) in that small intestinal transit was slower in CF mice than wild type controls. De Lisle (2007a) then went on to show that treatment of CF mice with laxative reduced the inflammation, mucus accumulation and bacterial load in the small intestine as well restoring intestinal transit to normal levels. Analysis of inflammatory markers has provided evidence to support the claim that CF patients have increased intestinal inflammation and that this inflammation can be reduced by administration of probiotics (Bruzzese et al. 2004). Furthermore, a pilot, random,

placebo-controlled, cross-over study on 19 CF patients established that treatment with probiotics reduced the number of pulmonary exacerbations and hospitalisations than placebo treated patients and increased FEV_1 and body weight than those treated with the placebo suggesting that intestinal status in CF patients in related to pulmonary health (Bruzzese et al. 2007).

1.4 Respiratory immune dysfunction

Pulmonary disease in CF has been linked to several factors relating to CFTR dysfunction. Impaired activity of CFTR causes reduced Cl^- transport into the lumen of the bronchi which results in osmotic potential that drives water out of the airway lumen and in to the epithelium (Quinton 1990). This is exacerbated by unregulated influx of Na^+ through the ENaC pump causing the movement of water out of the airway lumen (Stutts et al. 1995). Both CFTR and ENaC transporters play an important role in the maintenance of the airway surface liquid (ASL) which is ~7 μm high in ciliated cells of healthy individuals and consists of two distinct layers (Tarran et al. 2001). The purpose of the lower or periciliary layer (PCL) and the upper mucus layers is to trap and transport inhaled bacteria out of the lower respiratory tract (LRT) assisted by "beating" of the cilia, a process known as mucociliary clearance (MCC). CF epithelial cell lines have shown that sufficient hydration of the ASL cannot be maintained. In CF, the mucus is severely dehydrated, due to unregulated ion transport, resulting in depletion of the PCL to a point where the mucus layer is extremely viscous causing the cilia to collapse resulting in cessation of MCC (Matsui et al. 1998). Furthermore, attachment of the dehydrated mucus layer to the

ciliated epithelium causes formation of mucus plaques which cannot be removed by cough clearance. The rate of MCC varies between CF patients that could be due to several factors such as the class of CFTR mutation, with patients having the less severe class IV and V mutations having greater MCC function (Boucher 2007). Other problems resulting from the cessation of MCC include cleavage of antimicrobial peptides that would normally aid bacterial clearance (Rogan et al. 2004) and migration of neutrophils (Matsui et al. 2005). Furthermore, CFTR can internalise *P. aeruginosa* from the epithelial cell surface which prevents the chronic colonisation that is seen in CF lung disease (Pier 2000). However, many CF patients will not constitutively express CFTR at the epithelial cell surface due to degradation by the proteosome, thus, allowing *P. aeruginosa* to colonise the airways. Other research has shown that acidification of lysosomes is insufficient in CFTR deficient mice (Di et al. 2006; Teichgräber et al. 2008). The first example of this phenomenon has shown that alveolar macrophages from CFTR deficient mice display normal internalisation but defective intracellular killing of *P. aeruginosa* (Di et al. 2006). Acidification of the lysosome in alveolar macrophages is known to be CFTR dependent (Barasch et al. 1991). Di *et al.* (2006) posited that the defective/absent CFTR present in CF sufferers would cause reduced acidification of the lysosome, resulting in survival of any internalised pathogen. Furthermore, it has been suggested that the anaerobic nature of the mucous plaques present in the CF lung prevent the generation of reactive oxygen species by leukocytes, resulting in further impairment in bacterial killing (Döring et al. 2011). The second mechanism regarding acidification in CF mice is due to alkalinisation of vesicles due to absence of CFTR (Teichgräber et al. 2008). The alkalinisation causes a disruption in the balance between formation and breakdown of ceramide leading to accumulation of ceramide

in the airways. The ceramide deposits result in an increase in inflammation resulting in epithelial cell death which, in turn, leads to DNA deposits in the respiratory tract. The DNA deposits can be used by respiratory pathogens, namely *P. aeruginosa*, for adherence which increases the risk of pulmonary infection (Teichgräber et al. 2008).

1.5 Sampling strategies for polymicrobial communities in CF

All studies to date that have been used to analyse the microbiota of the CF lung have used whole sputum samples or bronchoalveolar lavage fluid (BALF). The likelihood is that these samples are far too large to sample individual microbial communities and it is more probable that many communities are pooled making changes in the microenvironment difficult to tease out. Gutierrez *et al.* (2001) found, during analysis of BALF samples, that the bacteria isolated were not homogeneously distributed between lobes and advised that multiple lobes be sampled in order to get a comprehensive view of the microbiota. Likewise, research utilising computed tomography to identify disease in the lungs of children with CF demonstrated that during a pulmonary exacerbation, airway inflammation is confined to a particular area of the lung as opposed to being widespread (Davis *et al.* 2007). These data suggests that only a small proportion of the total microbial community throughout the lung is the causative agent of exacerbations.

A recent paper by Rogers *et al.* (2010) highlighted the need for more thorough sampling strategies to be applied to sputum from CF patients. In this study, they took an initial expectorated sputum sample then induced sputum four times at five minute intervals and found that each sample individually only harboured ~58% of the

overall microbial community observed from all of the samples in combination. It is, therefore, important to fully assess the merits of each sample type that can be obtained. BALF samples are advantageous because they bypass the oral cavity and directly sample the lung, thus reducing the risk of contamination from oral bacteria. However, these samples are invasive, unpleasant for the patient and not routinely sought due to easy expectoration of sputum. Most molecular studies based upon 16S rRNA gene have utilised expectorated sputum and identified metabolically active anaerobes commonly disregarded as merely contaminating mouth flora (Rogers et al. 2005). Rogers *et al.* (2006) went on to demonstrate no significant difference between sputum and mouthwash samples obtained from a set of CF patients. Similarly, Tunney *et al.* (2008) showed that anaerobic bacteria could be isolated, enumerated and identified by culture of sputum from the CF lung. They demonstrated that a mixed anaerobic population could be isolated from 64% of samples and, by quantitative culture, that these bacteria were present in similar or sometimes greater numbers than common CF pathogens such as *P. aeruginosa*. Furthermore, it has been demonstrated that anaerobes can persist in the lung and are not susceptible to common antibiotics used in the management of CF respiratory disease (Worlitzsch et al. 2009).

Both RNA and DNA can be used to analyse microbial communities in CF sputum, both of which have their relative merits. Traditionally, RNA extraction would be considered difficult due to its instability at room temperature which would need a stringent sample handling protocol to get the sample from the ward to the laboratory without degradation or a shift in expression pattern. However, the advent of RNA preservation agents has made the analysis of RNA much simpler. The major advantage of RNA extraction allows characterisation of the metabolically active

community and when, for example, aetiology is under examination, the causative agent is most likely to be metabolically active. However, in the case of microbial infection in CF it is the immune response which adds to the pathology and causes symptoms so there is a strong argument for analysing both DNA and RNA (Balough et al. 1995; Rapaka & Kolls 2009). Similarly, to achieve full coverage of respiratory viral communities then both DNA and RNA need to be examined. However, there is also a case for this when analysing bacteria because an organism that is present but not metabolically active may merely be dormant and when environmental conditions shift they can become active and play a part in the community function (Prosser et al. 2007). One method of tackling this problem in clinical samples has been demonstrated (Rogers et al. 2008; Rogers et al. 2010). Prior to DNA extraction, propidium monoazide (PMA) was incubated with the sample which intercalates with the DNA of dead cells and extracellular DNA (eDNA), live cells are not affected by the treatment because PMA cannot traverse the cell membrane. Rogers *et al.* (2008) show that untreated samples have extra taxa when compared to PMA treated suggesting that eDNA or DNA from non-viable cells is contributing to the microbial community fingerprint and thus causing an over estimation of microbial diversity at that time. The PMA treatment strategy was taken forward and it has also been proven that non-viable cells contribute to quantification of microbial communities using qPCR (Rogers et al. 2010).

In many animal and plant communities there are few species of many individuals and many more species of fewer individuals. This can also be said of microbial communities (Dunbar et. al. 2002). Dunbar *et al.* (2002) sampled bacterial populations from four Arizona soils in an attempt to assess the number of species present. In all four soil samples *acidobacteria* accounted for approximately 50 % of

clones where as there are many divisions which account for less than 5 % of clones. This research also highlights important issues about sampling strategy for assessment of microbial communities. Dunbar *et al.* (2002) estimate that in order to reproduce the top 50 % of bacteria in a population containing 4,000 species then a samples size of 285,400 individuals would be required to obtain a ≥ 95 % confidence limit.

1.6 Microbial community assembly in CF

The oral mucosa and upper respiratory tract are huge reservoirs of bacteria that, with the failing of MCC, can bypass the host defence system and colonise the lung (Bittar, Richet, et al. 2008; Rogers et al. 2004; Harris et al. 2007). Traditionally, children with CF will most likely suffer from transient infections caused by *S. aureus*, *H. influenzae* and *P. aeruginosa* respectively (Cystic Fibrosis Foundation 2009). However, a recent molecular study on BALF from children with CF (mean age ~9 y.o.) has contradicted this data and shown a complex microbial community is already established in the lung (Harris et al. 2007). Similarly, Bittar *et al.* (2008b) developed a bacterial community data set that showed children as young as one colonised with up to 8 different species of bacteria. These studies demonstrate that bacteria can invade and colonise the lung in young CF patients and that early colonisation of the CF lung may not be restricted to 'classic' CF pathogens. Duan *et al.* (2003) demonstrated in a single patient that *P. aeruginosa* and the "normal oral flora", which includes *Staphylococcus* and *Streptococcus* spp., are the only constant colonisers over an 11 year period. However, specific members of the "normal oral

flora" were not elucidated and it, therefore, cannot be concluded which members, if any, are chronic colonisers of the CF lung.

Routine work in pathology suggests that *S. aureus* and *H. influenzae* are early colonisers and are succeeded by mucoid phenotypes of *P. aeruginosa* by age 13 and members of the *Burkholderia cepacia* complex (Bcc) later in life (Cystic Fibrosis Foundation 2009). However, a study conducted that looked at intensity of anti-Staphylococcal therapy on *P. aeruginosa* acquisition showed that patients were more likely to acquire *P. aeruginosa* if they had received constant therapy that would eradicate *S. aureus* in contrast to patients who had no or impromptu therapy (Ratjen et al. 2001). An *in-vivo* study on rats suggests that *P. aeruginosa* can lyse *S. aureus* and use it as a source of iron in low iron environments (Mashburn et al. 2005). This could be a key finding relating to the development of chronic *P. aeruginosa* infection in the CF lung. Although the CF lung has a high iron content it remains uncertain whether the amount of available iron present is indeed elevated or depleted (Reid *et al.* 2002; Reid & Kirov 2004; Kim *et al.* 2003; Zeng & Kim 2004).

Two key publications examining the microbial communities of the CF lung have shed some light on the effects of intrinsic and extrinsic factors in community development (Klepac-Ceraj *et al.* 2010; Cox *et al.* 2010). The first of which demonstrated that younger CF suffers had a more diverse microbial community than older CF suffers and that this was, in part, due to the younger individuals being culture negative for *P. aeruginosa* (Klepac-Ceraj et al. 2010). The authors cite the time hypothesis that the reduced diversity seen in more elderly patients is due to chronic antibiotic therapy regimens selecting for taxa which are more suited to this type of habitat and eliminating those which are susceptible. The second paper supports the temporal finding of Klepac-Ceraj *et al.* (2010) but they find an increase

in the bacterial diversity up to 11 years of age followed by a decrease thereafter (Cox *et al.* 2010).

There are two ecological theories that have been applied to microbial community assembly, neutral theory and niche (deterministic) theory. Neutral theory was first used to examine species diversity on oceanic islands (MacArthur & Wilson 1963; MacArthur & Wilson 1967). Neutral theory assumes all taxa to be of equal fitness and that diversity is driven by random immigration and extinction of species derived from a meta-community. MacArthur and Wilson (MacArthur & Wilson 1963; MacArthur & Wilson 1967) used this theory to explain the number of avian species on oceanic islands and found that islands with a greater area had a larger species number than islands which were smaller. This theory came to be known as the species-area relationship. Microbial ecologists have recently started using neutral theory to examine bacterial communities and have found that a taxa-volume relationship exist in several systems (van der Gast *et al.* 2006; Woodcock *et al.* 2007). However, this theory has not been applied to the bacterial communities associated with infectious diseases.

In a study examining metabolically active bacterial communities, patients had diverse communities consisting of different bacterial taxa which suggests that a deterministic community assembly between CF patients has not been observed (Rogers et al. 2005). Therefore, the microbial communities present in the LRT of CF patients may be assembled in a stochastic manner which means that neutral theory could possibly be applied to polymicrobial infection. Further studies examining the microbial diversity of the CF lung have also found that a patient specific microbial community exists (Harris *et al.* 2007; Bittar *et al.* 2008b). Although neutral theory provides a theoretical framework in which to examine microbial communities one of

its major caveats is to provide a useful indication of how and why the major taxa associated with infectious diseases come to the fore. For example, why is there an extremely high prevalence of *P. aeruginosa* amongst CF patients?

Niche or deterministic community assembly states that taxa exist in a community because they have specific traits which allow them to exploit the available resources. The inclusion of the individual's fitness in niche theory means that predictions can be made on the composition of the community at a given time point or under certain conditions. In CF, evidence for the CFTR genotype affecting microbial distribution in the CF lung has been demonstrated (Klepac-Ceraj et al. 2010). CF patients in this study were assigned in to three groups; F508del homozygous; F508del heterozygous and patients without an F508del allele. The findings of this analysis showed that patients who were either F508del homozygous or heterozygous had more similar communities than those who had no F508del allele. Likewise, CFTR genotype has also been implicated in susceptibility to fungal infections with F508del homo- and heterozygous patients being more susceptible to infection by *G. argillacea* (Giraud et al. 2010). Evidence emerging from investigations into the G.I. tract also supports niche theory where, in a mouse model, the major histocompatibility complex genotype has an effect on the bacterial flora (Toivanen et al. 2001). Furthermore, recent work on the infant gut microbiota suggested a largely deterministic community assembly process at the phylum level (Trosvik et al. 2010).

However, CFTR genotype is not the only factor which affects the microbial diversity in the CF respiratory tract. Unsurprisingly, antibiotic therapy too affects the microbial diversity of the CF lung (Klepac-Ceraj et al. 2010). Patients who were receiving long term antibiotic therapy displayed a decrease in overall species richness when compared to patients who were not regardless of *P. aeruginosa*

colonisation. However, patients who were receiving long term antibiotic therapy and were colonised by *P. aeruginosa* had a reduced phylogenetic diversity compared to those who were not as well as having a more distinct microbial community composition from each other.

One of the major flaws of niche theory is the large amount of factors that need to be considered in order to make an accurate prediction in complex microbial ecosystems.

1.7 Interaction in microbial communities

Examining interactions in microbial communities using evolutionary theory has received a lot of attention recently, and provides a useful framework for designing experiments that examine the social mechanisms of microbes. In broad terms social interactions have fitness consequences for the 'actor' and the recipient and can be assigned to one of four classes (West *et al.* 2007). Mutualism is where there is a benefit for both the actor and the recipient which is an act contrary to spite where both suffer a loss (West 2006). Selfishness is where the actor benefits to the detriment of the recipient and altruism is where the actor sacrifices fitness to the benefit of the recipient (West *et al.* 2006). An example of a spiteful behaviour is bacteriocin production because it has a negative effect on the producer (actor) and the recipient (Gardner *et al.* 2004). Bacteriocin producing strains of *P. aeruginosa* have been isolated from the CF lung and it is thought, with a greater understanding of microbial interaction, that the frequency of bacteriocin producing strains in the population could be manipulated in CF to reduce bacterial virulence (Inglis et al. 2009).

There are numerous types of competition which can be competitive or cooperative in nature (Duan et al. 2009). Passive competition is the simple interaction that can occur between competing for space in an ecosystem or a particular carbon source. Active competition is where one species releases a compound that interferes with the process of another to gain an advantage (Duan et al. 2009). Recent work has shown that interactions can occur between *Candida albicans* and *P. aeruginosa* through various signal mediated pathways (Hogan et al. 2004; Cugini et al. 2007). Farnesol, a signalling molecule produced by *C. albicans*, has the ability to repress pyocyanin production by *P. aeruginosa* (Hogan et al. 2004). The ability of *P. aeruginosa* to sequester iron is paramount to its survival and therefore this interaction could be exploited to reduce the fitness of *P. aeruginosa in-vivo*. Cooperative behaviours can also be passive or active and are difficult to explain like those found in social evolution theory. In both cases, kin selection theory (Hamilton 1964) is cited as the main cause. Kin selection theory states that the more closely related two organisms are the more likely they are to behave favourably to one another to pass on their genes, albeit indirectly, to future generations. Syntrophic relationships are formed between bacteria to facilitate the metabolism of a resource from which both bacteria benefit (Duan et al. 2009). It may be the case that syntrophic relationships are formed between different bacterial taxa in the CF lung which has an effect on the spatial distribution of microorganisms in a biofilm resulting in for example, the waste products of one organism being utilised by another. In a relevant environmental biofilm model of commensalism with *Burkholderia* and *Pseudomonas* spp., it has been established that the carbon source supplied to the bacteria has an effect on the biofilm architecture (Nielsen et al. 2000). When a carbon source that could be metabolised by both organisms was supplied, two

distinct biofilms were formed. In contrast, when one organism was dependent on the other for a metabolic by-product from the initial carbon source the organisms formed a mixed biofilm. The ability of metabolites to shape microbial communities is becoming an increasingly important area of research in microbial ecology and could help us to fully understand which members of the community interact to produce a negative impact on overall patient health.

Recently a medium was synthesised that reflects the nutrients present in the CF lung (Palmer et al. 2007). The Synthetic Cystic Fibrosis Sputum Medium (SCFM) was formulated by analysis of CF sputum by chromatography to calculate the average amount of ions, free amino acids, glucose and lactate from the sputum of 12 adult CF patients. This medium could be utilised, with addition of mucin, in both aerobic an anaerobic conditions to look at gene expression and or protein expression profiles when organisms exist in monoculture and then compared with co-culture and even as a whole community. This would give an insight into how microorganisms may interact with each other in the CF lung to determine, for example, whether other organisms that may colonise the lung can exacerbate virulence in a neighbouring species. However, care would need to be taken as there would be no immune factors present in the media which may be a factor in the cause of pulmonary exacerbations. This could be combated by using a Drosophila model that has been developed to simulate CF lung disease (Sibley *et al.* 2008a). This study established that *P. aeruginosa* strain PA01 was able to cause infection in a Drosophila model. The authors then went on to examine the ability of human oropharyngeal flora (OF) isolates to cause disease in the flies in monoculture followed by co-culture with PA01. Three classes of OF isolate were described, class 1; virulent isolates which could kill the flies in monoculture and enhance killing in co-culture with PA01, class

2; avirulent strains which could not kill the flies or enhance killing in co-culture and class 3; could not kill the flies in monoculture but could enhance killing in co-culture with PA01.

1.8 Current knowledge of CF microbial pathogenesis

1.8.1 Bacterial colonisation

Although classical culture methods have identified a subset of organisms that are isolated from the CF lung the advent of molecular techniques that exploit conserved regions of the 16S rRNA subunit have unearthed a wealth of organisms that have been missed by culture (van Belkum *et al.* 2000; Rogers *et al.* 2003; Rogers *et al.* 2004; Rogers *et al.* 2005; Harris *et al.* 2007; Bittar *et al.* 2008b; Sibley *et al.* 2008b) The molecular studies show some concordance with culture and find *S. aureus*, *P. aeruginosa*, *S. maltophilia* as some of the most commonly isolated bacteria from the CF lung (Bittar *et al.* 2008b). However, they also show the increased prevalence of oral *Streptococci* such as the *Streptococcus milleri* group (SMG), *Prevotella* spp. and *Neisseria* spp. as common isolates (Harris et al. 2007). The molecular studies based upon 16S rRNA gene have also identified metabolically active anaerobes commonly disregarded as merely contaminating mouth flora picked up during expectoration of sputum (Rogers et al. 2005). The presence of a large number of microbial taxa from the respiratory tract of CF patients, including emerging organisms, such as non-tuberculosis Mycobacteria, *Nocardia spp*. and *Inquilinus limosus* suggests that further work remains to determine their role as potential pathogens (Tomashefski *et al.* 1996; Barrio *et al.* 2008; Bittar *et al.* 2008a). Members of the *Burkholderia*

cepacia complex (Bcc) are frequently isolated from CF respiratory samples of older patients and have been shown to have a negative impact on survival rate (Jones *et al.* 2004; Kalish *et al.* 2006). This group of organisms were first isolated from CF sufferers in 1984 (Isles et al. 1984) and are now found in 2.9% of patients (Cystic Fibrosis Foundation 2009). However, this number may be a gross underestimate due to difficulties in distinguishing between the high number of Gram-negative rods isolated from CF based on their colony morphology and difficulties in identifying Bcc species based upon their biochemical profiles which means they are commonly misidentified or completely overlooked (Larsen et al. 1993; McMenamin et al. 2000). Infections with Bcc in people with CF are highly transmissible which makes it even more important that they are identified early (LiPuma et al. 1990). It is, therefore, timely that a multi-locus sequence tag method capable of both species and strain identification was devised to ensure that patients colonised with Bcc can be isolated from those who are not to reduce the risk of patient to patient transmission (Baldwin et al. 2005). The applicability of this technology has been further improved with the discovery that it can be applied direct to sputum samples rather than isolated colonies, allowing early identification of Bcc in patients who were previously deemed uninfected (Drevinek et al. 2010). The severity of Bcc infection varies depending on the genomovar. Jones *et al.* (Jones *et al.* 2004) showed that acquisition of *B. cenocepacia* (genomovar III) was associated with chronic infection and shorter survival time post infection than patients infected with *B. multivorans* (genomovar II) or *P. aeruginosa*, although both groups infected with Bcc had deaths attributed to 'cepacia syndrome'. 'Cepacia syndrome' is characterised by pyrexia, progressive respiratory failure, leukocytosis and an increased erythrocyte sedimentation rate (Isles *et al.* 1984). It was initially thought that 'cepacia syndrome' had an early onset

after initial colonisation but Blackburn *et al.* (Blackburn et al. 2004) have demonstrated that 'cepacia syndrome' can occur up to 9 years after initial isolation meaning that effective treatment strategies for Bcc are urgently needed.

1.8.2 Fungal colonisation

Many fungal species are frequently isolated from the sputum of CF patients with *Candida and Aspergillus* species being most common (Bouchara et al. 2009). This study showed that 70% of patients were colonised with at least one fungal species with up to four species being identified in one patient. Duan *et al.* (2003) have shown that recurrent *C. albicans* infection occurs over a ten year period, suggesting that fungi may be capable of chronically colonising the lung. This data is supported by the small amount of longitudinal data produced in a later study (Bouchara et al. 2009). It is now the case that more unusual fungal species are being isolated from the CF lung (Cimon et al. 2005). Sudfield *et al.* (Sudfeld et al. 2010) demonstrated from a population of 614 CF patients 10 species of filamentous fungi and two species of yeasts, as well as an undefined subset, were isolated. The most prevalent of the filamentous fungi was *Aspergillus fumigatus* which was present in 36.3% of patients followed by non-fumigatus species of *Aspergillus* (26.1%), yeasts were present in 28.5% of patients but a breakdown of the yeast data was not given due to a change in laboratory practice.

1.8.3 Respiratory viral infection

Several viral pathogens have been identified in the respiratory tract of CF patients. Respiratory syncitial virus (RSV) Influenza virus (A and B), parainfluenza virus, adenovirus and rhinoviruses have been detected in several studies and in some cases implicated as causative agents of pulmonary exacerbations (Ong *et al.* 1989; Pribble *et al.* 1990; Armstrong *et al.* 1998; Hiatt *et al.* 1999). A recent metagenomic study looking at DNA viruses has shown in a small patient cohort that certain eukaryotic and prokaryotic viral taxa are associated with CF when compared to healthy individuals (Willner et al. 2009). There has also been work done which shows that bacteriophage are also present in the sputum of CF patients colonised with *P. aeruginosa* (Tejedor et al. 1982). This study found that seven distinct bacteriophage were present in the sputum of seven CF sufferers plus one non-CF sufferer with one CF patient having four different bacteriophage isolated. The high diversity of the CF lung suggests that these organisms are able to act both synergistically and antagonistically to gain an advantage over each other, and possibly more importantly the hosts immune defence.

1.9 Biofilm formation in the CF lung

Biofilm formation by bacteria may be a key stage in CF lung disease which ultimately leads to chronic infection and respiratory failure due to the protection they provide against antimicrobial chemotherapy and the immune system (Stewart & Costerton 2001; Leid et al. 2005). *P. aeruginosa* is a key pathogen in CF that is able to attach to CF airway epithelial cells more readily than in healthy individuals (Saiman et al. 1992). *P. aeruginosa* is also able to attach to mucin, which is abundant

in the CF lung (Arora et al. 1996; Arora et al. 1998). *P. aeruginosa* forms biofilms with a distinct architecture when bound to mucin as opposed to DNA or glass showing reduced motility and increased tolerance to tobramycin which could be due to the increased thickness and biomass in the mucin derived biofilm (Landry et al. 2006). Furthermore, research has found that *P. aeruginosa* forms tight microcolonies when grown in an artificial CF sputum media (Sriramulu et al. 2005). Several genes were required for tight microcolony formation and that amino acids were important for development of colony morphology, lipopolysaccharide alterations and hyper expression of outer membrane pumps (Sriramulu et al. 2005). The architecture of *P. aeruginosa* biofilms has been described as "mushroom" shaped with a hollow *pileus* filled with live motile bacteria and autolysed dead cells and a *stipe* which consists mostly of a solid biofilm matrix (Ma et al. 2009). Thus, data only show *P. aeruginosa* biofilm structure in monoculture and it has been demonstrated that in co-culture with other CF isolates the architecture of the biofilm can be dramatically different. In a flow chamber biofilm model co-culture with *S. maltophilia*, *P. aeruginosa* biofilm architecture became filamentous like that of *S. maltophilia* (Ryan et al. 2008). The presence of both *P. aeruginosa* and *S. maltophilia* in a multispecies biofilm demonstrated an increased resistance to polymixins (Ryan et al. 2008). Furthermore, it has been shown that *P. aeruginosa* biofilms from CF airways are attached to mucus in the intraluminal space as opposed to attaching directly to the epithelium (Worlitzsch et al. 2002).

H. influenzae also has the ability to bind to mucins present in the airways and it is suggested that the mucins protect the respiratory epithelium from invasion (Davies et al. 1995). It has also been demonstrated that clinical isolates of *H. influenzae* have the ability to form biofilms on airway epithelial cells with increased resistance to

antibiotics (Starner et al. 2006). However, it has been revealed that different clinical isolates of *H. influenzae* form biofilms with varying degrees of success (Murphy & Kirkham 2002). The eDNA released into the biofilm matrix during the formation of *H. influenzae* biofilms adds structural stability to the biofilm, but more importantly spans channels in the biofilms, through which water and potentially substrates and signalling molecules can be transported into the biofilm and waste products transported out demonstrating that microbial biofilms have complex structures designed to maximise survival (Jurcisek & Bakaletz 2007).

Fungi have also been shown to have the ability to form biofilms in a human host. *C. albicans*, a common CF isolate, has been shown to form biofilms that increase its resistance to antimicrobials (Chandra et al. 2001). The biofilms formed by *C. albicans* are shown to have a similar make-up to those formed by bacteria with a cellular component encased in a matrix of extracellular cell-wall like polysaccharides (Chandra et al. 2001). *A. fumigatus* has also been shown to form biofilms with reduced susceptibility to antifungal agents on bronchial epithelial cells (Seidler et al. 2008). With all of these common CF isolates plus many others having the ability to form biofilms it would be prudent to investigate how these organisms are spatially distributed in the airways of CF patients in order to examine their social behaviour.

Recently a retrospective study on CF biofilms has demonstrated the need for better antibiotic sensitivity testing for CF isolates (Keays et al. 2009). The participants in this study were divided into two groups and antimicrobial chemotherapy was assigned using susceptibility testing results from planktonically grown cells or cells from the same sample in a biofilm. Using the planktonic antibiotic sensitivities 60% of patients were treated with combinations that inhibited bacterial growth. However,

when the same bacteria were tested using biofilm method only 22% of patients had all of their isolates remain susceptible to antibiotics (Keays et al. 2009). Furthermore, the patients who had at least one susceptible isolate compared to none using the biofilm method had shorter periods of hospitalisation (Keays et al. 2009). This study highlights the necessity for routine screening of CF isolates grown in biofilms which better match the *in-vivo* conditions and improve clinical outcomes.

1.10 Acute pulmonary exacerbations?

A pulmonary exacerbation in CF can be defined as changes in many factors such as patient wellbeing, cough, dyspnoea, loss of appetite and/or weight and changes in spirometric tests (Goss & Burns 2007). It is this vague definition of pulmonary exacerbations in CF that makes concordance between studies tackling aetiology and treatment difficult. Many studies have attempted to find an absolute definition for an exacerbation in CF, a criterion commonly used by authors is administration of intravenous (I.V.) antibiotics either in hospital or at home, but a general consensus is yet to be reached (Dakin *et al.* 2001; Rosenfeld 2001; Rabin *et al.* 2004; Marshall 2004). In the clinic it is desirable to prevent an exacerbation to reduce the burden on hospital facilities and on patient health. Recent research has found that exacerbations cause not only a short bout of ill health, but also that over time; they contribute to lung function decline (Amadori *et al.* 2009; Sanders, Bittner, *et al.* 2010). Pre-emptive treatment of patients thought to be in the early stages of an exacerbation without severe symptoms has been vindicated in adults and children (Sanders *et al.* 2010a; Sanders *et al.* 2010b). Recently, several biomarkers were assessed for the

ability to predict the start and resolution of pulmonary exacerbations (Gray et al. 2010). Serum calprotectin decreased significantly following the resolution of an exacerbation and also gave an indication of the duration until the next exacerbation (Gray et al. 2010). Routine testing for markers such as this can help to inform the treatment given by clinicians and hopefully improve patient outcomes.

Pulmonary exacerbations in CF are attributed to a number of phenomenon including increase in bacterial load, decrease in susceptibility to antimicrobials and acquisition of a new organism. In a study on infants with CF, respiratory viruses were identified in 52% of cases that required hospitalisation. RSV was the most common agent identified (44%) as cause of respiratory disease (Armstrong *et al.* 1998). Similarly, Hiatt *et al.* (Hiatt et al. 1999) have linked RSV infection in infants with pulmonary exacerbations in CF. This study showed that there was an increased incidence of hospitalisation in CF patients who presented with RSV compared to controls. Although RSV is the most commonly associated viral pathogen in CF pulmonary exacerbations, other respiratory viruses have been identified in the respiratory tract of CF patients. Influenza virus (A and B), parainfluenza virus, adenovirus and rhinoviruses have been detected in several studies (Ong *et al.* 1989; Pribble *et al.* 1990; Armstrong *et al.* 1998; Hiatt *et al.* 1999). Pribble *et al.* (1990) looked at pulmonary exacerbations in older patients with CF and identified Influenza virus (A and B) as the most common viral pathogen. Pribble *et al.* (1990) also went on to demonstrate that infection with Influenza virus was associated with decrease in lung function compared with patients with non-Influenza viral infection or patients with no viral infection. Willner *et al.* (2009) has shown that the functional genes present in the viral communities were distinct between CF and non-CF patients suggesting that the available resources and selective pressures may be a cause of respiratory

problems in CF. It would be interesting to note the differences in functional genes between clinically stable specimens and those collected when a patient is having an acute pulmonary exacerbation.

Another theory for the cause of pulmonary exacerbations in CF is increased density of *P. aeruginosa* (Regelmann et al. 1990). In this study, it was shown that during an exacerbation, treatment with antibiotics prevented proliferation of *P. aeruginosa* which resulted in increased lung function, a similar story to that seen in the clinic. Evidence suggests that viral infection facilitates bacterial colonisation of the lung in CF patients. Armstrong *et al.* (1998) found that 35% of hospitalised patients who had an exacerbation acquired *P. aeruginosa* in the following 60 months, compared to 6% of non-hospitalised patients. Similarly, Collinson *et al.* (1996) found that 32% of children acquired a new bacterial species following a viral infection. Most of these new bacterial species (10/12) were isolated during or within three weeks after an upper respiratory tract infection. The organisms isolated were *P. aeruginosa* (6/12), *H. influenzae* (4/12), *S. aureus* (1/12) and *B. cepacia* (1/12).

If viral infection is able to disrupt the microbial community it is a logical possibility that a new bacterial or fungal pathogen may also be able to disrupt the flora of the CF lung. A recent paper has possibly identified such a group of organisms, the SMG (Sibley *et al.* 2008b). It was recently shown that the organisms that make up the SMG have been found in CF patients only when presenting with an exacerbation and that treatment of these organisms can alleviate the symptoms upon eradication of the SMG (Sibley *et al.* 2008b). However, this was only a small study and further investigations in larger patient cohorts are required. It has been shown that the gene expression pattern of *P. aeruginosa* can be altered when present in co-infection with a member of the OF (Duan, 2003). This study demonstrated that a member of the

genus *Streptococcus* isolated from a CF sputum sample that would be considered OF in the presence of *P. aeruginosa* had the capacity to significantly increase lung damage without increasing *P. aeruginosa* load. The genes that were increased include several virulence factors such as lasB which encodes the virulence factor elastase. All of these theories regarding pulmonary exacerbations have credence and it may be the case that each of these mechanisms individually has the potential to trigger an exacerbation event. Alternatively, several of these factors may occur simultaneously or as part of a series of events that results in the initiation of an exacerbation. For example, recent research has found that rhinovirus infection causes biofilm dispersal in mucoid isolates of *P. aeruginosa* which stimulates a greater proinflammatory response which may result in an exacerbation (Chattoraj et al. 2011).

Shifts in microbial diversity may be able to cause exacerbation in CF. Similarly, in the vaginal ecosystem an increase in the diversity of the vaginal microbial community is associated with bacterial vaginosis (BV) with members of the phyla *Actinobacteria* and *Bacteroidetes* strongly associated with the disease community (Oakley et al. 2008). A change in microbial diversity causing disease is not sequestered to BV; it is also prevalent in oral and gastrointestinal disease states. In patients with ulcerative colitis (UC) the microbial community contained greater numbers of *Bacteroides* and *Prevotella* species than healthy individuals (Lucke et al. 2006). Furthermore, a change in microbial diversity may be facilitated by invasion of the lung by a reservoir of pathogenic species in the oral cavity. Poor oral hygiene practices have been found to cause colonisation of the lower respiratory tract with potential pathogens in cases of pneumonia (Pugin et al. 1991; Abe et al. 2005). Therefore, a rigorous oral hygiene routine would reduce the abundance and diversity

of oral microbial taxa potentially reducing the likelihood of LRT infection and possibly pulmonary exacerbations in CF patients. However, care needs to be taken when choosing oral hygiene practice as some oral hygiene products are not sterile (Kutty et al. 2007). Kutty *et al.* (2007) have shown that alcohol free mouthwash was contaminated with *B. cenocepacia* which has a negative impact on the life expectancy of CF patients (Isles et al. 1984).

1.11 Treatment of exacerbations

Currently, CF patients suffering from pulmonary exacerbations are hospitalised and administered I.V. antibiotics based upon the results of planktonically grown isolates. A recent study has identified that antibiotic sensitivity testing using planktonic cultures provides an inaccurate analysis of the susceptibilities of the bacterial cells *in-vivo* (Keays *et al.* 2009; see above). The patients in this study who were suffering an exacerbation and had at least one biofilm susceptible isolate had better clinical outcomes than those who had no biofilm susceptible isolates (Keays et al. 2009). The Calgary biofilm device used in this study has been recently validated for use on whole sputum rather than individual isolates (Spasenovski et al. 2010). This development, if combined the serial sputum induction method (Rogers et al. 2010) could allow for 'community antimicrobial susceptibility assays' which could make allowances for the interactions that occur between bacteria when put under stress.

1.12 Microbial ecology techniques

Most microbial ecology techniques utilise PCR of the 16S rRNA gene, which contains highly conserved regions across the domain bacteria and hypervariable regions that can be used to resolve the differences between species of bacteria in some cases. These techniques were first used to describe the bacterial communities inhabiting environmental samples such as soil (Muyzer et al. 1993; Liu et al. 1997). More recently, however, terminal restriction fragment length polymorphism (T-RFLP), length heterogeneity PCR (LH-PCR), clone libraries, phylochip and next generation sequencing have all been applied to investigate the polymicrobial nature of the CF lung (Rogers et al. 2003; Bittar, Richet, et al. 2008; Klepac-Ceraj et al. 2010; Armougom et al. 2009). T-RFLP, LH-PCR and DGGE are examples of communities fingerprinting techniques. T-RFLP analysis of bacterial communities requires the 16S rRNA gene to be amplified by PCR using dye labelled primers and the resultant fragment to be digested using a restriction enzyme. The digested fragments are then passed through a gel along with a size standard and the length of the labelled (terminal) end of the fragment can be measured (Liu et al. 1997). The fragments produced are of differing length due to the hypervariable regions of the 16S rRNA gene, allowing for the discrimination of different species. The advantage of this technique is that *in-silico* analysis can be used to select for the restriction enzyme with the best resolving power. Furthermore, T-RFLP is seen to be a relatively in-expensive technique after the initial outlay required for the equipment and *in-silico* analysis can provide the user with putative species identities based upon the fragment length. However, in some cases, different bacteria from the same genus

and sometimes different genera can produce fragments of equal length, thus, preventing the allocation of definitive species identities (Rogers et al. 2004).

Clone library studies remain the gold standard in microbial ecology studies as they can be used to amplify fully length16S rRNA fragments which can then be cloned into a suitable vector using competent *E. coli* followed by Sanger sequencing to give greater confidence when calling taxa to the species level when compared to fingerprinting techniques which only utilise a portion of the 16S rRNA gene (Harris et al. 2007). However, a major drawback to clone library studies is determining the proportion of the community that has been sampled. For example, Dunbar *et al.* (2002) sampled bacterial populations from four Arizona soils using clone libraries. In all four soil samples *Acidobacteria* accounted for approximately 50 % of clones where as there are many divisions which account for less than 5 % of clones. Dunbar *et al.* (2002) estimate that in order to define the most abundant 50 % of bacteria in a population containing 4,000 species then a clone library would need to sample 285,400 individual clones per soil to obtain a $\geq$ 95 % confidence limit. The drawback of this type of analysis is that the financial costs to fully elucidate a community of this size would be astronomical. One potential way of reducing the cost is to screen the clones prior to sequencing using restriction enzymes (Harris et al. 2007). This method allows the researcher to assess the relative abundance of particular taxa by counting the number of clones with a given RFLP pattern but only sequencing one clone from each RFLP type. However, due to the conserved nature of large portions of the 16S rRNA gene and the choice of restriction enzymes this method can still underestimate the total community diversity.

Recently, new molecular methods such as microarray and pyrosequencing have been utilised for microbial ecology studies. DNA microarrays, such as the Phylochip,

have been produced that can resolve up to 8,000 bacterial species based upon positive and negative binding of oligonucleotide probes complementary to the 16S rRNA gene (DeSantis et al. 2007). These studies can identify up to two orders of magnitude greater species diversity in a given sample than molecular finger printing techniques and at the fraction of the cost of a comprehensive clone library (Cox et al., 2010). However, microarrays represent a 'closed system' of identification where the potential member species are selected *a priori*. Furthermore, many researchers use these microarray studies for microbial community analysis but still feel that the taxa can only be resolved to the genus or family level. In pyrosequencing reactions, 16S rRNA genes are amplified using primers with an 8bp barcode sequence attached to the 5' end so that the sequences can be assigned to a given PCR reaction post pyrosequencing (Acostamartinez et al. 2008). These bar-coded fragments are bound to beads and dNTPs are added sequentially to the reaction mix and are incorporated into the target strand by DNA polymerase (Ronaghi 2001). This reaction produces a pyrophosphate which is equivalent to the number of dNTPs incorporated into the complementary strabd by the DNA polymerase. The pyrophosphate is then converted to ATP by an ATPase and the ATP is used to convert luciferin to oxyluciferin which produces light. The amount of light produced is proportional to the amount of pyrophosphate and therefore the number of dNTPs incorporated into the sequence. The excess dNTPs are removed by an apyrase enzyme and then the next dNTP is added and this cycle is repeated until the reaction is complete (Ronaghi 2001). This technique has similar drawbacks to clone library where the number of fragments to be sequenced to fully identify the community can only be determined after the reaction. Furthermore, the size of the fragments that can be analysed by the currently available instrumentation is only ~500bp meaning that fully length analysis

of the 16S rRNA gene is not possible, resulting in taxa being identified to the genus level (Armougom et al. 2009). One advantage of pyrosequencing to microarray is that it is an open system so currently unidentified taxa can be detected using this technique. Furthermore, a greater amount of data can be gained by pyrosequencing at a fraction of the cost of clone libraries which, with further development of the technology may allow for full length 16S rRNA sequencing which means that pyrosequencing may replace clone libraries as the gold standard in the years to come.

2. Aims and objectives

The aim of this research was to characterise the intrinsic and extrinsic factors which have an effect on bacterial and fungal communities present in the CF lung in a cohort of patients who were homo- or heterozygous for the F508del CFTR allele. Furthermore, the aim was to follow these patients longitudinally to determine the stability of the CF lung microbiota, to determine the effects of antibiotic therapy, and to assess if any changes occurred in the CF lung during times of pulmonary exacerbation which could be identified as the causative agent.

3. Methods

3.1 Culture of common CF isolates

Common bacterial isolates from CF patients were cultured in order to construct a "ladder" of known organisms that could be loaded onto a DGGE gel along with 16S rRNA fragments from sputum samples. The isolates were taken from the National collection of typed cultures (NCTC), Laboratory of Microbiology, University of Ghent (LMG) or fully identified wild type strains from the Freeman hospital (FRH).

No.	Species	Reference
1	*Pseudomonas aeruginosa*	NCTC 10662
2	*Staphylococcus aureus*	NCTC 6571
3	*Stenotrophomonas maltophilia*	NCTC 10257
4	*Haemophilus influenzae*	NCTC 11931
5	*Burkholderia cepacia*	LMG 17997
6	*Burkholderia multivorans*	LMG 17588
7	*Burkholderia cenocepacia*	LMG 18828
8	*Burkholderia stabilis*	wild strain FRH 18870
9	*Burkholderia vietnamiensis*	LMG 10929
10	*Burkholderia dolosa*	LMG 18941

11	*Achromobacter xylosoxidans*	wild strain FRH 604914
12	*Pseudomonas fluorescens*	NCTC 10688
13	*Ralstonia pickettii*	NCTC 11149
14	*Peptostreptococcus anaerobius*	NCTC 11460
15	*Streptococcus oralis*	NCTC 11427

H. influenzae was cultured on NADsens agar (Appendix 2) at 37 ºC in a static incubator with 5 % CO_2 saturation overnight. All other organisms were inoculated onto Luria-Bertani (LB) agar (Appendix 1) and incubated overnight at 37 ºC in aerobic conditions except for *S. oralis* which was grown in anaerobic conditions. The colony morphologies were checked by eye to ensure pure growth and a single colony was inoculated into LB broth overnight at 37 ºC except *H. influenzae* which was inoculated into NADsens broth and incubated overnight in a microaerophilic environment. The broth was checked for pure growth by inoculating agar plates (as described above) with 10 µL of culture. The broths were subsequently used for DNA extraction.

3.2 Collection of sputum samples

Spontaneously expectorated sputum samples were collected from patients attending an adult CF clinical at the Royal Victoria Infirmary, Newcastle upon Tyne and taken directly to the microbiology department at the Freeman Royal Hospital, Newcastle upon Tyne where they were immersed in RNA*later*® (Ambion) and stored at 4 ºC prior to nucleic acid extraction. The samples were washed three times with

phosphate buffered saline to remove any contamination from oral flora (Rogers et al. 2006) and the viscous bolus of sputum was digested with Sputasol (Oxoid) by mixing equal volumes of sputum and Sputasol.

3.2.1 Sample collection for sample handling study

Five spontaneously expectorated sputum samples were collected from adult patients attending a CF clinic and were immediately divided into two equal aliquots. One set of aliquots were immediately treated with RNA*later*® (Ambion), which is bacteriostatic, and stored at 4 °C. The second set of aliquots were initially incubated at room temperature (20 °C) for 24 hours. After 24 hours these aliquots were treated with RNA*later*® (Ambion) and RNA extraction was then performed on all aliquot samples.

3.2.2. Sample collection for assessment of intrinsic and extrinsic factors study

Twenty-nine spontaneously expectorated sputum samples were collected from a clinically stable adult population were stored at -20˚C prior to DNA extraction and routine microbial culture. Information regarding patient gender, age, CFTR genotype, forced expiratory volume in one second and the percentage predicted (FEV_1 % predicted), body mass index (BMI) and oral antibiotic therapy. Subsequently, results from routine microbiology were also obtained (Appendix 11).

3.2.3 Sample collection for temporal stability of the CF lung microbiota

From the initial 29 patient cohort, eighteen patients, who were considered to be frequently presenting at the clinic with exacerbations, were enrolled in the study and gave a total of 149 (Mean 8.3 sample/patient; Range 4 – 16) spontaneously expectorated sputum samples across a 20 month time period from October 2008 to July 2010. After expectoration the samples were stored at -20˚C until processed. Information regarding the patients' age, gender, CFTR genotype, clinical status, FEV_1 % predicted, oral and I.V. antibiotic therapy and results from microbial culture were collected. The patients' clinical status was classified as Stable, exacerbation and Routine I.V. (Appendix 12). An exacerbation was defined as deterioration in their clinical status with an increase in cough, sputum or chest symptoms, and/or a fall in FEV_1 and was subsequently treated with I.V. antibiotics, except in one instance where the patient received oral antibiotics for treatment of the exacerbation. Furthermore, when using cluster analysis to determine if patients who presented with an exacerbation had a characteristic bacterial community, routine I.V. samples were classified as stable because they had not qualified as exacerbation as defined above. All patients survived until the end of the study period except patient 13.

3.3 DNA extraction (UltraClean™ Microbial DNA isolation kit)

A 1.8 mL aliquot of broth or Sputosol treated sputum was pipetted into a sterile microfuge tube and centrifuged at 10,000 x g for 30 seconds. The supernatant was discarded and then centrifuged for a further 30 seconds at 10,000 x g. The remaining

supernatant was aspirated making sure that the cell pellet was undisturbed. The pellet was reconstituted in 300 µL MicroBead solution and vortexed gently for 10-15 seconds and then transferred to a MicroBead tube. Microbead solution is a cell lysis buffer which contains guanidine thiocyanate. A 50 µl aliquot of solution MD1 was added then the mix was heated at 65 ºC for 10 minutes. Solution MD1 is a cell lysis buffer which contains SDS to breakdown fatty acids and lipids which make up the cell membrane. The MicroBead tube was secured in a flat pad vortex head (MoBio) and vortexed at full speed for 10 minutes to mechanically lyse the bacterial cells. The MicroBead tubes were centrifuged at 10,000 x g for 30 seconds at room temperature and the supernatant (~300-350 µL) was transferred to a sterile microfuge tube. 100 µL of solution MD2 was added, the mix was vortexed for 5 seconds, and then incubated at 4ºC for 5 minutes to precipitate cell debris and protein. The resulting suspension was centrifuged at room temperature for 1 minute at 10,000 x g to separate the liquid phase (DNA) from the solid phase (cell debris). The entire volume of supernatant (~450 µL) was pipette into a sterile microfuge tube ensuring that the pellet is not disturbed then 900 µL of solution MD3 was added to the supernatant and vortexed for 5 seconds. Solution MD3 is a high salt solution which facilitates DNA binding to the silica membrane of the spin filter. Then 700 µl of the supernatant mix was passed through a 0.22 µm filter and pipetted into a spin filter and centrifuged at 10,000 x g for 30 seconds and the flow through discarded. The remaining supernatant was added to the spin filter and centrifuged for a further 30 seconds at 10,000 x g and the flow through discarded. 300 µL of solution MD4 was added to the spin filter and centrifuge at 10,000 x g for 30 seconds to clean the DNA bound to the spin filter membrane by removing excess salt and other contaminants. The flow through was discarded and the empty spin filter was

centrifuge at 10,000 x g for 1 minute to remove any residual MD4 that may remain. The spin filter was transferred to a sterile microfuge tube and 50 µl of solution MD5, a low salt solution, was pipetted directly onto the centre of the spin filter membrane and centrifuged for 30 seconds at 10,000 x g to elute DNA from the spin filter membrane. The eluate was stored at -20 ˚C before use in downstream reactions.

3.4 RNA Extraction from sputum (UltraClean™ Microbial RNA isolation kit)

A 1.8 mL aliquot of Sputasol (Oxoid) treated sputum was added to a microfuge tube and centrifuged at 10,000 x g for 30 seconds. The supernatant was decanted and centrifuge at 10,000 x g for a further 30 seconds. The remaining supernatant was aspirated ensuring that the cell pellet remained undisturbed. The pellet was reconstituted in 300 µL of solution MR1 and vortexed gently for 10-15 seconds and the resulting mix was transferred to a MicroRNA bead tube. Solution MR1 is a lysis buffer used to lyse cells and inhibit RNases. 15 µL of solution MR2 was added to the mix and vortexed briefly to homogenise. Solution MR2 contains dithiothreitol which will further inhibit RNase activity. The solution was heated to 65 ºC for 10 minutes. Heating at 65˚C makes the cells more amenable to lysis and causes shearing of the genomic DNA to prevent contamination of the final RNA eluate. The MicroRNA bead tube was secured in a flat pad vortex head and vortexed at full speed for 10 minutes to mechanically lyse the cells. The MicroRNA bead tubes were centrifuged at 10,000 x g for 30 seconds and the supernatant (~300-350 µL) was transferred to a sterile microfuge tube. 500 µL of buffer MR3 was added to the supernatant and vortex for 5 seconds then 250 µL of buffer MR4 was added, the solution vortexed

for 5 seconds, then incubated at 4 ºC for 5 minutes. Solution MR3 and MR4 precipitate proteins and facilitate binding of the RNA to the silica membrane of the spin filter. The mix was centrifuged at 10,000 x g for 1 minute to separate the solid phase from the liquid phase. The liquid phase was passed through a 0.22 µm filter in to a sterile microfuge tube ensuring that the solid phase is not disturbed to remove any potential pathogens that have not been lysed. A 650 µL aliquot of the liquid phase was added to a spin filter and centrifuged at 10,000 x g for 30 seconds. The flow through was discarded and the previous step was repeated with the remainder of the liquid phase. 300 µL of Solution MR5 was added to the spin filter and centrifuged for 30 seconds at 10,000 x g to removes excess salts and some contaminants. The flow through was discarded and the spin filter was centrifuged for 1 minute at 10,000 x g to remove any residual solution MR5. The spin filter was placed into a sterile microfuge tube and 50 µL of RNase free water was pipetted directly onto the centre of the spin filter membrane which was then centrifuged at 10,000 x g for 30 seconds. The spin filter was discarded and the eluate was stored at -20 ºC, before use in downstream reactions.

3.5 Reverse transcription

A 10 µL aliquot of RNA was pipetted into a PCR tube along with 1 µL of dNTPs (New England Biolabs; 25 mM) and 1 µL of random hexamer primers (QIAGEN; 0.4 µg/µL) and heat the mixture to 65 ºC for 5 minutes. Then chill the mixture on ice and add 4 µL of 5x first strand buffer, 2 µL of Dithiothreitol (0.1 M) and 1 µL of RNaseOUT™ (Invitrogen; 40 U/µL) and mix by pipette. Incubate the mixture at 25

ºC for 2 minutes and add 1µL of Superscript™ II RT (200 U) and mix by pipette. Incubate the mixture at 25 ºC for 10 minutes, 42 ºC for 50 minutes and 70 ºC for 15 minutes. The cDNA is now ready to be used in a PCR reaction.

3.6 Routine culture of CF sputum samples

Sputum samples were cultured by routine pathology using the Health protection agency recommendations (Health protection agency 2009). Briefly, a bolus of sputum was incubated with an equal volume of Sputosol (Oxoid) and vortexed until liquid. A 10 µL aliquot of the liquid sputum was diluted in 2 mL of sterile saline and used to inoculate blood, chocolate and chocolate bacitracin agar plate and incubated at 37 ˚C in a microaerophilic atmosphere for 48 hours to detect *H. influenzae, Moraxella catarrhalis, S. aureus and Streptococcus pneumoniae*. The sputum-saline mix was also used to inoculate a mannitol trehalose salt and a cysteine lactose electrolyte deficient agar plate and incubated at 37 ˚C for detection of *S. aureus* and Enterobacteriaceace or Pseudomonads respectively. The early identification of members of the Bcc requires inoculation of the sputum onto a *Burkholderia cepacia* selective agar plate which is incubated at 30 ˚C for five days. Sputum samples from CF patients are routinely screened for fungi by inoculating a Sabouraud agar plate and incubating it at 35 ˚C for five days. Any clinically relevant isolates were sub cultured for antibiotic sensitivity testing using the disc diffusion method.

3.7 Polymerase chain reaction

3.7.1 Bacterial 16S rRNA amplification

PCR amplification of the V3 region of the bacterial 16S rRNA gene was performed to analyse the bacterial community of CF sputum samples. The primers used are V3f (5'- CCT ACG GGA GGC AGC AG-3') and V3r (5'- ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993). In order to run the products on DGGE the PCR products from the initial reaction are re-amplified in a second round of PCR using the above forward primer with a 40 bp GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG G GCC-3') attached to the 5' end. The reaction was performed with 0.5 mM each primer 1x EX-Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, 1.25 U Ex-Taq (Takara) and 1 µL of gDNA or cDNA template made up to 50 µL with sterile 18.2 MΩ/cm H_20. The cycling conditions used were an initial denaturation for 5 min at 95 ˚C followed by 20 cycles of 95 °C for 1 min, 65 °C (-0.5 °C per cycle) for 1 min and 72 °C for 30 s followed by 15 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min with a final extension at 72 °C for 30 min. A negative control was setup for all PCR reactions to ensure that the reaction mix and components were not contaminated. The 30 minute final extension step was included in all PCR cycles that were to be analysed by DGGE to prevent visualisation of spurious double bands which would hinder accurate analysis of the gel images and make excision of bands difficult (Janse et al. 2004).

3.7.2 Fungal 28s rRNA amplification

PCR amplification of the fungal community of sputum was amplified using PCR primers specific for the 28s rRNA region of the fungal genome. The primers used were U1 (5'-GTGAAATTGTTGAAAGGGAA-3') and U2-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGA CTC CTT GGT CCG TGT T -3') (Sandhu et al. 1995). The reaction was performed with 0.5 µM each primer 1x Ex Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, 1.25 U Ex Taq (Takara) and 1 µL of cDNA template, the reaction was made up to 50 µL with sterile 18.2 MΩ/cm H_20. The cycling conditions used were an initial denaturation for 5 min at 95 ˚C followed by 10 cycles of 95 °C for 1 min, 60 °C (-1 °C per cycle) for 1 min and 72 °C for 30s followed by 25 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 30 s with a final extension at 72 °C for 30 min (Janse et al. 2004). A negative control was setup for all PCR reactions to ensure that the reaction mix and components were not contaminated.

3.7.3 Confirmation of successful PCR

A 1% (w/v) agarose gel was prepared to confirm that PCR had been successful by electrophoresis of the PCR product. 0.3 g of agarose (Melford) was added to 30 mL 1x TAE (Appendix 3) and boiled until fully dissolved. The agarose was cooled to approximately 50 ºC then 5 µL of SYBR Safe (Invitrogen; 10,000x) was added to the molten agarose, mixed, and poured into the gel cast and left to set. The agarose gel was submerged in 1x TAE and a 5 µL aliquot of PCR product was added to 1 µL

of 6x bromophenol blue (Appendix 4), mixed by pipetting, and then loaded in to the wells of the agarose gel alongside a 5 µL of Hyperladder 1 (Bioline). The electrophoresis tank was connected to a power pack and ran at a constant current of 120 mA for 20 minutes. The gel was removed from the cast and viewed under U.V. light on BIO-RAD gel doc 2000 using quantity one™ software (v4.1.1.) to confirm presence of pure PCR product of correct size by comparison with fragments from Hyperladder 1. Hard copies were produced using Mitsubishi Video copy processor (Model P91 attached to gel doc).

3.8 Denaturing gradient gel electrophoresis (DGGE)

3.8.1 Assembly of gel cast

Glass plates used for DGGE were cleaned using ethanol before use to remove any residue that may interfere with the gel casting. The spacers used to separate the glass plates had a thin film of silicon grease applied to the inner edges to prevent leakage of the denaturant during the run (Brinkhoff & van Hannen 2001). The large glass plate (20 cm x 16 cm) was placed on the bench and spacers were aligned with the outside edge of the plate. The small glass plate (16 cm x 16 cm) was placed on top of the spacers ensuring that they remain aligned. The plates were secured in plastic brackets with the arrows facing towards the glass plates. The brackets were stood upright and the alignment card was inserted between the glass plates to ensure the spacers were correctly aligned. The brackets were adjusted so that there is resistance when the alignment card is removed. If two gels were to be electrophoresed on the

same run then repeat as described above, if only one gel was ran then a balance plate was setup as above without the spacers to prevent the buffer leaking from the upper chamber during electrophoresis.

3.8.2 Casting the gel

The gel cast was clamped in the gel cast stand and a 19 gauge needle fitted with a tube and Y-fitting was attached to the centre of the plates. Two plastic 50 mL tubes were stood in a rack and labelled high and low and the reagents described below were added to the tubes, the lids secured, and inverted to mix.

Reagent	**High**	**Low**
DCODE dye (Appendix 4)	100 µL	0
Denaturing solution (Appendix 5)	25 mL	25 mL
APS (10% w/v)	216 µL	216 µL
TEMED	21.6 µL	21.6 µL

A syringe with rubber tubing was used to draw 16 mL of solution from the tube labelled high and was then attached to the Y-fitting, this was repeated with a different syringe for the low solution. The syringes were secured in a Model 475 gradient former and the cam wheel was turned to dispense the solutions into the gel

cast ensuring that the gel stream runs in a straight line for an equal gradient. Once the solutions had been dispensed into the cast the needle was removed and a 16 well comb was put into the gel cast.

3.8.3 Running the gel

The buffer used for DGGE was made up with 140 mL of 50x TAE (Appendix 3) in 7 L of distilled water in the buffer tank. The DGGE control unit was placed in the buffer tank, the thermostat was set to heat the buffer to 60 ºC and the stirrer was switched on. Once the buffer had come to temperature the control unit was removed and the gel casts were lowered into the buffer chamber ensuring the red mark is on the right hand side and then the upper chamber was filled with the buffer. A 15 µL aliquot of sample was mixed with an equal volume of 2x DGGE loading buffer (Appendix 4) and loaded onto gel. The DGGE control was replaced and left until the temperature had returned to 60 ºC. The power pack was set to 200 V for 4.5 hours for bacterial community analysis or 70 V for 17 hours for fungal community analysis.

3.8.4 Staining

The gel cast was removed from the buffer tank and the cast was disassembled. A 25 µL of SYBR green I (Invitrogen, 10,000x) was diluted in 250 mL of 1x TAE in a staining tank. The gel was removed from the glass plates and submerged in the staining solution for 30 minutes on an orbital shaker then the solution was decanted.

The gel was de-stained in dH2O to remove any excess stain and viewed under U.V. light on BIO-RAD gel doc 2000 using Quantity One™ software (v4.1.1.) and the resulting image was captured.

3.8.5 Excision of bands

Bands were excised from DGGE gels whilst exposed to U.V. light once an image of the gel had been taken for analysis. The bands of interest were excised using a scalpel and placed in a microfuge tube. The bands were immersed in 10 µL of 18.2 MΩ water and vortexed for 1 minute. The bands were stored at 4 ºC overnight to allow DNA to elute from the gel, then vortexed and a 1 µL aliquot of the eluate was used in a PCR reaction as described above.

3.9 Sequencing of PCR products

3.9.1 Calculating PCR product concentration

An estimate of PCR product concentration was calculated when confirming successful PCR by agarose gel electrophoresis. Hyperladder 1 (Bioline) was used to estimate concentrations as it produces bands of a known size and concentration when 5 µL is loaded onto an agarose gel (Appendix 6). The intensity of the PCR products when viewed under U.V. light was compared to the equivalent band in Hyperladder 1 (Bioline) to get an estimate of the concentration of the PCR products loaded onto

the agarose gel. This value was used to calculate the concentration of the PCR product in ng/µL and the concentration required for sequencing (Appendix 6) was adjusted by either drying to concentrate or addition of water to dilute taking into account the subsequent dilutions.

3.9.2 BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems)

Excess dNTPs and primers were removed using ExoSAP-IT (USB) by mixing 5 µL of PCR product with 2 µL of ExoSAP-IT and incubated at 37 ˚C for 15 minutes to remove dNTPs and primers followed by an inactivation step at 80 ˚C for 15 minutes. The product of this reaction was then was used in a cycle sequencing reaction as described below (Table 3.1).

Positive control was set up using pGEM-3Zf (+) as a template and -21 M13 control primer (forward) both of which were supplied by the manufacturer. The cycling conditions used for the sequencing PCR were an initial denaturation at 96 ˚C for one minute followed by 25 cycles of 96 ˚C for 10 seconds, 50 ˚C for 5 seconds and 60 ˚C for 4 minutes. The Products from the cycle sequencing reaction were purified using ExoSAP-IT (USB) as described above.

3.9.3 ABI PRISM® 3130 Genetic Analyzer sample electrophoresis

The purified products were desiccated in a vacuum centrifuge so that the correct concentration for the sequencing reaction was achieved. The samples were pipetted in to 1.5 mL microfuge tubes with lids open into a vacuum centrifuge and dried at 60 ºC for approximately 30 minutes (until volume is 1-2 µL).

Table 3.1. Cycle sequencing reaction setup

Reagent	**Positive control (µl)**	**Negative control (µl)**	**PCR product (µl)**
Ready reaction mix	0.5	0.25	0.5
BigDye sequencing buffer	1.75	1.875	1.75
Primer (1 pmol/µl)	2.0	2.0	3.2
Template	2.0	0.0	3-10 ng
Sterile 18.2 Ω/cm H_2O	5.25	5.875	To 10 µl

A 10 µL aliquot of Hi-Di™ Formamide (Applied Biosystems) was added to the almost dry sequencing PCR product. The sample was pipetted into a MicroAmp® optical 96 well reaction plate (Applied Biosystems) secured in a Plate base 96 well (Applied Biosystems). Once loaded a 96 well Plate Septa (Applied Biosystems) was used to seal the plate to prevent evaporation of the products during the sequencing electrophoresis. The samples were run using a 36 cm capillary array and polymer POP-7™ (Applied Biosystems) under manufacturers recommended conditions. The sample electropherograms were analysed on SeqScape® software (v2.5) with integrated KB™ Basecaller algorithm to generate pure and mixed signal base calls with quality values.

3.9.4 NCBI BLASTn search

Any sequences obtained from DGGE band excision and re-amplification was compared with known sequences using the NCBI BLASTn tool. All sequences were input in FASTA format and the nucleotide collection was used for comparison.

3.10 Statistical analysis of DGGE gels

3.10.1 Bio-Rad Quantity One

The image that was captured in TDS 1-D scan file format using Quantity One software was opened. The application tab was then selected and relative quantity to

the percentage of bands in lane and relative front (Rf) were selected so that reports were generated with the appropriate information. At this stage the image was converted so that black bands were set against a white background to obtain a clearer image. The background was subtracted from the inverted image manually using a rolling ball algorithm so that only peaks relating to bands and not artefacts were used for band detection. The lanes of the gel were framed manually so that 'smiling' or 'leaning' of the lanes could be corrected by creating anchors to shift the frame to centralise the bands. Once the frames of the lanes have been manipulated the auto-detect bands option was used and lane width set to 4.100 mm to prevent overlap between lanes for darker bands. The detected bands were then assessed by eye and either added or removed using the Create/Remove bands tool. The bands were then matched against the DGGE ladder created using reference strains with extra bands not included in the ladder being matched manually and repeated until all bands had been assigned a band number. An 'All Lanes Report' was then created which contains Lane Number, Band Number and relative quantity which was subsequently input into a Microsoft Excel spreadsheet to create a band matrix.

Once the band matrix was input into the spreadsheet the total intensity for each lane was calculated using the autosum formula. The relative intensity of each band was normalised by dividing the relative intensity by the total intensity of the lane in which the band was present to give a value between zero, indicating no band was present in that position and 1, indicating that the band in question accounted for the entire intensity found in that lane (i.e. there was only one band present).

3.10.2 Quantity One statistical analysis

To determine the variance present in the samples the band matrices were input into PAST and a principle component analysis was performed (Hammer et al. 2001).

3.10.3 TotalLab Phoretix 1D and 1D Pro

Images captured using Quantity One were exported in tagged image file format (.TIFF) at 276 dots per inch (DPI). A new experiment file was created and the appropriate .TIFF files were added. The lanes of the gels were framed using the automatic lane creation function and the frames adjusted so that all bands were central in the lanes then the changes were accepted. The background from the lanes was subtracted using a rolling ball method with a radius of 200 pixels. The detect band setting was applied to the gels using the preset conditions then bands were added/removed by eye, the band width was also adjusted by eye. The most important stage when aligning multiple gels is the Rf calibration function (Tourlomousis et al. 2010). As stated previously a ladder of known organisms was loaded on each DGGE gel in the experiment so that successful gel alignment could be achieved. The bands from the DGGE ladder were assigned standard Rf values based upon the distance they had migrated through the gel and these were the same for each gel in the experiment. A minimum of five bands per standard has been suggested for accurate interpolation of multiple gels which was maintained or exceeded throughout our bacterial analysis (Tourlomousis et al. 2010). The Rf lines were then manually

manipulated to correct for any ‘smiling’ or ‘leaning’ present in the gel image. The final experiment was then saved and closed.

A new database was created in Phoretix 1D Pro and then the gels analysed in the Phoretix 1D programme were added. The alignment of the gels was checked by eye using the ladders from multiple gels using the compare lanes function. If the alignment was deemed satisfactory then the gels were matched using the ladder lanes and the match gel function. Once matched, the software created a band matrix using the Rf values of all bands and the relative intensities which is exported to Microsoft Excel to be used for further statistical analysis. The band matrix was normalised in the same way as the matrix produced using Quantity One.

3.10.4 Ecological analysis

To examine the structure of the microbial communities present in the CF lung, species richness, evenness and Shannon diversity were calculated using the DGGE band matrix. Species richness (R) was calculated by simply counting the total number of bands present per sample from the DGGE band matrix. The Shannon diversity index (*H’*) was calculated using the formula;

$$H' = -\sum (p_i \log[p_i])$$

The symbol *pi* is the relative intensity of each band in a lane. This was calculated using the function in Microsoft Excel. Firstly, the log of the relative intensity was multiplied by the relative intensity for every band in all of the lanes ($p_i \log[p_i]$). Then

the sum of these values for each lane was taken and multiplied by -1 (-∑) which gives the Shannon diversity of the sample. Species evenness (E) was calculated using Microsoft Excel by dividing *H'* by the log of R.

3.10.5 Raup-Crick Similarity index

The Raup-Crick (S_{RC}) measures the presence/absence of bands between two samples where a value of 1 refers to two identical samples and a value of 0 refers to two completely unrelated samples. The advantage to the Raup-Crick similarity index is that the output generates an indicator of statistical significance where a value of ≤ 0.05 indicates that the two samples are less similar than would be expected by chance and a P-value of ≥ 0.95 indicates that the samples are more closely related then would be expected by chance. The probabilities are calculated by 200 pair-wise Monte Carlo simulations using PAST programme (Hammer et al. 2001).

3.10.6 Canoco analysis

The band matrix for both bacterial and fungal community analysis was further analysed using Canoco (v4.5.1) and images were produced using Canodraw (v4.14). Firstly, the normalised band matrices were copied from the Microsoft Excel spreadsheet to the clipboard. The information from the clipboard was input into Canoco using WCanoImp (4.5.2.0) to convert the data from the band matrices in to a format compatible with Canoco. The discrete patient variables were assigned positive (1) or negative (0) values and the continuous patient variables were not

manipulated before input into the spreadsheet. The band matrices were first analysed by detrended correspondence analysis (DCA) using normalised relative intensities followed by either redundancy discriminate analysis (RDA) or canonical correspondence analysis (CCA) in combination with Monte Carlo permutation testing depending on the DCA axis length (<3.5 RDA; >3.5 CCA). The results from the Monte Carlo permutations were deemed statistically significant if the P-value was < 0.05. The analysed data was then opened in Canodraw and a biplot was created for samples and environmental variables. In order to distinguish between discrete and continuous variables, discrete variables were assigned a centroid whereas continuous variables were represented by an arrow. The other visual parameters that were changed on the biplot were that the shape and colour of sample points were changed according to important variables.

3.10.7 Sensitivity

The sensitivity for analysis of the applicability of molecular and culture techniques was determine using the formula true positive divided by the true positives plus the false positive (Sensitivity = TP/ TP + FP).

3.10.8 Dice coefficient measures

The Dice (Sorensen) coefficient was used to compare the temporal stability of bacterial communities and was calculated using PAST (Hammer et al. 2001). The Dice index gives values between 0 and 1 where a value of 1 between two samples

means the two samples are identical. The Dice coefficient measures presence absence and does not therefore take into account relative abundance of species. The following formula was used to calculate the Dice coefficient;

Dice Similarity = 2M/(2M+N)

Where M is the number of matches and N is the number of taxa in just one column.

3.11 Cloning

3.11.1 Preparation of competent cells

The method for preparation of competent cells was derived from the method of Hanahan (Hanahan 1983). 100 µL of JM109 competent cells were inoculated onto M9 minimal media supplemented with Vitamin B1 (Appendix 7) and incubated overnight at 37 ºC. A single colony was taken from the agar and a starter culture of 5 ml of LB broth was inoculated with the colony. A 0.5 mL aliquot of the starter culture was removed and used to inoculate 50 mL of LB broth (1% v/v). The culture was grown in an orbital shaking incubator at 37 ºC and 200 rpm until an $OD_{550\ nm}$ of 0.55 was reached at which point the culture was placed on ice for 30 minutes. The culture was centrifuged for 10 minutes at 4 ºC and 2,000 x g after which the supernatant was discarded. The cell pellet was resuspended in 4 mL ice cold FSB solution (Appendix 8) and incubated on ice for 15 minutes and then centrifuged for

10 minutes at 4 ºC and 2,000 x g after which the supernatant was discarded. The pellet was resuspended in 720 µL of ice cold FSB solution and 26 µL of Dimethyl sulfoxide was added in a drop-wise fashion. The JM109 cells were checked using positive control from pGEM-T kit (Promega). Successful preparations found to be competent were dispensed into 50 µl aliquots and stored at -80ºC.

3.11.2 Ligation

Ligation reactions were performed as set out according to Table 3.2. The ligation reaction(s) were mixed by pipetting and incubated at room temperature for 1 hour or alternatively overnight at 4 °C to increase the number of transformants.

3.11.3 Transformation

Tubes of JM109 High efficiency competent cells were removed from storage (-80 °C) and thawed on ice for approximately 5 minutes. Subsequently, 2 µL of ligation reaction were transferred to a sterile microfuge tube on ice and 50 µL of JM109 cells were added to the ligation reaction and mixed by gentle flicking. The tubes were put on ice for 20 minutes and then Heat-shocked at 42 °C for 50 seconds and then returned to ice for a further 2 minutes. Following this, 950 µL of room temperature SOC medium (Appendix 9) was added to the tube and incubated for 1.5 hours at 37 °C in an orbital incubator with shaking at ~150 rpm. Two JM109 LB plates (Appendix 2) were warmed in an incubator at 37 °C for 30 minutes prior to spreading a lawn from a 100 µL aliquot of the ligation reaction onto each of the two plates which were incubated overnight (16-24 hours) at 37 °C. The presence of white

Table 3.2. Ligation reaction setup

Reagent	**Positive control**	**Standard reaction**
2x Rapid Ligation Buffer	5 µl	5 µl
pGEM-T® Easy Vector	1 µl	1 µl
PCR product	-	3 µl
Control insert DNA	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl
H2O	1 µl	-

colonies was used as a marker of successful incorporation of the PCR product into the plasmid and this was confirmed by restriction digest.

3.12 Plasmid purification - PureYield™ Plasmid Miniprep kit

Antibiotic selection broth (5 mL) (Appendix 2) was prepared and individual broths were inoculated with a single white colony from the transformation stage and incubated overnight at 37 ºC. Prior to beginning the experiment the cell lysis buffer was warmed to 37 ˚C and inverted to dissolve any precipitate. To ensure maximum yield was achieved the alternative protocol for larger volumes was used. A 1.5 mL aliquot of overnight culture was added in to a microfuge tube and centrifuge at 13,000 x g for 30 seconds and the supernatant discarded, this step was repeated once to process a total volume of 3 mL. The cell pellet was resuspended in 600 µL of sterile 18.2 MΩ/cm water and 100 µL of cell lysis buffer was added. The cell lysis buffer contains SDS to lyse cell membrane and sodium hydroxide which breaksdown the cell wall but also causes DNA to linearise by breaking hydrogen bonds. The tube was inverted 6 times until the solution changed from opaque to clear blue. Within 2 minutes 350 µL of neutralization solution was added and the solution was mixed by inversion and a precipitate was formed. The neutralisation solution contains potassium acetate which reduces the alkalinity of the solution causing renaturation of the plasmid DNA but leaving gDNA insoluble allowing it to be separated from the plasmid DNA by centrifugation. The solution was centrifuged at 13,000 x g for 3 minutes to pellet the precipitate and the supernatant was recovered and placed in to a PureYield™ Minicolumn. The column was centrifuged at 13,000 x g for 15 seconds

and the flow through discarded. A 200 µL aliquot of Endotoxin removal wash was added to the column and centrifuged for 15 seconds, followed by addition of 400 µL of column wash solution to the column and a 30 second centrifugation at 13,000 x g. The column was removed from the receiver tube and placed in a microfuge tube and 30 µL of elution buffer was added to the column membrane which was then left to stand for 1 minute. The column was centrifuged for 15 seconds and the eluated DNA was split in to 10 µL aliquots and stored at -20 ˚C.

3.13 DNA quantification

The DNA concentration of the plasmid extraction(s) was quantified so that analogous amounts could be used to calculate the amount required to generate an appropriate standard curve for the qPCR reaction and to calculate the amount required for a restriction enzyme digest to confirm the correct size fragment was incorporated in to the plasmid. The spectrophotometer was blanked with an appropriate solution and 5 µL of sample was pipetted in to a cuvette with 65 µL of 10 mM Tris-HCL pH 7.5. The spectrophotometer was set to read the wavelength between 200 nm and 300 nm and the absorbance value at 260 nm was taken to calculate the concentration of DNA using the following formula;

[DNA] (µg/mL) = A260nm x DNA coefficient x Dilution factor

3.14 Restriction enzyme (RE) digest

Between 0.2-1.5 µg of DNA was used in the RE digest along with 5 U of EcoR1 (Promega) per µg of DNA; 1x RE buffer; 0.1 mg/mL BSA and made up to 20 µl with sterile 18.2 MΩ/cm H_2O. The reaction was incubated at 37 ºC for 90 minutes followed by a heat inactivation step at 65 ºC for 10 minutes. The glycerol concentration was kept below 10% in the final reaction mix so that it did not affect enzyme specificity.

3.15 Quantitative PCR (qPCR)

3.15.1 Preparation of qPCR standard curve

The control strains used to prepare qPCR standard curves were cultured and had DNA extracted as described above. Conventional PCR was carried out on each strain using 1x Buffer I (NEB), 0.5 µM primer, 0.2 mM each dNTP, 1 mM $MgCl_2$, 1 µL gDNA made up to 50 µL with sterile 18.2 MΩ/cm H_2O under the following conditions 97 °C at 3 min for one cycle; 97 °C for 30 s, Annealing temperature (AT; Table 3.3) 30 s, 72 °C at 30 s for 30 cycles and a final extension step of 72 °C for 10 min with successful PCR confirmed by 1% agarose gel electrophoresis as described above. The PCR products were cleaned to remove unincorporated dNTPs and primers using Exo-SAP-IT as described above and cloned using the p-GEMT easy vector cloning kit (Promega).

Absolute quantification of the whole or divisions of the microbial community was achieved through generation of a standard curve. The first step in the generation of the standard curve was to calculate the combined length (bp) of the plasmid and the PCR product. The weight of the plasmid was calculated by multiplying the size of the plasmid (bp) by the average weight of one bp which is 1.096×10^{-21} (g/bp). The average weight of one bp was calculated by dividing the average molecular weight of a double stranded DNA molecule (660 g) by Avagadro's number (6.023×10^{-23}). The next step was to calculate the mass of plasmid containing the copy number needed to achieve a suitable standard curve. The standard curve was prepared to quantify between 300,000 and 30 copies/ml in 10-fold dilutions for all primer sets except for Eub338/Eub518 which was prepared to quantify between 6,000,000 and 60 copies/ml also in 10-fold dilutions. Therefore, to calculate the mass of plasmid containing the copy number of interest, the mass of the plasmid calculated in stage two was multiplied by the desired copy number for the standard curve. Once this figure had been achieved the final concentration of the plasmid DNA could be calculated by dividing the mass of plasmid required by the volume in the final PCR reaction mix. It was then possible to prepare a standard curve by using the formula $M_1V_1 = M_2V_2$ where M_1 is the final concentration of the plasmid which was calculated in the previous step, V_1 is the final volume required to achieve the concentration which at this stage is unknown, M_2 is the concentration of the stock plasmid and V_2 is the volume in which the plasmid will be diluted. Therefore, the equation needs to be rearranged so that V_1 can be calculated.

Table 3.3. Primers used for qPCR assay

Primer	**Sequence**	**Target Gene**	**AT (ºC)**	**Reference**
Eub 338	ACT CCT ACG GGA GGC AGC AG	16S rRNA	65	Lane 1993
Eub 518	ATT ACC GCG GCT GCT GG			Muyzer *et al*. 1993
Ps-f	GRM CGC TAA TAC CGC NTA CGT	16S rRNA	50	Baxter and Cummings 2008
Ps-r	TCC TCT CAG ACC AGT TAM GGA			
HI-IV	ACT TTT GGC GGT TAC TCT GT	Outer membrane protein P-6	55	Van Ketel *et al.* 1990
HI-V	TGT GCC TAA TTT ACC AGC AT			

3.15.2 Setup of qPCR reaction mix

Once the standard curve had been prepared using the above protocol the reaction mix for the standard curve and reactions could be made. All of the reaction components were kept on ice during the reaction setup which was prepared in a PCR Workstation™ (C.B.S. Scientific) and pipette filter tips were used to limit the potential for contamination. No template controls (NTCs) were setup to ensure that all of the reagents, plastics and pipettes used in the reaction were sterile. The standard curve, NTCs and unknowns were all setup in triplicate to ensure that the protocol had been performed accurately. The qPCR method used herein was as described (Baxter & Cummings 2008). Firstly, the plasmid DNA and that of the unknowns was heated to 95 °C and held for 10 minutes to ensure that any tertiary structures which may have formed had been eliminated so that efficient amplification was possible. The plasmid DNA was then used to prepare the standard as described above using sterile distilled water as the diluent and filter tips (SLS) to reduce the risk of contamination. Once the standard curve dilutions had been prepared the reaction mix was prepared ensuring that ABsolute™ QPCR SYBR mix (Thermo Scientific) had limited exposure to light to prevent its degradation. The final reaction mix contained 1x ABsolute™ QPCR SYBR® Green Mix (Thermo-Start™ DNA Polymerase, 3 mM $MgCl_2$) , 0.35 mM each primer, 12.5 µg BSA and 5 µl of DNA in a final volume of 25 µL. The cycling conditions used were an initial enzyme activation step at 95 °C for 15 min, then 50 cycles of 95 °C 10 seconds, annealing temperature (Table 3.3) for 15 seconds and extension at 72 °C for 20 seconds on RotorGene RG-3000 instrumentation (Corbett life sciences). Target copy

numbers for each reaction were calculated from the standard curve and were used to ascertain the number of copies per ml of sputum then log transformed.

3.15.3 Normalisation of qPCR data

The mean copy numbers from the triplicate reactions were calculated using the qPCR standard and the value for the refrigerated samples was paired with the same room temperature sample and the two values were added together. The concentrations for the two temperature conditions were then divided by the two values added together to normalise the values and obtain a value between 0 and 1 to reduce the noise from biological variance.

3.15.4 One-Way Analysis of variance (ANOVA)

The normalised concentrations from qPCR were then input into Minitab (v16.1.0) along with patient number and treatment type input as a number, 1 for fridge and 2 for room temperature. A One-Way ANOVA was selected with normalised concentration as the response and treatment as the factor. This generated a report that contained the mean and standard deviation for each treatment as well as a p-value depicting the difference between the two means incorporating range. A p-value of <0.05 was deemed to be significant.

3.15.5 Pearson product moment correlation

Continuous variables (Age, FEV_1 % Predicted, Bacterial Shannon diversity, species number, community evenness and fungal species richness) associated with qPCR were ranked then analysed by Pearson product moment correlation and given P-values using Minitab 16. Discrete variables (CFTR Genotype, Sex, Pseudomonas colonisation and administration of inhaled antibiotics) associated with qPCR, fungal or bacterial species number were analysed by One-way ANOVA also using Minitab 16. A P-value of <0.05 was deemed significant for both tests.

3.16 Ethical Approval

Ethical approval for the project was granted by the School of Applied sciences ethics committee at Northumbria University. This included storage of human specimens in the secure tissue bank facility within the university. However, this was never utilised and samples were stored and processed at the Freeman Hospital.

Ethical approval for the Freeman Hospital was obtained from County Durham and Tees Valley research ethics committee REC (ref 07/H0908/68). The criteria are;

- Such samples would be only those that are sent to the laboratory for routine investigation i.e. no additional samples would be requested specifically for the study.
- The additional test(s) performed on the sample would always be within the scope of the test that has been requested by the clinician.

Test that require specific informed consent e.g. HIV testing would not be performed.

- The fact that extra tests may be performed on patient samples would in no way impact on the routine processing of the samples using the standard operating procedures of the laboratory or the reporting of results
- Any data obtained from additional tests would not be reported and would not be allowed to impact on patient management in any way. Data would be used solely for the evaluation of methods within the laboratory
- On occasions evaluations may be sponsored by commercial companies interested in the performance of new diagnostic products. On such occasions, no patient identifiable data will be released to such companies.

This approach has been approved by our Trust R&D Department and the Chair of the Local Ethics Committee.

4. Assessment of sample handling practices on microbial activity in sputum samples from cystic fibrosis patients

Abstract

Aim: The aim of this study was to quantitatively and qualitatively assess the effect of sample storage on the metabolically active microbial community found in sputum samples from patients with cystic fibrosis (CF).

Methods: Sputum samples were collected and split in two equal aliquots one of which was immersed in RNA*later* and refrigerated immediately, the second stored at room temperature for 24 h and RNA*later* was subsequently added. mRNA was extracted and RT-PCR-DGGE and qPCR analysis of the bacterial and fungal communities was carried out using CA, RDA, CCA, and Raup-Crick for DGGE and One-way ANOVA for qPCR data.

Results: Significant differences in the bacterial communities between the two protocols were observed but there were no significant difference seen in the fungal community analyses. Analysis by qPCR demonstrated that room temperature storage gave statistically significant increases in Eubacteria and *Pseudomonas* spp. and a statistically significant decrease in those of *H. influenzae*.

Conclusions: The analysis of metabolically active microbial communities from CF sputum using molecular techniques indicated that samples should be stored at 4˚C upon addition of RNA*later* to obtain an accurate depiction of the CF lung microbiota. Also, storing respiratory samples at room temperature may cause an over representation of *P. aeruginosa* and mask the presence of other clinically significant organisms.

4.1. Introduction

The airways of cystic fibrosis (CF) patients have been shown by both culture dependent (Baltimore et al. 1982; Tunney et al. 2008) and independent methods, which do not require prior cultivation of microorganisms, (van Belkum et al. 2000; Rogers et al. 2003) to be a complex microbial environment containing many different taxa. Analysis of these microbial communities by culture dependent methods shows that CF patients are most likely to suffer from infections due to *P. aeruginosa*, *S. aureus* and *H. influenzae* respectively (Cystic Fibrosis Foundation 2009). Culture independent studies are largely in agreement with culture; however, molecular studies also show the increased prevalence of *Prevotella* spp., *Neisseria* spp. and oral streptococci such as the SMG (Harris *et al.* 2007; Bittar *et al.* 2008b; Sibley *et al.* 2008b). Comparisons of culture dependent and independent techniques have highlighted the limitations of routine microbial culture to unearth the true diversity of this environment. This may be due to the fastidious nature of many anaerobic bacteria (Bittar, Richet, et al. 2008) or due to heavy growth of *P. aeruginosa* during aerobic culture making isolation of clinically significant organisms difficult. Whilst validation and the improving selectivity of routine culture still make this the 'gold standard' for identification of many CF isolates, the use of culture independent techniques is becoming increasingly important as a clinical tool. Therefore, it is important to assess the effects of sample storage on microbial nucleic acids because many studies are multicentre and require shipping of samples from the ward to the laboratory which may be on different sites.

The effect of sample handling and storage on the results of routine microbial culture has previously been assessed. It has been demonstrated that storing samples at 4 °C

for 48 h yields reproducible results by culture in up to 75 % of samples (Gould et al. 1996). A recent study showed that these results held true for the predominant organism in samples when cultured immediately versus samples stored at 4 °C and at 20 °C (room temperature) (Pye et al. 2008). However, quantitative culture of the samples demonstrated that in 24 % of samples stored at 4 °C there were at least ten-fold fewer viable organisms compared to 8 % in the samples stored at 20 °C (Pye et al. 2008). The findings of Pye *et al.* (2008) recommends storage of samples at room temperature rather than 4 °C, which is now becoming common practice in many studies using both culture dependent and independent methods. The effect of sample storage on the results of molecular studies is yet to be defined which may have a significant effect on the results of such studies, especially when looking at metabolically active members by way of mRNA due to the short half life of this molecule.

In this study we hypothesised that storing samples at room temperature skews the data set from what would truly be seen in the CF lung. We propose a simple method for processing sputum samples to give a more accurate depiction of how the microbial community is composed *in-vivo*.

4.2 Methods

A total of five CF patients were selected for this study based upon the volume of sputum they had produced so that aliquots were available for both storage conditions and routine microbiological culture. The patient group consisted of three males and two females with a mean age of 28 at the time of sampling.

4.3 Results

4.3.1 DGGE Ladder

The initial test of the DGGE ladder for bacteria revealed that a denaturing gradient of 35-55% was insufficient to encompass the organisms regularly isolated from CF sputum by culture (Fig. 4.1). Therefore, the denaturing gradient was increased to 35-65% and the ladder was run again using the same conditions as before (Fig. 4.2). This image revealed that the bands from several organisms denatured at the same Rf so organisms were removed from the final ladder based upon their suspected clinical relevance leaving seven organisms in total (Fig. 4.3). This ladder was run alongside the CF sputum samples to give putative identities to the bands present.

4.3.2 DGGE analysis of microbial communities

The DGGE analyses of the bacterial community produced 32 distinct band positions (12-22 bands per lane). Qualitative observation showed that refrigerated samples had more bands in the top third of the gel (lower GC content) compared to those stored at room temperature (Fig. 4.3). Firstly, correspondence analysis demonstrated that the samples appeared to be separated by the first axis (Appendix 10). A DCA axis length of 0.745 was observed for the bacterial communities. To determine if this finding was significant statistical analysis of the band profiles by RDA including Monte Carlo permutation testing demonstrated that samples were significantly grouped ($P < 0.002$) according to sample storage (Fig. 4.4). Furthermore, the refrigerated sputum samples show a greater variance over the y-axis than the samples stored at room temperature. In order to investigate the effects on ecological analysis of the bacterial

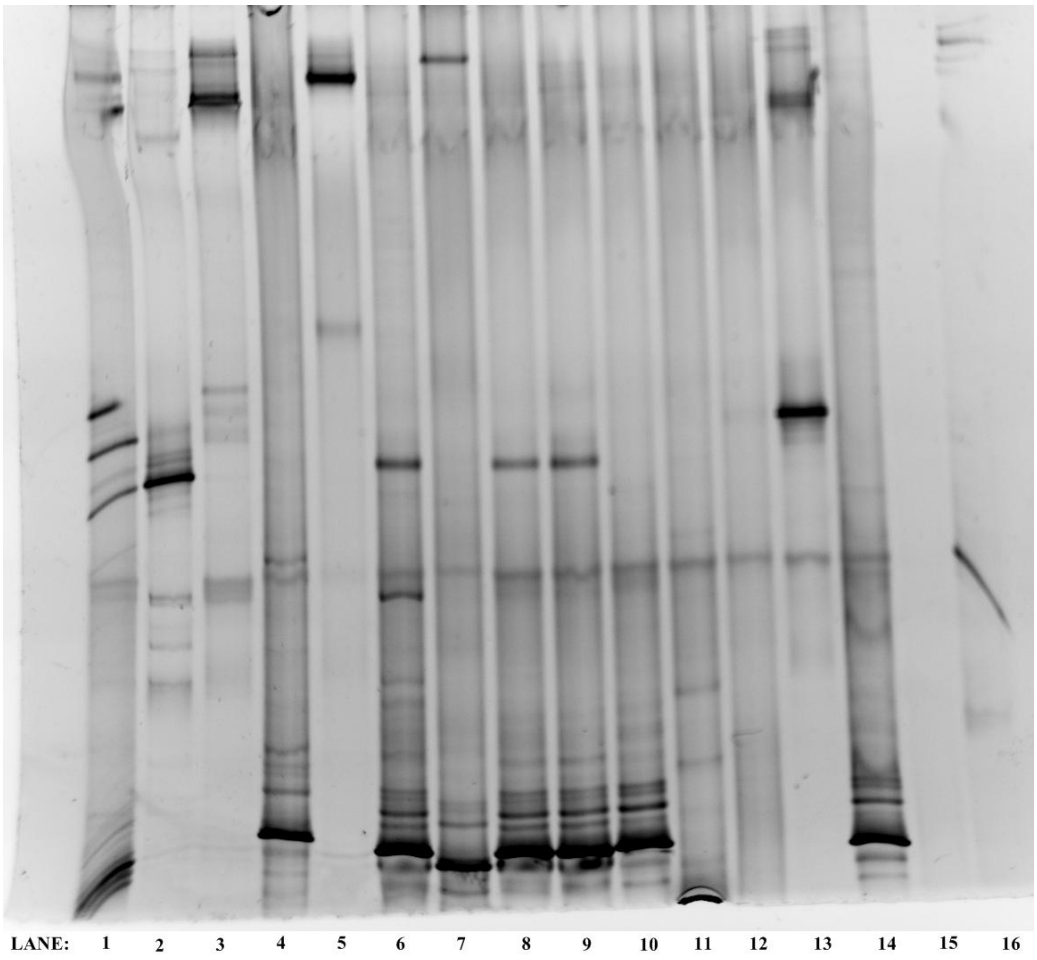


Figure 4.1. Preliminary analysis of the separation of common CF isolates on a 35-55% denaturing gradient

Lane 1 ladder Lane 2 P. aeruginosa Lane 3 S. aureus Lane 4 Steno. maltophilia Lane 5 H. influenzae Lane 6 B. cepacia Lane 7 B. multivorans Lane 8 B.cenocepacia Lane 9 B.stabilis Lane 10 B.vietnamiensis Lane 11 B. dolosa Lane 12 A. xylosoxidans Lane 13 P. fluorescens Lane 14 R. pickettii Lane 15 P. anaerobius Lane 16 Strep. oralis

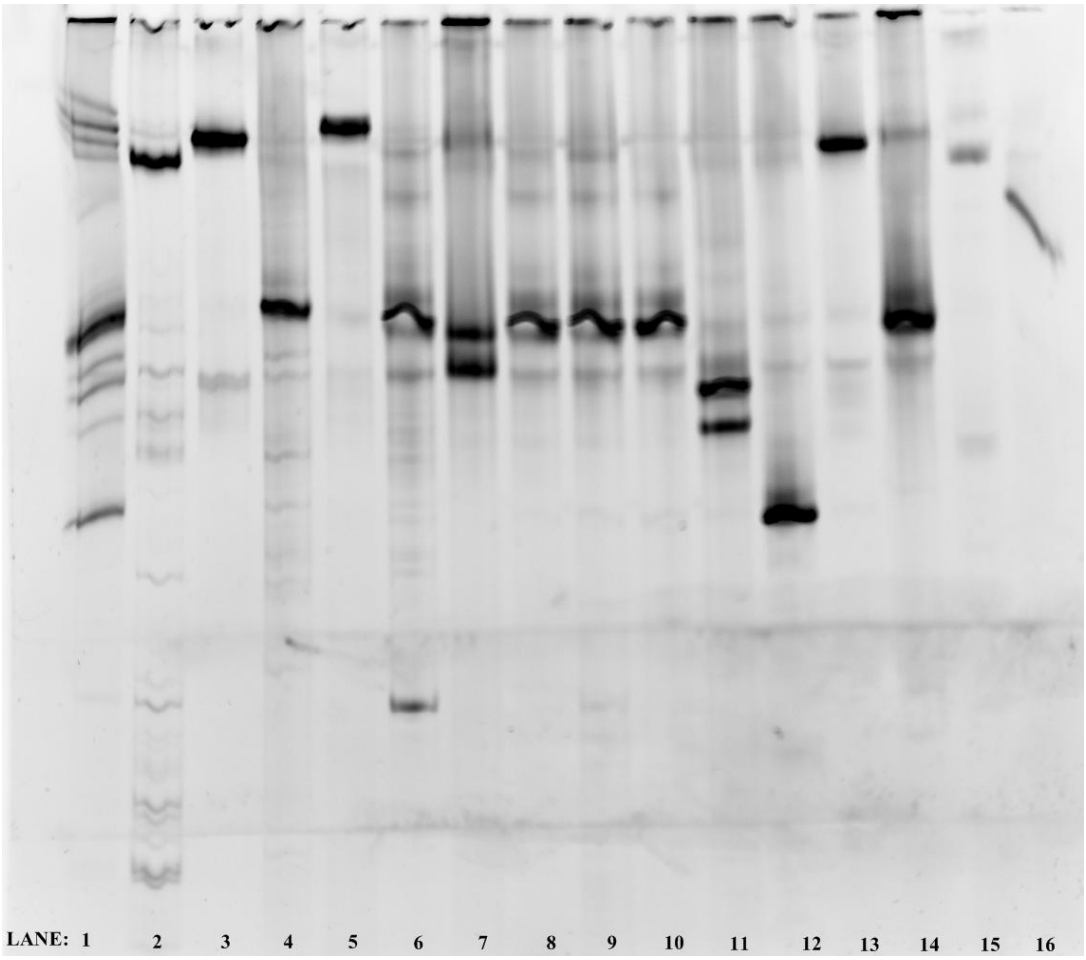


Figure 4.2. Further test of DGGE ladder and single isolates on a 35-65% denaturing gradient

Lane 1 ladder Lane 2 P. aeruginosa Lane 3 S. aureus Lane 4 Steno. maltophilia Lane 5 H. influenzae Lane 6 B. cepacia Lane 7 B. multivorans Lane 8 B.cenocepacia Lane 9 B.stabilis Lane 10 B.vietnamiensis Lane 11 B. dolosa Lane 12 A. xylosoxidans Lane 13 P. fluorescens Lane 14 R. pickettii Lane 15 P. anaerobius Lane 16 Strep. oralis

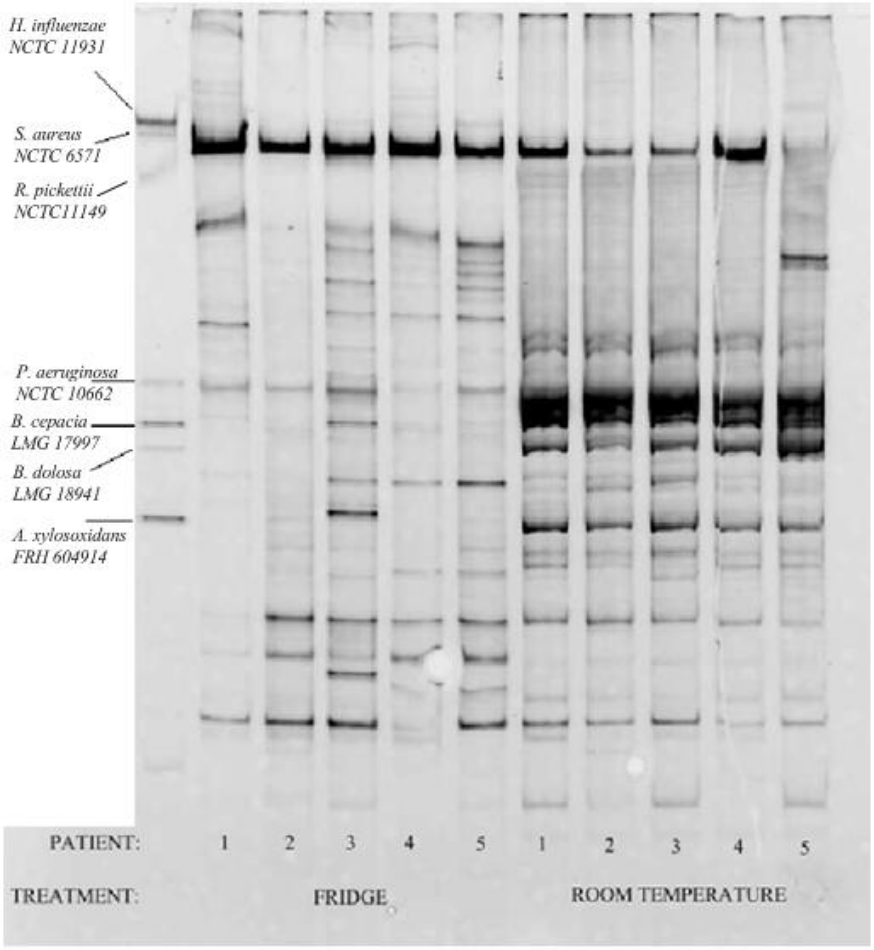


Figure 4.3. DGGE analysis using a 35-65% denaturing gradient showing the effect of storage on the bacterial communities of the five patients

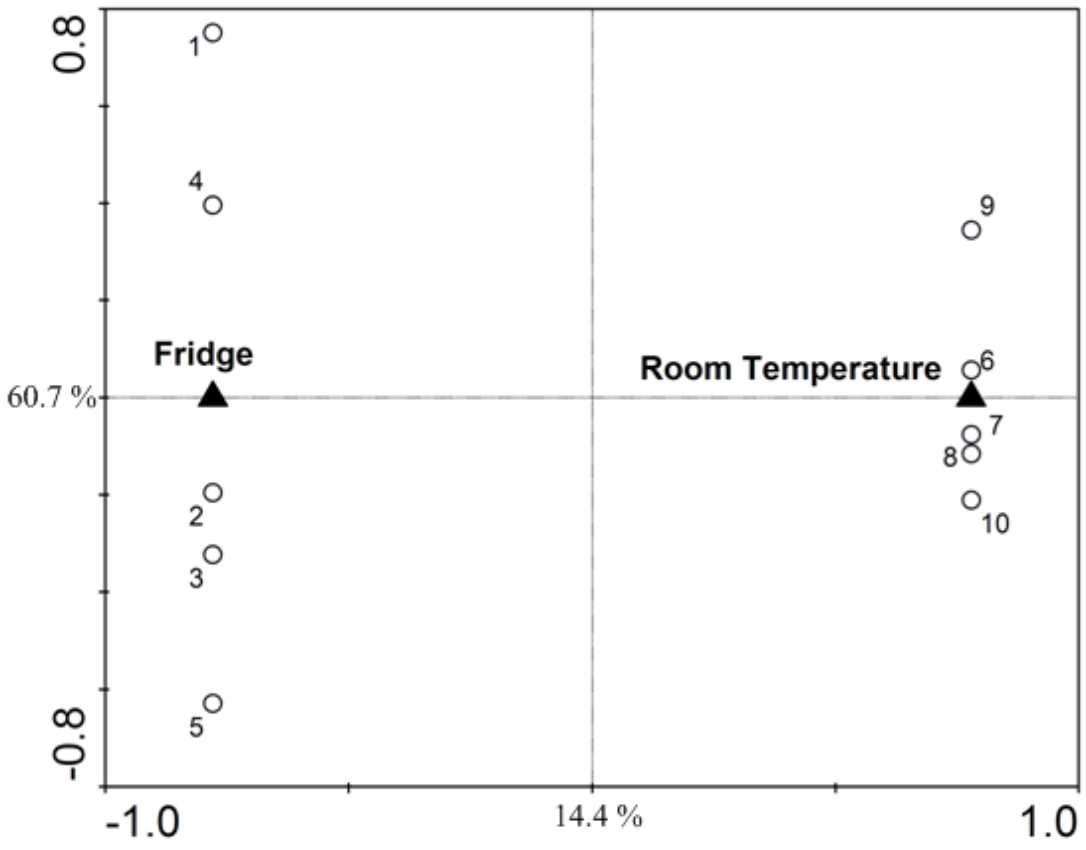


Figure 4.4. RDA analysis of the bacterial DGGE profiles for the samples stored in the fridge and at room temperature

Table 4.1. Comparison between samples handling protocol using Raup-Crick similarity index

		Fridge					**Room Temp.**				
		1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Fridge	**1**		0.84	0.46	**0.95**	0.38	0.63	0.61	0.73	0.58	0.43
	2			0.77	0.71	0.63	0.85	**0.99**	0.87	0.57	0.65
	3				0.84	0.65	0.22	0.28	0.90	0.10	0.26
	4					0.81	0.62	0.30	0.66	0.56	0.34
	5						0.47	0.49	0.71	0.23	0.75
Room Temp.	**1**							**1.00**	**1.00**	**1.00**	0.89
	2								**0.99**	**1.00**	**0.99**
	3									**0.99**	0.84
	4										0.93
	5										

community Raup-Crick similarity index was calculated for both storage conditions. This analysis found that when comparing the samples stored at 4˚C with each other that only one (n = 10) was significant in comparison to seven (n = 10) for the samples stored at room temperature (Table 4.1). Furthermore, when

comparing between the two sample handling protocols only one was significant (n = 25).

DGGE analysis of the fungal community gave a total of 17 distinct bands (4-10 per lane; Appendix 10). The DCA analysis of fungal DGGE gave an axis length of 4.450. There was no significant difference between sputum collection protocols as determined by CCA (P = 0.180; Appendix 10) but the band intensities were less in the samples that were stored at room temperature as compared to those that were refrigerated (Fig. 4.5). This difference was demonstrated by CA of the fungal DGGE profile which showed a separation of the samples by handling protocol rather than by patient (Fig. 4.6). Four bands (F1-F4) were excised, sequenced and deposited in GenBank (accession numbers GU001640, GU065334-36 respectively). The NCBI BLASTn tool was used to search for closest deposited sequence match. The closest related sequences were *Candida dubliniensis* (FM992695.1) 99%, *C. albicans* (GQ495089.1) 100%, *C. parapsilosis* (AY497686.1) 99% and *Aspergillus fumigatus* (FM197606.1) 99% respectively.

4.3.3 Real-time PCR results

Quantification of metabolically active total bacteria and *Pseudomonas* spp. using the mean copy number of the 16S rRNA gene, indicated a greater than 2-fold increase of *Pseudomonas* spp. 16S rRNA gene in the samples stored at room temperature

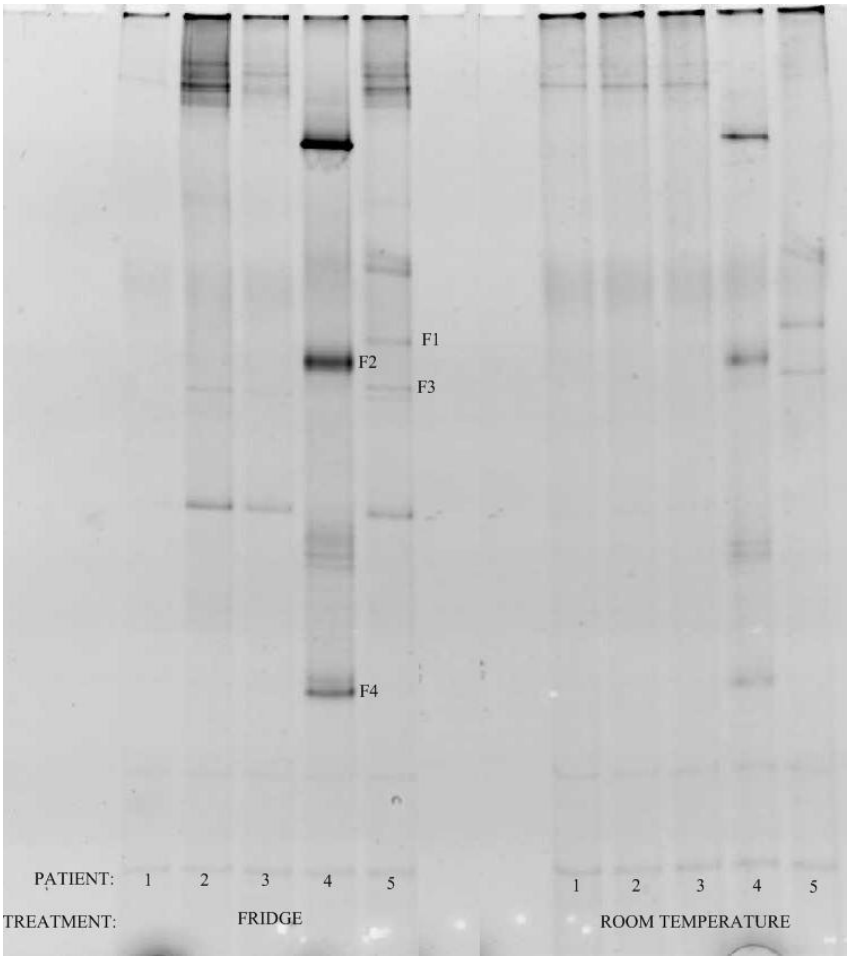


Figure 4.5. DGGE analysis using a 30-55% denaturing gradient showing the effect of storage on the fungal communities of the five patients.

F1-F4 represent excised bands which were subsequently sequenced

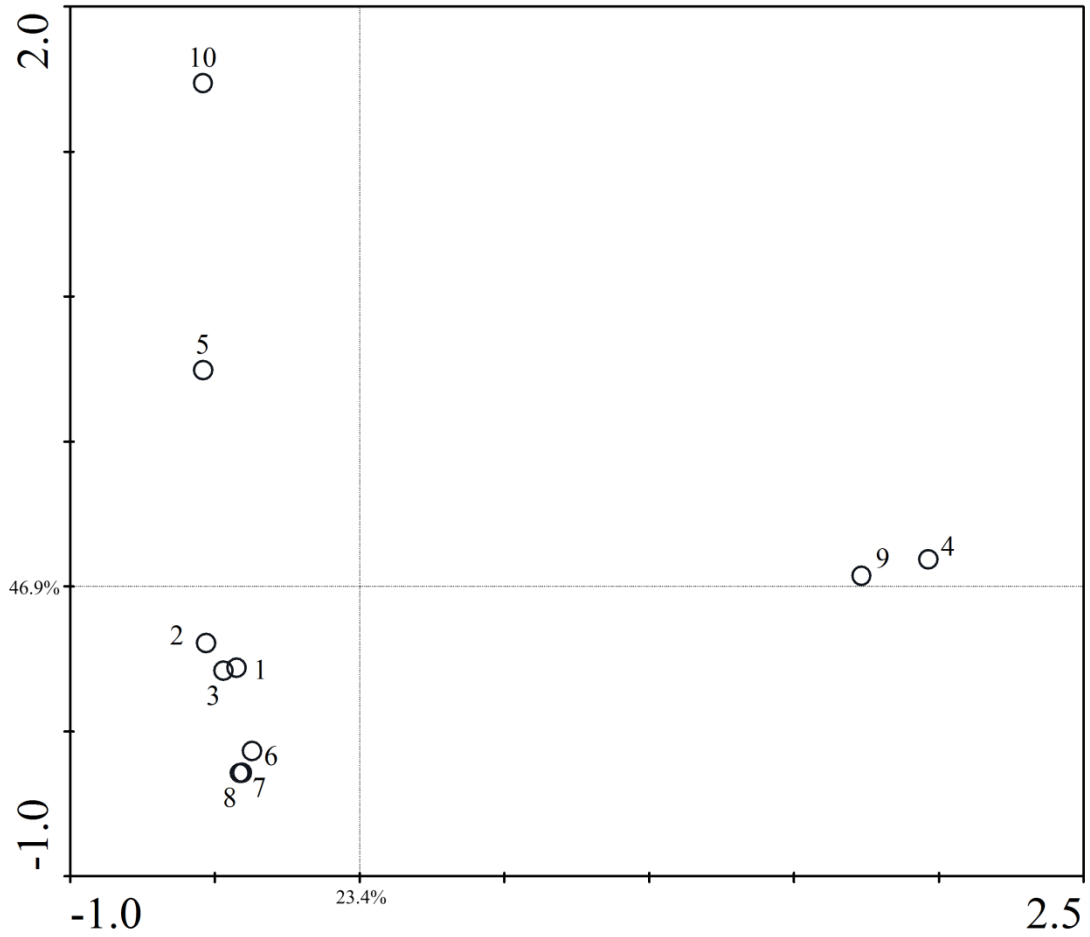


Figure 4.6. CA analysis of the fungal DGGE profiles from the five patients samples stored in the fridge and at room temperature

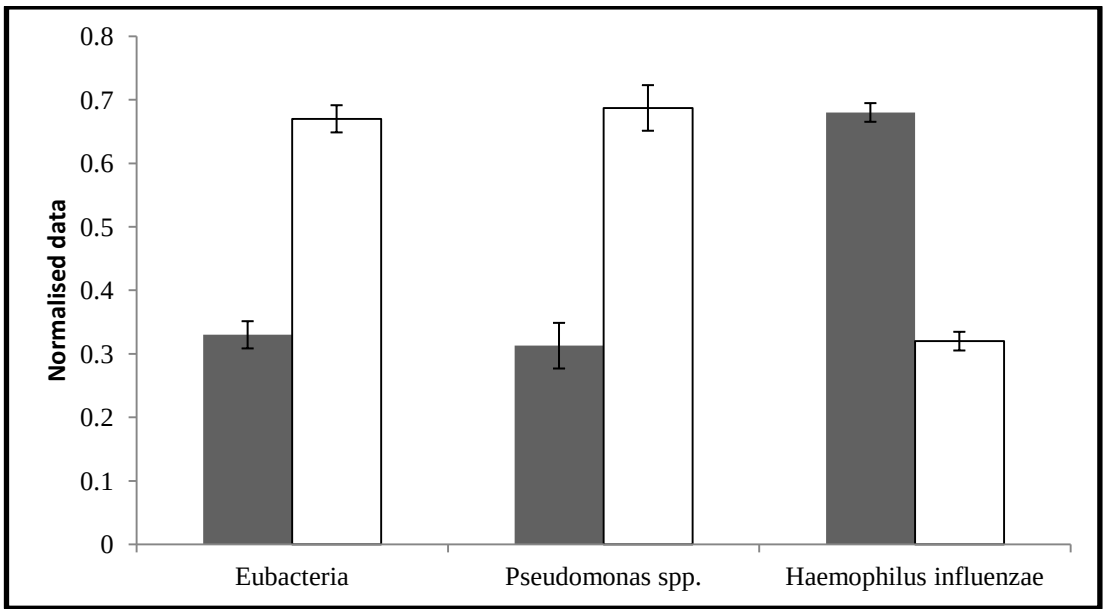


Figure 4.7. Normalised qPCR data to demonstrate the mean effect of sample storage on bacterial 16S rRNA**.**

Grey bars indicate samples stored at 4 ^{O}C; white bars indicate samples stored at room temperature

compared to the refrigerated samples (Fig. 4.7). This finding was statistically significant for both taxa (P <0.001). In contrast, quantification of the mean *H. influenzae* P6 outer membrane protein copy number revealed a greater than 50%decrease in P6 gene copy numbers in the samples stored at room temperature compared to the sample stored at 4°C that was statistically significant (P <0.001).

4.4 Discussion

Preparation of a DGGE ladder of known organisms revealed one the the caveats of 16S rRNA PCR-DGGE in that bands from different organisms can posess the same migration rate (Jackson et al. 2000). Furthermore, it was noted from the construction of the DGGE ladder that an individual organism can contain several heterogeneous copies of the 16S rRNA gene which has been noted previously (Dahllöf et al. 2000). The caveats identified from the construction of the DGGE ladder show that obtaining an accurate depiction of the bacterial communities present is difficult at best. However, it has been shown that PCR-DGGE can be utilised to identify the major changes that occur due to pertubation or identify difference between bacterial communities in different habitats (van der Gast *et al.* 2008). Therefore, this technique, in combination with qPCR should give a clear indication of any changes which may occur due to the differing sample storage.

Many studies on CF therapies are multicentre and involve both qualitative and quantitative bacterial analysis. This usually entails shipping of samples from study sites to a central processing laboratory. Here we show that the storage of sputum samples can have a powerful effect on the results of culture independent techniques

for detection of bacteria. In most situations, it is not feasable or in some cases possible to process sputum samples immediately especially if they need to be transported from the ward to the laboratory or from one site to another. In this study we take samples that have been immersed in RNA*later* and stored at 4˚C to be an accurate representation of the microbial community as it would be observed in the lung. We have based this assuption on the preservatative property of RNA*later* preventing RNA degradation as well as the bacteriostatic properties it possess preventing any change in the RNA profile of the sample. DGGE analysis of the bacterial community indicated that storage of samples significantly affected the bacterial community in the sputum ($P < 0.002$). It appears from the change in the DGGE profiles that storage at 4°C is more favorable for visualising organisms with a lower 16S rRNA GC content (Fig 4.3). Statistical analysis of the profiles showed that the samples stored at room temperature clustered together more along the y-axis than the samples stored at 4°C suggesting that storage at room temperature masks intersample variability (Fig 4.4). Further evidence of this was demonstrated using Raup-Crick similarity index where samples stored at room temperature appeared to be statistically similar on seven occasions compared to only one when the samples stored at 4˚C were analysed (Table 4.1). If the samples stored at room temperature were taken to be true then this would suggest a mostly deterministic bacterial community assembly for the CF lung whereas analysis of the samples stored at 4˚C suggests that community assembly is, in fact, stochastic. The stochastic community assembly seen in the samples stored at 4°C is in line with that found in previous studies (Rogers et al. 2005).

Culture independent techniques have previously been shown to idenify many more taxa in CF sputum samples than the corresponding sample analysed using culture

dependent techniques our data supports those of previously published work (van Belkum et al. 2000). Moreover, quantification using qPCR data showed a significant increase ($P < 0.001$) in the number of total bacteria and *Pseudomonas spp.* in the sputum samples stored at room temperature (Fig 4.7). These observations may indicate that growth of some microbial taxa, particularly *P. aeruginosa*, are favoured at room temperature in comparison to 4 °C. The increase in numbers of *Pseudomonas* spp. after storage at room temperature may explain the overgrowth of *P. aeruginosa*, commonly seen in routine microbiology. This might result in the clinical significance of *P. aeruginosa* being overestimated in such samples. It was also shown that *H. influenzae* numbers are significantly decreased after storage at room temperature when compared to storage at 4 °C ($P < 0.001$). It has been shown previously that *H. influezae* has been difficult to recover from transport swabs due to its fastidious nature (Rishmawi et al. 2007). It has also been observed that *H. influenzae* is more difficult to recover from sputum samples after postage than from fresh samples processed immediately (May & Delves 1964). However, our data contradict those of Pye *et al.* (2008) who suggest that storage at room temperature produces more favorable conditions for the recovery of *H. influenzae*.

The numbers of active bacteria present in CF sputum has not previously been assesed using culture independent methods. Using culture dependent techniques, between 10^8 and 10^9 cfu/ml were observed for the predominant organism in the sample (Pye et al. 2008). In contrast, the total bacterial 16S rRNA copy number/ml in our sample set is between 10^7 and 10^9 with a mean of 1.00×10^9 and between 10^5 and 10^7 copies/ml of this relates to *Pseudomonas* spp.. With most organisms having more than one copy of this gene our account of the active bacteria in CF sputum is significantly lower than the number of viable cells as determined by culture when

using this gene as a target. Pye *et al.* (2008) also showed that *H. influenzae* was present at 10^9 cfu/ml when it is the predominant organism, whereas, *H. influenzae* was not isolated by culture from any of our patient cohort but was identified using molecular techniques. This highlights the increased sensitivity of molecular techniques compared to culture which supports the findings from previous studies (van Belkum et al. 2000; Dalwai et al. 2007). However, the fact the *H. influenzae* was not cultured from our cohort could be due to several factors. Firstly, all of our patients were culture positive for *P. aeruginosa* which may have overgrown on the culture media and masked the growth of *H. influenzae*. This finding is supported by our qPCR data which suggests that most of the metabolic activity in our samples was due to *Pseudomonas spp.* even when the samples were stored at 4°C in RNA*later* when compared with the total number of bacteria.

There has been no previous work done by culture dependent or independent methods showing the effect of storage on fungi from CF sputum. The results of this study show that, although there was a decrease in band intensity for fungi when stored at room temperature, there was no significant difference between the sample collection proceedures ($P = 0.180$). Due to there being a slightly lower band intensity for the samples stored at room temperature our data suggests that samples required for fungal analysis should be stored at 4°C to preseve the nucleic acids in the sample. Sequence analysis of DGGE bands allowed the identification of *A. fumigatus*, a fungal pathogen that is known to adversley effect lung function (Amin et al. 2010). Also several species of Candida were found by PCR-DGGE analysis which were absent from the culture data. Due to the increased recovery rate of metabolically active fungi in CF sputum by molecular techniques it may be fruitful to utilise these techniques to identify colonising fungal species and then to asses their role as

potential pathogens. There is currently very little information pertaining to the fungal members of the CF lung microbiota and how they persist in the CF lung. Further work is required to fully analyse this community.

4.5 Conclusions

In conclusion, this study, although small, supports handling protocols where respiratory samples being used in molecular studies should have RNA*later* added immediately and then to be subsequently stored at 4 °C until required for processing if an accurate depiction of the community is to be observed and accurate quantification of bacterial numbers is to be achieved. However, for fungi, sample handling proceedures are less crucial.

5. Bacterial and fungal communities present in the lungs of cystic fibrosis patients with a severe genotype are affected by genetic and environmental factors

Abstract

Aim: The microbiology of the CF lung has been well characterised by both culture and molecular techniques and have been shown, in both instances, to be polymicrobial. The aim of this study was to investigate the CF lung microbiota in an adult population to determine the extent that environmental and genetic factors affect the microbial communities in patients with the most common CFTR mutation, F508del.

Methods: DNA was extracted from sputum samples of 29 adult patients who were either homo- or heterozygous for the F508del mutation. Universal bacterial and fungal primers were used to amplify the 16S rDNA and 28S rDNA. These fragments were analysed by DGGE. Universal bacteria primers were used to quantify the total bacterial load from the sputum using qPCR.

Results: Bacterial communities present in the CF lung were found to be stochastically assembled. Furthermore, CCA analysis of the bacterial DGGE profiles found that sex (P = 0.046) and being colonised by *P. aeruginosa* as determined by culture (P = 0.034) were driving forces that shape changes in the bacterial communities of the CF lung. Further investigation found that bacterial diversity and bacterial community evenness were significantly reduced in females (P = 0.006 and 0.003 respectively). These diversity indices were also reduced in patients colonised by *P. aeruginosa* but this was not significant. An inverse relationship was seen between age and FEV_1 in our patient cohort which has been demonstrated in previous studies (P = 0.012). Fungal community analysis found that patients

homozygous for F508del had a greater number of fungal species than those who were heterozygous (P = 0.007).

Conclusions: This study has demonstrated that many factors affect microbial community assembly in the CF lung. Recent work has found that the CF lung consists of core and satellite communities of bacteria in a CF population. Understanding the factors that cause these communities to develop will hopefully lead to more effective treatment strategies, especially for patients with a more severe disease phenotype.

5.1 Introduction

Cystic fibrosis is a lethal autosomal recessive condition most prevalent in Caucasians. Most of the morbidity and mortality in CF can be attributed to the chronic lung pathology caused by microbial infection and the subsequent hyperinflammatory response. The microbiology of CF has been well characterised over recent years by both molecular and culture based methodologies (Burns et al. 1998; Emerson et al. 2010; Rogers et al. 2003; Rogers et al. 2005; Harris et al. 2007). Common CF pathogens routinely identified by conventional microbiology include *P. aeruginosa*, *H. influenzae*, *S. aureus*, *B. cepacia complex*, *C. albicans* and *A. fumigatus* (Burns et al. 1998; Emerson et al. 2010). However, more recently anaerobic bacteria such as those belonging to the genus *Prevotella* and *Veillonella* as well as facultative anaerobes such as Streptococci have been identified using both culture dependent and independent methods (Harris *et al.* 2007; Bittar *et al.* 2008b; Tunney *et al.* 2008; Worlitzsch *et al.* 2009). Furthermore, the interactions between

common CF isolates have been investigated (Duan, 2003; Sibley *et al.* 2008a). These include the increased virulence of *P. aeruginosa* in co-culture with oral streptococci and that production of the signalling molecule Farnesol by *C. albicans* represses pyocyanin production by *P. aeruginosa* (Cugini *et al.*, 2007; Duan *et al.* 2003). Although both culture dependent and independent methodologies can be utilised to investigate the microbiology of the CF lung, culture independent techniques such as microarray, T-RFLP and clone libraries suggest that there is a greater microbial diversity in the CF lung than is found by culture dependent techniques alone (Rogers et al. 2004; Harris et al. 2007; Klepac-Ceraj et al. 2010; Guss et al. 2011). However, the discrepancy in bacterial community richness between culture dependent and independent techniques will be due, in part, to the inability/difficulty in culturing some microorganisms (Rogers et al. 2004; Guss et al. 2011). Furthermore, single bacteria species may harbour several heterogeneous copies of the 16S rRNA genes which means that culture independent analysis could lead to an overestimation of the bacterial species richness.

Although culture independent analysis of the CF lung microbiology has been on-going for over a decade it was only very recently that multivariate analysis has been used to assess the effects of clinical factors on these microbial communities (Cox *et al.*, 2010; Giraud *et al.*, 2010; Klepac-Ceraj *et al.*, 2010). Klepac-ceraj *et al.* (2010) found that CFTR genotype, inhaled antibiotics and *P. aeruginosa* isolation by culture affected the composition of bacterial taxa in a group of paediatric CF patients. Similarly, CFTR genotype has also been implicated in susceptibility to fungal infections with F508del homo- and heterozygous patients being more susceptible to infection by *G. argillacea* than patients with no F508del mutation (Giraud et al. 2010). These studies suggest that microbial community assembly in the CF lung is

affected by environmental factors (i.e. use of inhaled antibiotics) which lends itself to the hypothesis that a niche based model may be useful to predict the colonisation patterns of the CF lung based upon patient specific factors. However, other evidence suggests that neutral community assembly, in which community assembly is due to random immigration from a meta-community, might be more applicable to CF lung microbiology. Firstly, Rogers *et al.* (2005) found that each CF patient in their study had a unique bacterial fingerprint suggesting that the microbial community assembles stochastically. Similarly, work examining the geographical relationship between the microbial communities of the CF lung found that patients residing in the United Kingdom (UK) and the United States of America (USA) did not have either significantly different or significantly similar bacterial communities within paired patients (Stressmann *et al.*, 2011). Instead the communities had no greater relationship than one would expect by chance. This observation supports a stochastic (neutral) community assembly model both within and between the cohorts suggesting that neither selective pressures within the lung or biogeography affects the microbial community composition (Stressmann *et al.* 2011).

However, recent work finds that a core bacterial community does exist for CF lung microbiology (van der Gast *et al.* 2010). This strongly suggests that some species are merely transient or found in low abundance and, therefore, may not play a key role in lung pathology. Intrestingly, however, the only routinely cultured CF pathogen to be identified as part of the core community was *P. aeruginosa*, whilst *S. aureus*, *H. influenzae* and *S. maltophilia* are members of the satellite community (van der Gast *et al.* 2010).

The aim of this research was to characterise the intrinsic and extrinsic factors which have an effect on bacterial and fungal communities present in the CF lung in a cohort

of patients who were homo- or heterozygous for the F508del CFTR allele. Furthermore, the aim was to investigate whether microbial community assembly in the CF lung is stochastic or deterministic.

5.2 Methods

The patients enrolled in this study were selected for the study by the perception of the clinician that they were stable at the time of sampling but were frequently presenting at the clinic with exacerbations. All of the patients selected were either homo or heterozygous for the F508del CFTR mutation. A summary of patient age, gender, CFTR mutation, FEV_1 (% Predicted), BMI, current routine antibiotics and microbial culture data are presented in Table 5.1.

5.3 Results

5.3.1 Culture based microbiology

Microbial culture was carried out by State-registered biomedical scientists using methods recommended by the HPA for the detection of pathogens in CF sputum (Health protection agency 2009). Bacterial pathogens were detected in 25/29 patients enrolled in the study with polymicrobial communities being present in 8 of those patients, as determined by culture, which gave a mean bacterial species number of 1.14 pathogens/patient. However, microbial culture identified ten patients who had more than one morphotype of *P. aeruginosa* which were investigated as separate species. With these morphotypes taken into consideration the mean number of bacterial pathogens rises from 1.14 to 1.52 pathogens/patient.

Table 5.1 Mean clinical demographics of the 29 patient cohort

Gender	Genotype	Age (years ± SD)	FEV1 % Predicted (± SD)	BMI (± SD)	Inhaled antibiotic therpay	Microbiological culture		
						P. aeruginosa	***Candida* spp.**	***A. fumigatus***
16 Male	17 F508del/F508del	29.55 ± 10.99	56.59 ± 22.94	21.94 ± 3.28	23	23	15	3

Microbial culture was also used to detect any fungal pathogens present in the sputum. Fungal pathogens were detected in 17/29 patients enrolled in the study which gave a mean of 0.62 pathogens/patient. Of the patients' positive for fungal pathogens by culture, 16/17 had monomicrobial fungal communities.

5.3.2 Ecological analysis

The DGGE analyses of the bacterial community produced 37 distinct band positions (Fig 5.1) with a mean of 12.24 bands per lane (Range 6-22 bands per lane; Appendix 11). Each individual DGGE band was presumed to be a single taxon and was used as a measure of species richness. The mean Shannon index was 1.87 (Range 0.91 - 2.65; Appendix 11) which was used to give an overall indication of bacterial diversity which takes into account both species richness and evenness. The mean evenness of the bacterial communities was 0.76 (Range 0.44 – 0.87; Appendix 11). The mean Dice similarity between all of the patients was 41%.

The DCA analysis gave an axis length of 3.862, therefore CCA was used to analyse the species and environmental data. Monte Carlo permutation testing under full model (499 permutations) was used to analyse the relationship between the discreet and continuous variables and the DGGE band matrix which included age, sex, CFTR genotype, FEV_1 % predicted, BMI, culture positive for *P. aeruginosa*. The factors that were least significant (Use of inhaled antibiotics, BMI and CFTR genotype, P = 0.990, 0.664 and 0.304 respectively) were removed to produce the final image (Fig. 5.2). Permutation testing found that *P. aeruginosa* colonisation by culture and gender was statistically significant with P-values of 0.032 and 0.046 respectively.

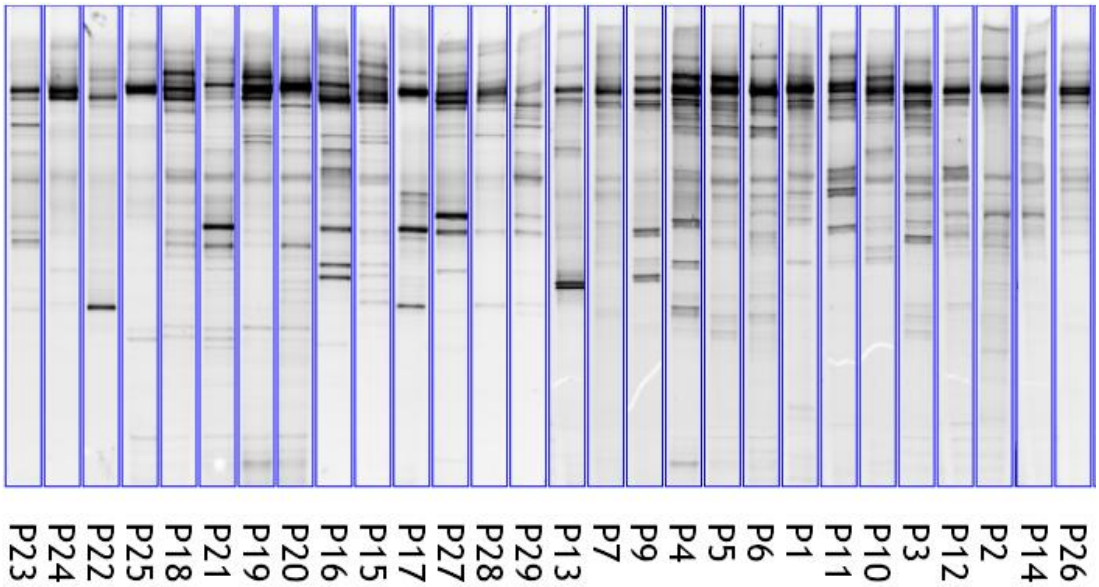


Figure 5.1 Bacterial DGGE profiles using a 35-60% denaturing gradient showing the bacterial community profiles from the 29 patients

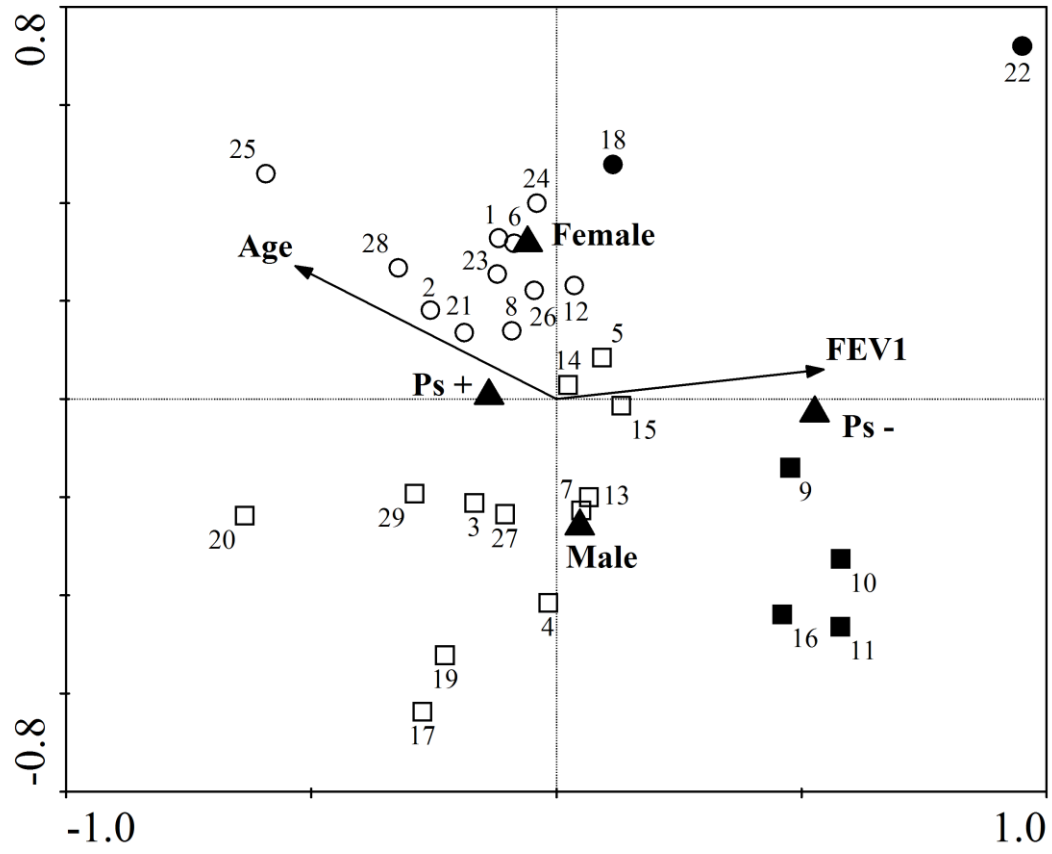
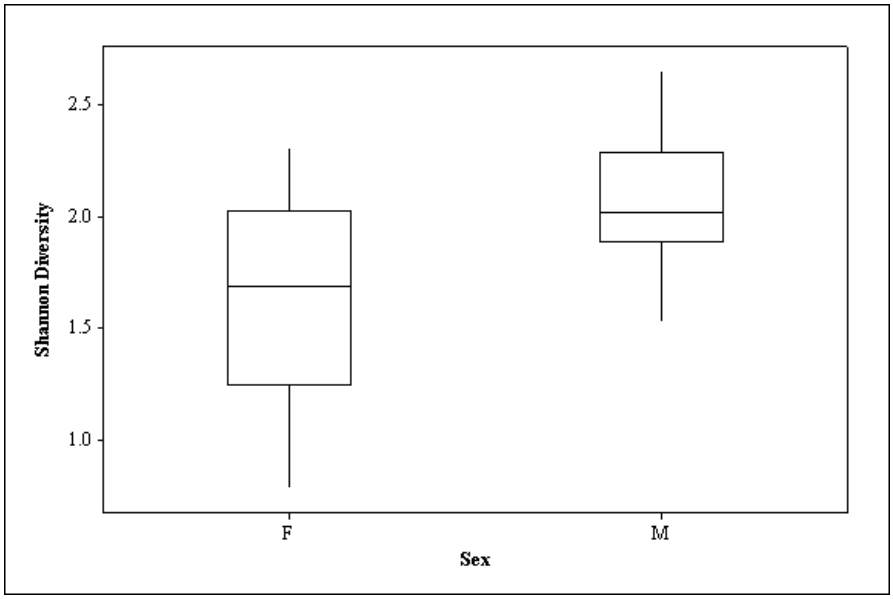


Figure 5.2 Canonical correspondence analysis of bacterial DGGE profiles including environmental variables

■ *P. aeruginosa negative males,* □ *P. aeruginosa positive males,* ● *P. aeruginosa negative females,* ○ *P. aeruginosa positive females*. ▲ Discrete variables (Gender and *P. aeruginosa* detected by culture). ↑ Continuous variables (FEV_1 % Predicted and age)

(a)



(b)

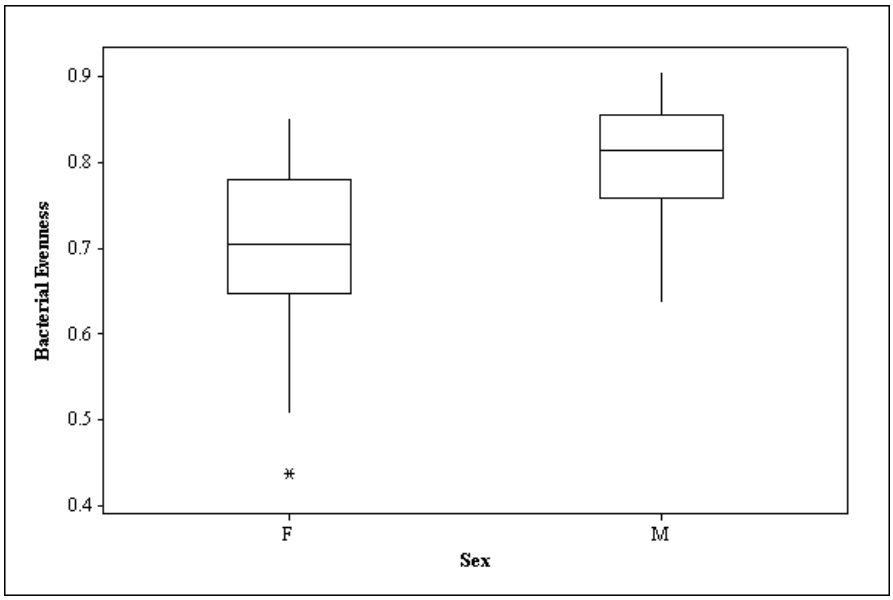


Figure 5.3 Comparison of the Shannon Diversity (a) and Bacterial community evenness (b) between male and female patients

This finding was further investigated to examine which aspects of the bacterial community differed between the sexes using one-way ANOVA analysis against the Shannon diversity, species richness and species evenness. The analysis found that Shannon diversity and community evenness were significantly lower in females than in males (P = 0.006 and 0.003 respectively; Fig 5.3 a & b). Further analysis was also carried out to investigate how *P. aeruginosa* colonisation affected the bacterial communities. This analysis found that the values for Shannon diversity, Richness and evenness were lower in patients colonised by *P. aeruginosa*, however, none of these values were significant (P = 0.166, 0.408 and 0.112 respectively. Both FEV_1 (% predicted) and age were not significant but still included (P = 0.086 and P = 1.94 respectively). Patients who were culture negative for *P. aeruginosa* were more likely to have a higher FEV_1 (% Predicted) than those who were positive as analysed by routine culture as indicated by the arrow for FEV1 included in the CCA analysis (Fig 5.2.).

5.3.3 Raup-Crick Similarity index

The DGGE band matrix was used to calculate Raup-Crick pair-wise similarities for the 29 patients in the study to test whether the bacterial communities displayed stochastically or niche driven community assembly (Table 5.2). A total of 406 pair-wise comparisons were carried out and the analysis found that 12 (3.0 %) of pair-wise comparisons yielded statistically different ($P \leq 0.05$) communities than would be expected by chance; 41 (10.1 %) of pair-wise comparisons were statistically similar ($P \geq 0.95$) and the remaining 353 (86.9 %) were neither statistically similar nor different ($P\ 0.05 \geq \leq 0.95$).

Table 5.2 Raup-Crick similarity index analysis of bacterial community assembly between the 29 patients

Number (%) of pair-wise comparisons			
≤ 0.05	**≥ 0.95**	**0.05 ≥ ≤ 0.95**	**Total No. (%) of S_{RC} comparisons**
12 (3.0)	41 (10.1)	353 (86.9)	406 (100)

5.3.4 Bacterial qPCR

The relationship between bacterial load (qPCR value) and discrete variables (sex, CFTR genotype, *P. aeruginosa* status according to culture data) was examined using one-way ANOVA analysis. Sex, CFTR genotype and *P. aeruginosa* colonisation according to culture data were not statistically significant (P = 0.500, 0.564, 0.254 respectively). The relationship between continuous variables (FEV_1 % predicted, BMI, Bacterial community H', R and E and fungal species richness) and bacterial load was examined using Pearson product moment correlation (Table 5.3). None of the continuous variables displayed a significant relationship with bacterial load. However, the analysis did demonstrate a significant negative correlation between age and FEV_1 % predicted (P = 0.030; Fig 5.4).

5.3.5 Fungal community analysis

Fungal DGGE gels were aligned using sequence analysis due to the lack of a standard ladder. DGGE analysis of the fungal community gave a total of 11 distinct band positions with a mean of 1.41 taxa per lane (Range 0 - 5 per lane; Fig 5.5). Bands present on the fungal DGGE gels were used as a measure of species richness where one band was presumed to be a single taxon. Eleven bands, from two DGGE gels were excised and sequenced. The NCBI BLASTn tool was used to search for closest deposited sequence match. The closest related sequences were from seven different species (Table 5.4)

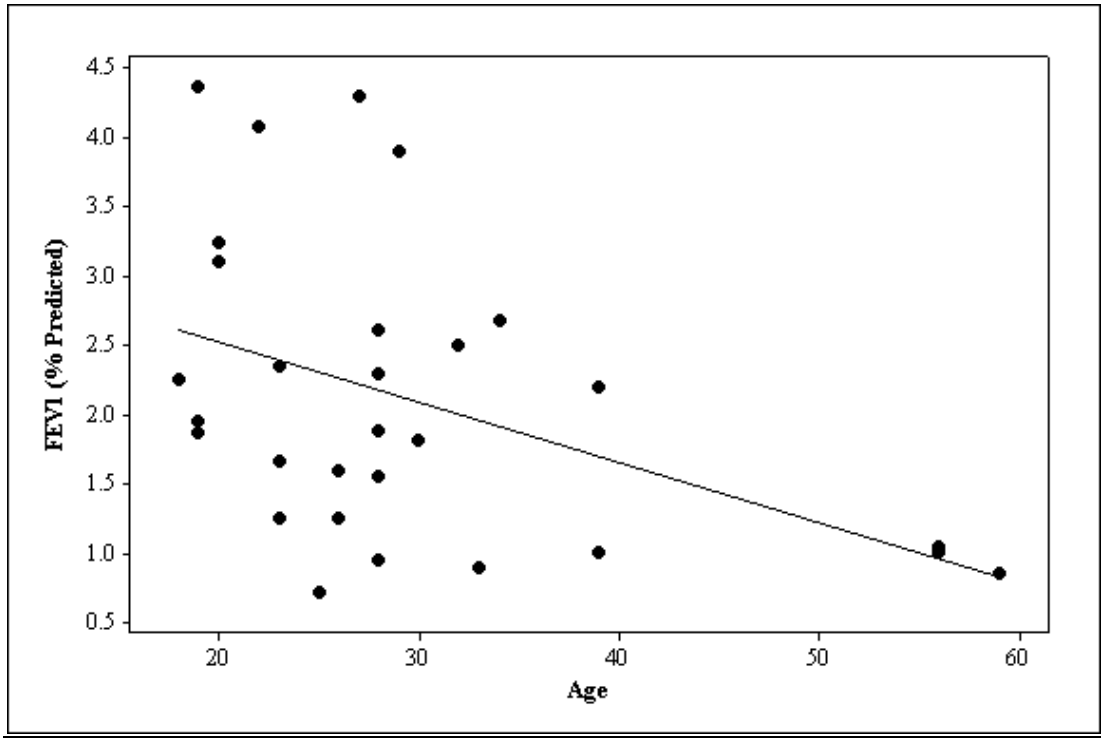


Figure 5.4 Relationship between lung function decline and patient age observed in the 29 patient cohort

Table 5.3 Pearson product moment correlation for comparison of bacterial load and continuous variables

	Age	FEV1 (% Predicted)	Shannon Diversity	Fungal Richness	Bacterial Load
FEV1 (% Predicted)	-0.404				
	P = 0.030				
Shannon Diversity	-0.194	0.274			
	P = 0.312	P = 0.150			
Bacterial Richness	-0.118	0.131			
	P = 0.544	P = 0.497			
Fungal Richness	-0.329	0.172	0.166		
	P = 0.082	P = 0.373	P = 0.388		
Bacterial Load	-0.076	0.288	-0.303	0.205	
	P = 0.696	P = 0.130	P = 0.110	P = 0.285	
BMI	0.337	0.063	-0.273	-0.085	0.189
	P = 0.074	P = 0.744	P = 0.152	P = 0.661	P = 0.327

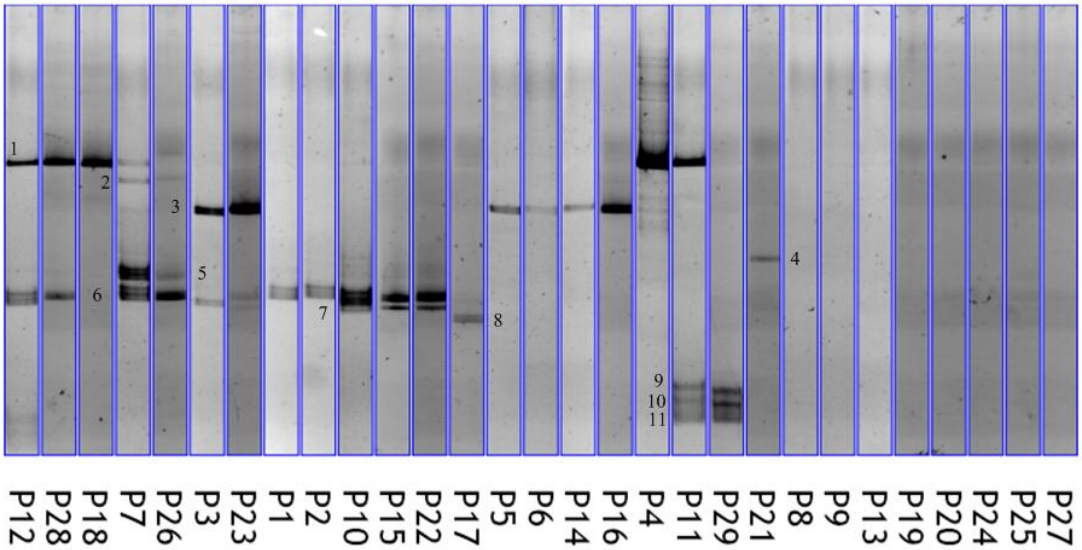


Figure 5.5 Aligned DGGE gel showing fungal diversity in the 29 patient cohort

Numbers correspond to fungal species. Band 1 C. parapsilosis, Band 2 Unknown, Band 3 C. dubliniensis, Band 4 D. hansenii, Band 5 Unknown, Band 6 C. albicans, Band 7 S. cerevisiae, Band 8, C. glabrata, Band 9, A. fumigatus Bands 10 & 11 Unknown

Table 5.4 Sequence data from the bands excised from the fungal DGGE analysis

Band number	Closest match	Max identity
1	*Candida parapsilosis* strain CBS 604 26S ribosomal RNA gene, partial	100 %
2	*Candida dubliniensis* CD36 chromosome R, complete sequence	100 %
3	*Debaryomyces hansenii* strain LL2 26S ribosomal RNA gene, partial	98 %
4	*Candida albicans* isolate CA-ALBIC-SW008 large subunit ribosomal	100 %
5	*Saccharomyces cerevisiae* strain YM3-1 26S ribosomal RNA gene, partial sequence	100 %
6	*Candida glabrata* strain QD3.3 26S ribosomal RNA gene, partial sequence	100 %
7	*Aspergillus fumigatus* partial 28S rRNA gene, strain INFU/Jc/KF/6	100 %

Bands representing *C. parapsilosis*, *C. albicans*, *C. dubliniensis* and *A. fumigatus* were present on both gels and it was these bands that were used to align the DGGE gels (Fig. 5.5).

The ability of PCR-DGGE and culture to detect fungal species present in the CF lung was assessed. In this study, fungal species were detected by culture in 59% (17/29) of patients whereas PCR-DGGE detected fungal species in 72% (21/29) of patients. Further investigation revealed that the data from both techniques found fungal species in a total of 83% (24/29) of patients. Therefore, the sensitivity for culture and molecular tests are 71% and 88% respectively. Culture analysis of fungal communities found that mixed fungal infection occurred in two patients (7%) whereas PCR-DGGE analysis found that ten patients (34%) had mixed fungal infections. The most predominant fungi found by PCR-DGGE was *C. albicans* (38%) followed by *C. parapsilosis* (24%), *C. dubliniensis* (24%), *A. fumigatus* (7%) *S. cerevisiae* (7%), *C. glabrata* (3%) and *D. hansenii* (3%) by matching with sequenced band positions.

Due to fungal communities being of low diversity, it was decided not to perform multivariate analysis. Therefore, fungal community richness was assessed against discrete variables (Sex and CFTR genotype) using one-way ANOVA analysis. Analysis of the fungal species number against the discrete variables demonstrated that patients who are homozygous F508del had a greater species richness (Mean = 1.9) compared to heterozygous F508del patients (Mean = 0.67) (Fig 5.6; P = 0.007). Fungal community richness was included in the Pearson product moment correlation described above and was not found to be significantly affected by any of the continuous variables tested (Table 5.3).

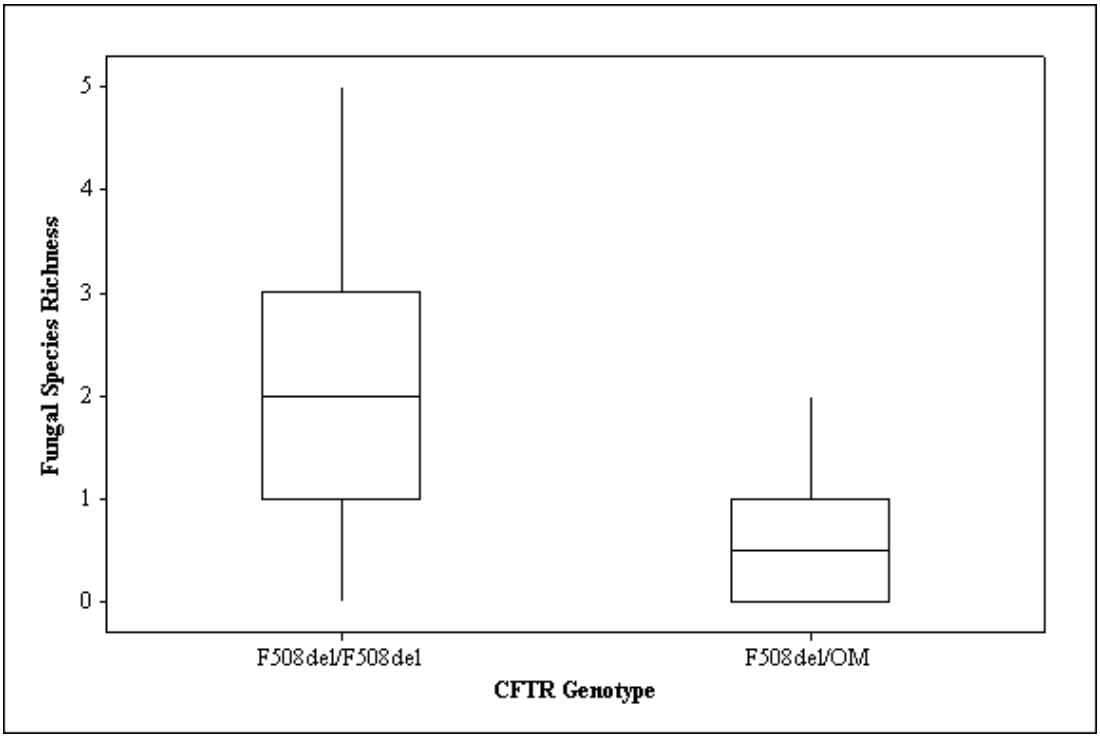


Figure 5.6 Fungal species richness in F508del homo- and heterozygotes

F508del/F508del =Homozygous F508del mutation, F508del/OM = Heterozygous F508del CFTR mutation

5.4 Discussion

Studies examining polymicrobial communities in the environment demonstrate that more diverse communities tend to be more resistant to perturbations from invaders or external stress than less diverse communities (Girvan et al. 2005). Therefore, in order to select the most effective therapeutic intervention, polymicrobial infections need to be characterised using both molecular techniques and cultivation techniques so that 'unculturable' microorganisms can be detected. Our aim was to analyse the microbial diversity observed within the lower respiratory tract of CF patients who were homo- or heterozygous for the F508del mutation. This mutation is by far the most common mutation seen in CF sufferers with 66% of mutations seen worldwide (Dorfman et al. 2008). Furthermore, CF patients who possess an F508del mutation have a more severe disease phenotype which is characterised by an earlier death. McKone *et al.* (2006) demonstrated that high risk CF patients had a mean survival age of 24.2 years and low risk patients had a mean survival age of 37.6 years.

5.4.1 Bacterial community analysis

Molecular microbial analysis of the CF sputum samples collected during this study gave a far larger estimate of microbial species diversity than did microbial culture. However, the culture methods used were designed to culture the traditional CF pathogens with other microbial species being selected against or discarded which may account for this disparity. Several recent studies have examined the ability of culture to detect a wide range of bacteria in CF. Rogers *et al.* (2009) used the HPA

guidelines to culture bacteria that were used in our study and compared them with T-RFLP on whole sputum. They found that molecular analysis found a mean of 16.3 unique profiles when sputum was directly analysed and a mean of 8.8 unique profiles when the analysis was culture dependent. In our study we found a mean of 1.14 pathogens per patient which is indicative of only reporting organisms perceived to be detrimental to patient health and highlights the fact that other bacteria can be identified using the HPA recommended guidelines. Using a non-selective culture method, Guss *et al.* (2011) found a mean bacterial species number of 5.75 per patient. As both studies (Rogers et al. 2009; Guss et al. 2011) used a variety of culture media and conditions this suggests that a large proportion of bacteria residing in the CF lung cannot be cultured using currently available media. Although the culture methods used in this study showed a lower microbial species diversity, further insights were made using this method which it is unlikely would be seen with the molecular methods employed. Firstly, antibiotic sensitivity testing was possible for cultured microbes and MRSA isolates were identified in 4/5 patients who were culture positive for *S. aureus*. However, further molecular investigation using PCR primers that were specific for MRSA may have allowed for identification of antibiotic resistant isolates present in the samples (Huletsky et al. 2004). Secondly, microbial culture allowed for the identification of different morphotypes of *P. aeruginosa* present in the same sample. The ability to differentiate these morphotypes is important for the management of CF patients. Foweraker *et al.* (2005) found that the antibiotic susceptibilities within morphotypes varied considerably which is important in clinical decisions regarding administration of the appropriate antibiotics.

Analysis of the bacterial community present in sputum from our CF cohort found a mean bacterial species richness of 12.24 ± 4.09 species (DGGE bands) per patient. This is in line with the techniques used by Rogers *et al.* (2004) for examining the polymicrobial communities present in the CF lung who found 13.4 ± 6.7 species per patient. However, this number is far less than those produced by the microarray analysis performed by Klepac-ceraj *et al.* (2010) and Cox *et al.* (2010) which could detect >1000 taxa per patient. However, early culture independent techniques, such as DGGE, are still able to provide valuable insights into microbial community structure (van der Gast *et al.* 2008). Patients who were culture positive for *P. aeruginosa* (*n* = 23) had significantly different bacterial community profiles from those who were culture negative (*n* = 6) (P = 0.032; Fig 8). This finding supports the finding of Klepac-ceraj *et al.* (2010) who found a similar link in a group of CF children. Klepac-ceraj *et al.* (2010) posit that the differences observed in *P. aeruginosa* colonisation may be due to long term antibiotic therapy reducing taxonomic richness allowing *P. aeruginosa* to flourish which had been previously demonstrated in ventilator associated pneumonia (Flanagan et al. 2007). Although our study shows a greater species richness in patients culture negative for *P. aeruginosa* (mean = 13.5) when compared to those who are culture positive (mean = 12.0) this was not significant (P = 0.408). Furthermore, all of the patients enrolled on our study are receiving at least one long term oral antibiotic therapy which was chosen based on culture results from pathology. Our data, taken together with those of Klepac-ceraj *et al.* (2010) clearly show that *P. aeruginosa* colonisation in both adults and children affects the bacterial community present in the CF lung. Further investigation comparing Shannon diversity, richness and evenness against *P. aeruginosa* colonisation found that although all of the ecological measures were

lower for patients colonised by *P. aeruginosa* none of these variables were significant. Another contributing factor for the effect of *P. aeruginosa* colonisation on CF microbial diversity could be the effect it has on the environment in the CF lung. Yoon *et al.* (2002) demonstrated that *P. aeruginosa* colonisation of the CF lung had the capacity to change the environment on the mucus from aerobic to either microaerophilic or anaerobic. Tunney *et al.* (2008) found, using microbial culture that patients who were colonised by *P. aeruginosa* were more likely to be colonised by anaerobic bacteria. Therefore, the observation that patients who were culture positive for *P. aeruginosa* had significantly different community profiles from those who were culture negative in our study may be due to a shift in environmental conditions in the lung from aerobic to anaerobic allowing anaerobes to replace aerobes.

Furthermore, CCA analysis of the bacterial community composition found that the profiles between the sexes was significantly different (P = 0.046; Fig 5.2). Females had a significantly reduced Shannon diversity and bacterial community evenness when compared to males (P = 0.006 and P = 0.003 respectively; Fig 5.3). Community evenness is used to describe the distribution of taxa. A low evenness is associated with a high dominance of one or a few taxa and a high evenness demonstrates that taxa are present in similar abundances. When applying ecological theory to bacterial communities, evenness is thought to be one of the most important measures (Wittebolle et al. 2009). High community evenness is thought to protect bacterial communities from perturbation due to the increase in functional redundancy (Wittebolle et al. 2009). However, these findings relate to the functionality of the ecosystem which we have not analysed in this study. Furthermore a reduction in diversity has been associated with a more severe disease phenotype (Flanagan et al.

2007). An increased rate of lung function decline and mortality in females with CF has been identified for decades, especially in those who are less than twenty years of age (Kerem *et al.* 1992; Corey *et al.* 1997; Rosenfeld *et al.* 1997). Furthermore, females colonised with multidrug resistant bacteria are more prone to pulmonary exacerbation than their male counterparts (Block et al. 2006). Increased incidence of pulmonary exacerbations is linked to premature mortality (Amadori et al. 2009). The disparity between the sexes has also been linked to colonisation by mucoid *P. aeruginosa* (Levy et al. 2008) which has significant effects on CF lung function decline and prognosis (Henry et al. 1992). These factors may be the reason that different bacterial communities are observed between males and females with CF. However, the reason for the increased susceptibility to infection in females is not clear, although immune response has been implicated. In a study examining the effect of gender in *P. aeruginosa* colonisation in a mouse model found that females were more susceptible to infection than males (Guilbault et al. 2002). Furthermore, it was also demonstrated that females produced a more profound inflammatory response than males (Guilbault et al. 2002). This may be due, in part, to the ability of gender specific hormones to regulate the immune response. Wang *et al.* (2010) found that male mice who were administered the female sex hormone E2 produced an increased inflammatory immune response to *P. aeruginosa* than controls. The increased inflammatory response observed in females may be the cause of reduced bacterial diversity and community evenness in females. Therefore, our data suggests that the gender difference observed in CF patients is apparent in entire bacterial community not just *P. aeruginosa* colonisation.

Our data shows that the majority (86.9%) of pair-wise comparisons using the Raup-Crick similarity index indicated that bacterial community assembly in the CF lung is

stochastic, not deterministic. Furthermore, a recent study examining the relationship between the bacterial community in American and British CF patients also found a stochastic community assembly in most cases suggesting that the microbial community is indeed stochastically assembled (Stressmann *et al.* 2011). However, recent work examining bacterial communities present in the CF lung found that fifteen bacterial species made up the core community whilst other species identified were categorised as part of the satellite community due to the infrequent isolation from patients and their low abundance in the system. This data suggests that the fifteen core bacteria have a selective advantage over other species which suggests that the core community is deterministically assembled. Further evidence for deterministic community in the CF lung has been identified, whereby the bacterial communities, over-time, display increased relatedness which suggests that species found in older CF patients have adapted to the environment of the CF lung (Cox *et al.* 2010). There is research that supports this finding in *P. aeruginosa*, one of the phyla found to be present in older CF patients and also to be part of the core CF bacterial community. It has been well established that during acute infection *P. aeruginosa* undergoes conversion from non-mucoid to mucoid strains which is thought to aide biofilm formation which, in turn, reduces the susceptibility to antibiotics and the immune response (Stewart & Costerton 2001; Leid et al. 2005). During chronic infection strains of *P. aeruginosa* have been monitored over an eight year period in the CF lung and found to reduce expression of virulence factors which will reduce the immune response (Smith *et al.* 2006). Furthermore, the structure of cell surface receptors which are known pathogen associated molecular patterns targeted by the immune response undergo structural changes during chronic CF lung infection which reduces cytokine production and leukocyte recruitment (Cigana et al.

2009). Raup-Crick similarity index data suggests that bacterial communities in the CF lung are stochastically assembled. However, the identification of a core community suggests that some species of bacteria are deterministically assembled. These datasets taken together suggests that certain bacterial species have a selective advantage when colonising the CF lung and that these species are able to exploit a niche or outcompete other species, whereas, others are stochastically assembled by random immigration of the meta-community. A study examining species of fish in the Bristol Channel supports this theory (Magurran & Henderson 2003). In this study they partitioned the species of fish into a core and satellite community based upon the species frequency and abundance over 21 years. They found that the core community were ecologically adapted to life in the estuary whereas satellite species were adapted to survive in deep water or rocky shores. The authors follow up this by suggesting that when applying niche based community assembly models that the satellite species are ignored as they are not adapted to survive and persist in the environment (Magurran 2007).

Our findings show that there was an inverse relationship between age and FEV_1 ($P = 0.030$; Fig 4) which supports the findings of Cox *et al.* (2010) who found the same relationship in an age stratified study of CF patients. However, Cox *et al.* (2010) also found that species diversity decreased with age whereas our findings only found a very tenuous inverse relationship between age and diversity. This is most likely due to DGGE only being able to identify the major taxa present in a given environment whereas the methodology employed by Cox *et al.* (2010) as a greater resolution. The fact that FEV_1 declines with age is due to the amount of time that the insults of infection and inflammation have had to damage lung tissue which is in line with common findings in the clinic. This phenomenon has been demonstrated in several

studies looking at differences in spirometry and lung tissue damage in CF patients of different ages. The utility of spirometry, especially FEV_1, to assess lung function in CF has been assessed on several occasions and proven to be the most indicative predictor of clinical status (Kerem *et al.* 1992; Corey *et al.* 1997). Kerem *et al.* (1992) found that a FEV_1 (% predicted) below 30% was linked to a high risk of mortality in CF patients. Similarly, Corey *et al.* (1997) found a link between age and the rate at which lung function declined in a longitudinal study. Furthermore, physical changes in lung architecture that are most likely to be the cause of long term lung function decline have also been identified (Helbich et al. 1999). Helbich *et al.* (1999) used a stratified study to examine three groups of patients (group 1; 0-5 years, Group 2; 6-16 years, Group 3; 17 years and over) and found that both the severity and extent of lung tissue damage increased with age using CT score. Furthermore, CT score and CT findings had a significant correlation with the spirometry results (Helbich et al. 1999).

5.4.2 Fungal community analysis

In our study 59% (n = 17/29) patients were culture positive for fungal isolates with a mean fungal species number of 0.62 per patient. Using culture techniques the only genera isolates were Candida (n = 15) and Aspergillus (n = 3) possibly because these organisms are screened for in CF sufferers and deemed potential pathogens. Only one patient in the cohort had polymicrobial fungal communities present in the culture data, which consisted of two species, *A. fumigatus* and *Candida* spp. On some occasions *Candida* spp. were fully elucidated to *C. albicans* based upon colour

change on ChromID Candida (Biomerieux) which may mean that communities that consist of several *Candida* spp. could be present but have not been fully elucidated. PCR-DGGE analysis found that 72% (n = 21/29) of patients were positive for fungi which shows improved sensitivity when compared to conventional methods which had a sensitivity of 59% (n = 17/29). On seven occasions PCR-DGGE identified the presence of fungi which were not detected using culture which is what would be expected based upon previous comparisons between culture and molecular techniques (Bouchara et al. 2009). Further investigation of this data revealed three instances where PCR-DGGE had failed to identify fungi in a sample where a patient was culture positive. This means that between the two datasets (molecular and culture) 83% (n = 24/29) of patients were positive for fungi. Therefore, sensitivity for culture and molecular tests are 71% and 88% respectively, suggesting that molecular techniques can better detect fungi than culture based techniques. Furthermore, the techniques were in disagreement regarding the identity of the fungi present in many cases, although all but one were due to *Candida* spp not being fully identified to the species level by culture methods. The other instance was where the molecular methods detected a band with the same Rf as *C. parapsilosis* in patient 4 whereas culture detected *A. fumigatus* as well as *Candida* spp.

A total of 41 fungal taxa were identified by PCR-DGGE across the twenty nine patients studied which is greater than the number detected by culture (n = 18). Furthermore, polymicrobial fungal communities were identified in eleven patients using PCR-DGGE and only one patient by culture. We found, by molecular analysis that the mean number of fungal species was 1.41 (Range 0 – 5) which is similar to the results of Bouchara *et al.* (2009) who found a mean of 1.74 species per sample using microarray analysis. This data suggests that PCR-DGGE is capable of a similar

resolution in terms of fungal diversity as the microarray produced by Bouchara *et al.* (2009) and has the advantage of being an open rather than a closed system so that unusual fungal isolates can be identified. However, the microarray approach has an advantage in that once conducted, there was no need for any further analysis whereas our method required DNA sequencing for identification. With further development the PCR-DGGE method could display similar advantages by running a ladder of known isolates alongside the unknown CF sputum samples allowing for identification of fungal species without any need for DNA sequencing. However, this would depend upon the heterogeneity of fungal 28S rRNA sequences as well as the coverage of the primers.

A total of 14 unique bands relating to seven fungal species which belonged to four genera were identified by PCR-DGGE followed by sequence analysis (Table 5.4). An attempt was made to sequence all eleven unique bands identified by PCR-DGGE but this was not possible due to the close migration distance between bands which yielded mixed PCR products even after five cycles of band excision, re-amplification and visualisation by DGGE analysis. As a ladder of known CF organisms had not been compiled for fungi, bands were excised and sequenced so that different gels could be matched. Bands from *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *A. fumigatus* were identified on both gels so these bands were used for alignment. In addition, bands relating to *C. glabrata*, *D. hansenii* and *S. cerivisiae* were also identified by band excision followed by sequence analysis. In our study, 38% (11/29) of patients had bands relating to *C. albicans* by PCR-DGGE analysis. *C. albicans* has been isolated in up to 80% of CF patients during one study which is not uncommon using either culture or molecular techniques (Haase *et al.* 1991; Peltroche-Llacsahuanga *et al.* 2002; Bakare *et al.* 2003; Bouchara *et al.* 2009).

Patients with pancreatic insufficiency, osteopenia and patients who are also colonised by *P. aeruginosa* are most likely to be colonised by *C. albicans* (Chotirmall et al. 2010). However, our data did not show a significant relationship between *P. aeruginosa* colonisation and *C. albicans* infection (P = 0.803). Recently *C. albicans* has been identified to be a cause of lung function decline and to increase the rate of hospital-treated exacerbation in CF suggesting that fungi may be more important in the lung pathology in CF than was originally thought (Chotirmall et al. 2010). Therefore, accurate identification is required so that appropriate treatment can be administered. In our study, one sample that was positive for *C. albicans* by culture was identified as *C. dubliniensis* by PCR-DGGE. This discrepancy may be due to both *C. albicans* and *C. dubliniensis* producing green colonies on ChromID Candida. It is commonly hypothesised that fungal colonisation of the CF lung is transient and does not contribute to lung function decline in CF. However, Duan *et al.* (2003) have shown that *C. albicans* is present in the majority of samples over a ten year period, suggesting that fungi are capable of chronically colonising the lung. In total, 24% (7/29) of patients had bands with the same migration distance as *C. dubliniensis* by PCR-DGGE analysis. Previously, *C. dubliniensis* has been isolated from CF sputum at a prevalence of 11.1% in a study utilising microbial culture techniques (Peltroche-Llacsahuanga et al. 2002). However, previous studies suggest that colonisation by *C. dubliniensis* does not lead to invasive infection and its role as a pathogen in CF is yet to be defined (Peltroche-Llacsahuanga et al. 2002). In our study 24% (7/29) and 3% (1/29) of patients were colonised by *C. parapsilsos* and *C. glabrata* respectively. Both *C. glabrata* and *C. parapsilosis* have previously been identified from the CF lung with relatively low frequency and neither species has been implicated in the deterioration of lung function or has a negative impact on the

prognosis of respiratory disease in CF (Bouchara et al. 2009). Furthermore, mixed Candida infections were present in 24% (7/29) of patients usually with only two *Candida* spp, although one patient did have three. The clinical significance of mixed Candida infection is unknown at present but requires further investigation if the prevalence is high as our data suggests. In contrast, *A. fumigatus*, found in 7% of patients by PCR-DGGE, is a fungal pathogen that is known to adversley effect lung function. Amin *et al.* (2009) found that patients colonised with *A. fumigatus* had significantly reduced lung function compared to controls and had increased risk of hospitalisation. Futhermore, patients who are receiving antibiotics are at increased risk of *A .fumigatus* infection compared to controls (Bargon et al. 1999). All of the patients enrolled in our study were receiving at least one group of antibiotics suggesting that *A. fumigatus* colonisation perhaps should have been greater than observed if prophylaxis is indeed a contributing factor. It is now the case that atypical fungal species are being isolated from the CF lung (Cimon et al. 2005). *S. cerevisiae* was identified in two patients by PCR-DGGE. *S. cerevisiae* is commonly known as bakers or brewer's yeast and has only previously been identified as a respiratory infectious agent in immuno-compromised patients (Tawfik et al. 1989). *D. hansenii*, halotolerant yeast usually found to produce the veins in blue cheese was also isolated from one patient. *D. hansenii* has previously been found to cause extrinsic allergic alveolitis in one elderly patient and was contracted from a contaminated humidifier (Yamamoto et al. 2002). It may be the case that these organisms merely contaminate the oesophagus after consumption and have contaminated the sputum sample during expectoration. A recent study found that *C. albicans*, *C. parapsilosis* and *S. cerevisiae* which were identified in our study, were also members of the healthy oral mycobiome (Ghannoum et al. 2010). Therefore, the

oral cavity may also act as a reservoir for opportunistic infection by fungal species in CF. The emergence of other fungal pathogens in CF suggests that close monitoring and more robust techniques are required to detect the prevalence of pathogenic fungi. The data from our study suggests that both molecular and culture based techniques can fail to isolate or identify fungi and that the techniques may need to be used in tandem to obtain full coverage.

Analysis of the fungal communities against discrete and continuous variables found that patients who were homozygous for F508del mutation had more diverse fungal communities than heterozygotes ($P = 0.007$). The relationship between CFTR genotype and fungal infection has been identified previously but only in regards to a single fungal species (Giraud et al. 2010). The increased susceptibility of patients who are homozygous F508del to fungal infection needs to be monitored closely now that fungi are being linked to lung function decline in CF (Amin et al. 2010; Chotirmall et al. 2010). Investigation into fungal infection in CF has been mostly limited to investigation of *C. albicans* and *A. fumigatus*. However, our findings along with those of previous studies reveal that fungal colonisation of the CF lung is not limited to the traditional CF pathogens and can involve multiple fungal species colonising the lung at once (Cimon et al. 2005; Giraud et al. 2010). Further characterisation of the CF lung 'mycobiome' is required along with further investigation to define their role as pathogens.

5.5 Conclusions

The CF lung houses a polymicrobial community consisting of both bacterial and fungal species. Bacterial community assembly in the CF lung is influenced by stochastic processes as well as genetic and environmental factors. We found the bacterial community present in the lungs of female patients displayed reduced diversity and evenness when compared to male patients. Furthermore, *P. aeruginosa* colonisation also significantly affected the bacterial communities present in the CF lung. Although bacterial diversity, richness and evenness were lower in patients who were colonised by *P. aeruginosa* this was not significant suggesting that other factors may be the cause. A possible explanation for the difference seen between patients who are colonised by *P. aeruginosa* and those who are not could be due to the change in environmental conditions, namely a change from an aerobic to an anaerobic environment, resulting in the replacement of bacteria that prefer an aerobic environment with anaerobic bacteria.

Analysis of the fungal communities present in the CF lung found that patients who were homozygous for the F508del CFTR mutation were more susceptible to fungal infection than heterozygotes. Furthermore, PCR-DGGE analysis of fungal communities was a more sensitive technique and revealed a greater diversity than culture based techniques. Therefore, further investigation into the utility of molecular techniques in the identification of fungal pathogens is needed.

6. Temporal dynamics of the microbial communities in the Cystic Fibrosis lung

Abstract

Aim: Recently, the effects of aging on the lung microbiota have been elucidated for CF patients, demonstrating that older CF patients have less diverse, rich and even bacterial communities. Furthermore, the relatedness of the organisms colonising the CF lung increases with age. However, there is very little longitudinal data on dynamics of bacterial and fungal colonisation in the CF lung. Therefore, we aimed to examine the temporal aspects of CF microbial diversity in individual CF patients to examine the stability of these communities and to examine if any key changes occur during presentation with an exacerbation.

Methods: A total of 149 samples were collected from 18 adult CF patients attending a clinic at the RVI hospital, Newcastle upon Tyne. The samples were subject to DNA extraction followed by bacterial and fungal community analysis as well as qPCR analysis of the bacterial load.

Results: Bacterial diversity, species richness and FEV_1 % predicted displayed a negative correlation with patient age. Analysis of the individual patients found that there was a constant turnover of bacterial taxa across the sampling period which was increased depending on the number of I.V. antibiotic therapies the patient received during the study. Fungal taxa identified in our study displayed both chronic and transient colonisation of the CF lung.

Conclusions: Our data suggests that the bacterial and fungal communities of the CF lung do not fluctuate to create a characteristic community profile that is associated

with acute exacerbations. Furthermore, the abundance of bacterial species and the bacterial load were not the cause of exacerbations in our cohort.

6.1 Introduction

Longitudinal studies in CF have provided useful insights into the risk of aging in CF with clinical parameters such as lung function decline and the increased incidence of cystic fibrosis related diabetes (Corey et al. 1997; Marshall et al. 2005). Furthermore, bacterial succession in the major CF pathogens has been well characterised by the CF foundation which shows that children are most likely to be colonised by *S. aureus* and *H. influenzae* and during adolescence these organisms will largely be replaced by *P. aeruginosa* (Cystic Fibrosis Foundation 2009). Many studies in CF focus on *P. aeruginosa* due to its association with an increased incidence of morbidity and mortality (Sagel et al. 2009). These studies find that *P. aeruginosa* strains alter their gene expression profiles, switch to a mucoid phenotype and invoke changes in cell surface receptors to evade the immune response (Stewart & Costerton 2001; Leid *et al.* 2005; Smith *et al.* 2006; Cigana *et al.* 2009).

Although the polymicrobial nature of the CF respiratory tract has been well established by culture and molecular techniques, only recently has the investigation into the temporal variation in the microbial communities has been undertaken using molecular techniques (Cox *et al.* 2010; Tunney *et al.* 2011). Cox *et al.* (2010) used an age stratified study of CF patients to determine how the CF lung microbiota may change over time. The advantage to a stratified study is that patients need only give one sample and are not followed over a period of years and therefore, patient compliance is not an issue. Using this technique, Cox *et al.* (2010) demonstrated that

bacterial diversity, community richness and evenness decreased with increasing age and, that members of the family Pseudomonaceae are most predominant in older CF patients. This finding suggests that eventually all CF patients may succumb to colonisation by *P. aeruginosa* regardless of genotype, sex or any other parameter that has been investigated thus far. However, the mechanisms regarding the ability of *P. aeruginosa* to colonise the vast majority of CF patients and why particular patients become colonised sooner than others cannot be elucidated using a stratified study. Tunney *et al.* (2011) examined the relationship between bacterial richness and load during times of pulmonary exacerbation. They found that, as expected, I.V. antibiotics reduced the numbers of viable aerobic and anaerobic bacteria present in the lung. However, very little change in the overall community richness was observed between exacerbation, post-I.V. antibiotics and stable bacterial communities. These data suggests that the bacterial communities present in the CF lung are resistant to perturbation by antibiotics which is supportive of earlier data (Mulcahy et al. 2010).

Many species of fungi have been identified that can colonise the CF lung but very little work has been carried out on the persistence of these organisms (Haase *et al.* 1991; Bouchara *et al.* 2009). The longitudinal analysis of fungal colonisation in individual patients is yet to be assessed over a long period. However, a short follow up during the assessment of a new microarray for detection of fungal pathogens in CF found that fungi could be chronic or transient coloniser over a short period (Bouchara et al. 2009). This was supported by the culture data they collected during the study.

Here, we aimed to assess the diversity and stability of both bacterial and fungal communities in a longitudinal study. We investigated whether changes in the

bacterial and fungal community structure and diversity have a demonstrable link to pulmonary exacerbations in CF and whether fungal species are chronic colonisers of the CF lung.

6.2 Methods

An 18 patient cohort was followed over a 20 month period, where both stable and exacerbation samples were collected. A total of 149 samples comprosed of 100 stable and 49 exacerbation samples with a mean of 8.2 samples collected from each patient (Range 4-15). Patient age (32.33 ± 13.00), gender (9 Males: 9 Females), genotype (10 F508del:F508del, 8 F508del:OM), FEV_1 (% Predicted), clinical status (100 stable:49 exacerbation), microbiological culture results, routine and I.V. antibiotics were collected at each sampling point (Appendix 12).

6.3 Results

6.3.1 Whole cohort analysis

Shannon diversity index, bacterial species richness and bacterial community evenness were calculated to assess the relationship between patients. These data along with FEV_1 % predicted, age and fungal species richness were analysed using Pearson product moment correlation to assess which of the continuous variables affected the microbial community (Table 6.1). Pearson product moment correlation found that FEV_1 % predicted, Shannon diversity and bacterial species richness had a significant negative correlation with age ($P = <0.0001$, 0.004 and <0.001 respectively; Table 6.1).

Table 6.1 Pearson product moment correlations demonstrating the relationship between the continuous variables collected from the patient cohort

	Age	**FEV_1 (% Predicted)**	**Bacterial load**
FEV_1 (% Predicted)	-0.607		
	P < 0.0001		
Bacterial load	0.005	0.045	
	P = 0.947	P = 0.587	
Shannon Diversity	-0.233	0.308	0.034
	P = 0.004	**P = <0.0001**	P = 0.680
Bacterial Richness	-0.270	0.245	0.014
	P = 0.001	**P = 0.003**	P = 0.865
Bacterial Evenness	-0.112	0.220	0.048
	P = 0.173	**P = 0.008**	P = 0.557
Fungal Richness	0.075	-0.032	0.125
	P = 0.361	P = 0.705	P = 0.130

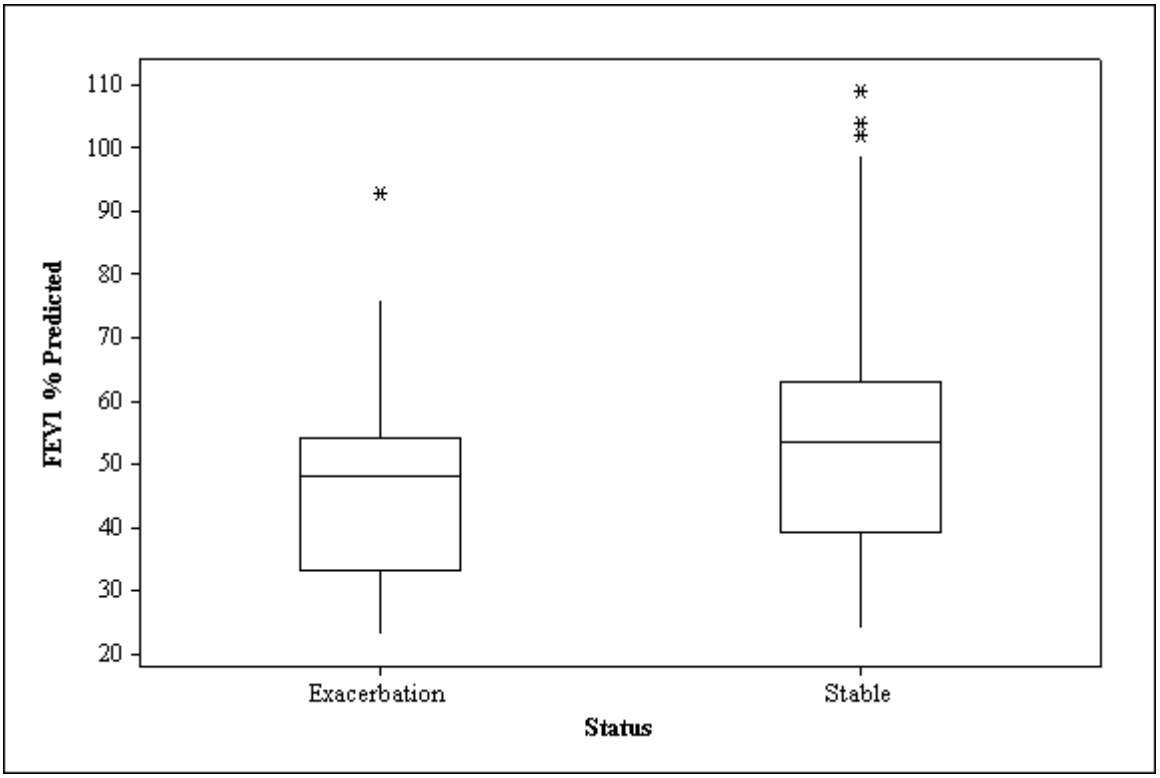


Figure 6.1 Whole cohort analysis of FEV1 (% Predicted) when stable and presenting with an exacerbation

Furthermore, Shannon diversity, bacterial species richness and bacterial community evenness displayed a positive correlation with FEV_1 % predicted (P = <0.001, 0.003 and 0.008 respectively).

One way ANOVA comparing clinical status with FEV_1 % predicted, Shannon diversity, bacterial species richness, bacterial community evenness, fungal species richness and bacterial load found that when patients presented with an exacerbation they had a significant reduction in FEV_1 % predicted compared to samples collected when stable (P = 0.023; Fig 6.1).

6.3.2 Stability of bacterial communities

Dice similarity was calculated using PAST (Hammer et al. 2001) to evaluate the percentage similarity of banding patterns over time between consecutive sampling points for individual patients. The mean pair-wise percentage similarity over the entire sampling period was 72.0% (Range 60.9 – 92.2%). Analysis on our cohort as a whole and the results from previous studies suggests that bacterial diversity declines with age. In order to determine if our study period was of sufficient length to determine this change, Pearson product moment correlation was calculated using Shannon diversity, bacterial species richness and bacterial community evenness and time across the study period (months). A significant reduction in bacterial diversity was observed in only one patient (Table 6.2). To assess the effect of I.V. antibiotics on bacterial community stability the number of I.V. antibiotic therapies was normalised against the total number of samples for each patient and compared to the mean percentage similarity. The normalised values and the mean similarities were then analysed using Pearson product moment correlation and a significant negative

Table 6.2 Correlation analysis of bacterial diversity, richness and evenness over the duration of the study

	Richness		**Shannon diversity**		**Evenness**	
Patient	**Correlation**	**P-value**	**Correlation**	**P-value**	**Correlation**	**P-value**
1	-0.328	0.298	0.384	0.218	0.732	**0.007**
2	-0.317	0.250	-0.455	0.088	-0.389	0.152
3	-0.721	0.067	-0.611	0.145	0.536	0.215
4	0.406	0.367	-0.160	0.731	-0.730	0.062
5	0.649	0.115	0.605	0.150	0.262	0.571
6	0.069	0.849	0.208	0.564	0.229	0.524
7	-0.504	0.202	-0.692	0.057	-0.699	0.054
8	0.891	**0.017**	0.502	0.311	-0.282	0.588
9	-0.769	**0.006**	-0.754	**0.007**	-0.474	0.140
10	0.067	0.853	0.430	0.215	0.458	0.183
11	0.088	0.821	0.401	0.285	0.492	0.178
12	0.538	0.213	0.633	0.127	-0.427	0.340
13	-0.111	0.834	0.747	0.088	0.844	0.035
14	0.775	0.225	0.855	0.145	0.622	0.378
15	0.098	0.817	0.025	0.954	-0.029	0.946
16	-0.553	0.198	-0.569	0.182	-0.153	0.744
17	0.866	0.058	0.332	0.586	-0.200	0.746
18	-0.103	0.776	-0.099	0.786	-0.091	0.802
Mean	0.058		0.099		0.039	

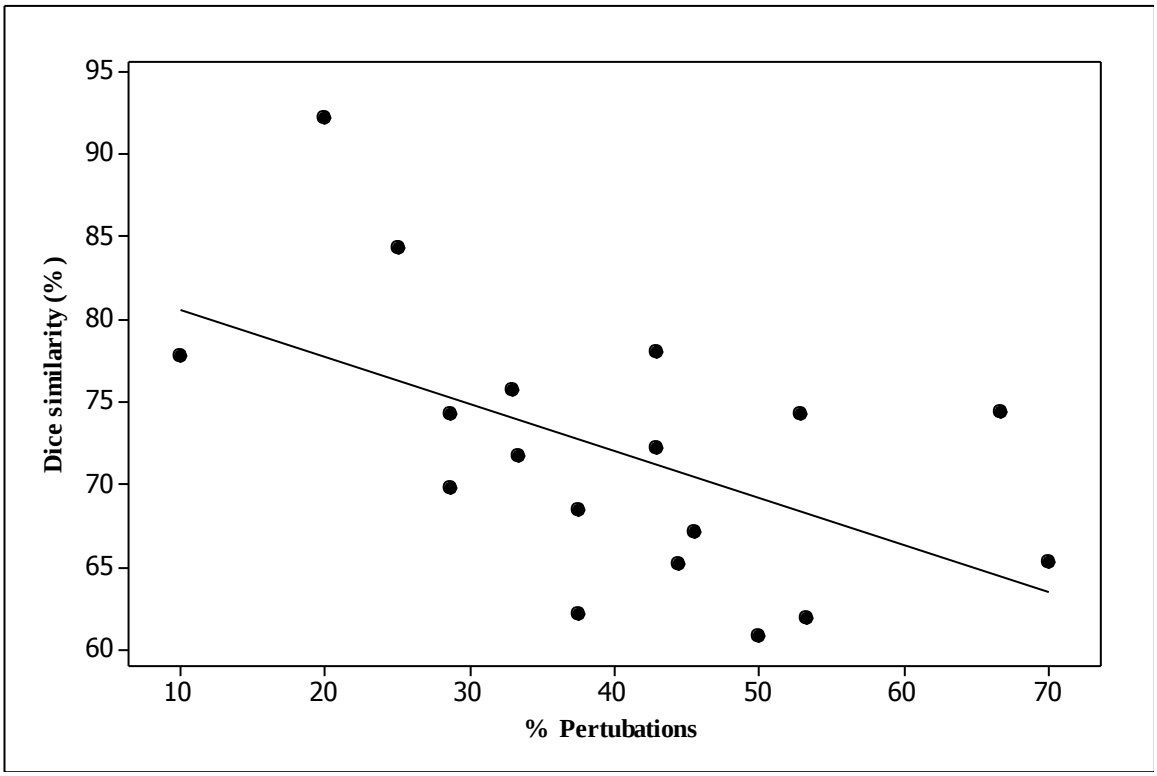


Figure 6.2 Bacterial communities are less similar when patients have had a greater number of I.V. antibiotic therapies during the course of the study

correlation was observed suggesting more I.V. interventions led to a decrease in the similarity of the bacterial communities (P = 0.02; Fig 6.2).

6.3.3 Relationship between bacterial communities and exacerbation in CF

To determine whether bacterial load was related to exacerbations one-way ANOVA analysis was carried out on the entire cohort. This analysis found that the total bacterial load was not significantly different between samples obtained when the

patients were stable or having an exacerbation (P = 0.580). This analysis was repeated for each individual to examine whether changes in bacterial load were being masked by the variation between patients. The bacterial load for individual patients was not significantly different between samples obtained when the patients were stable or presenting with an exacerbation (Table 6.3).

A cluster analysis was performed on the presence/absence (binary) band matrix to determine if samples obtained when the patients presented with an exacerbation group separately from those obtained when the patients were stable. The cluster analysis shows that there is no split between stable and exacerbation derived samples (Fig 6.3). Therefore, DCA analysis was used to determine whether RDA or PCA should be used to further examine the band matrices for presence absence and abundance of bacterial taxa. The DCA gave an axis length of 2.58 for the binary band matrix and 2.50 for the abundance band matrix which indicated that RDA analysis should be used for further analysis. The RDA analysis was performed for both band matrices with clinical status as the only environmental variable considered. The RDA analysis found that neither was significantly correlated to

Table 6.3 Individual patient analysis of bacterial load when stable and presenting with an exacerbation

Patient	Stable mean Bacterial load	Exacerbation mean Bacterial load	Bacterial load v Clinical status (P-value)
1	9.02	9.37	0.639
2	8.09	8.37	0.424
3	9.04	8.79	0.261
4	8.96	9.50	0.550
5	9.15	9.25	0.809
6	7.87	7.55	0.521
7	8.76	8.79	0.924
8	9.13	N/A	N/A
9	8.54	8.42	0.784
10	7.84	8.57	0.255
11	9.39	8.81	0.625
12	8.39	8.93	0.481
13	8.67	8.73	0.671
14	9.61	9.40	0.822
15	9.62	9.97	0.404
16	8.90	8.92	0.905
17	9.00	9.15	0.388
18	9.10	N/A	N/A

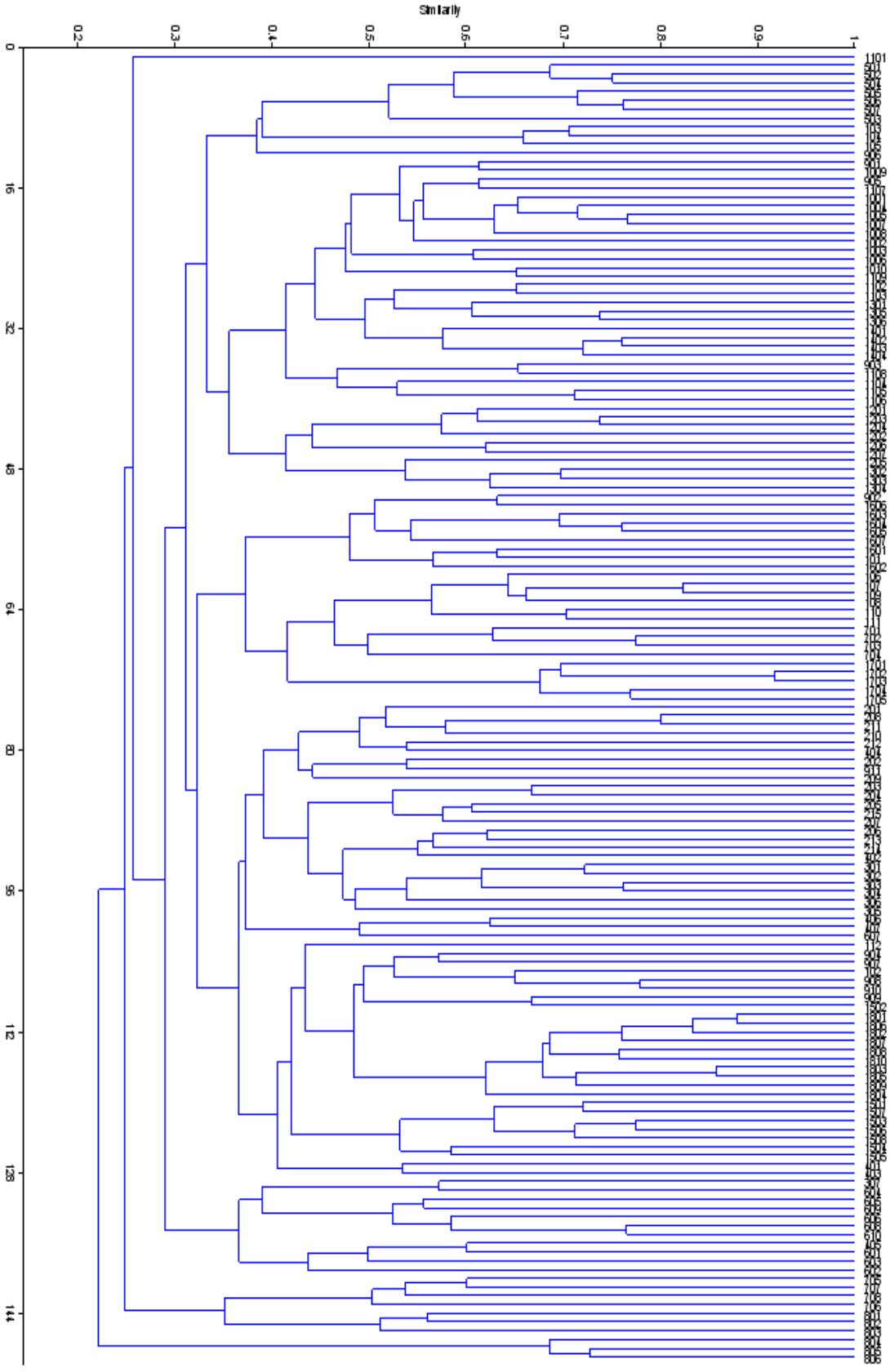


Figure 6.3 Cluster analysis of the entire patient cohort

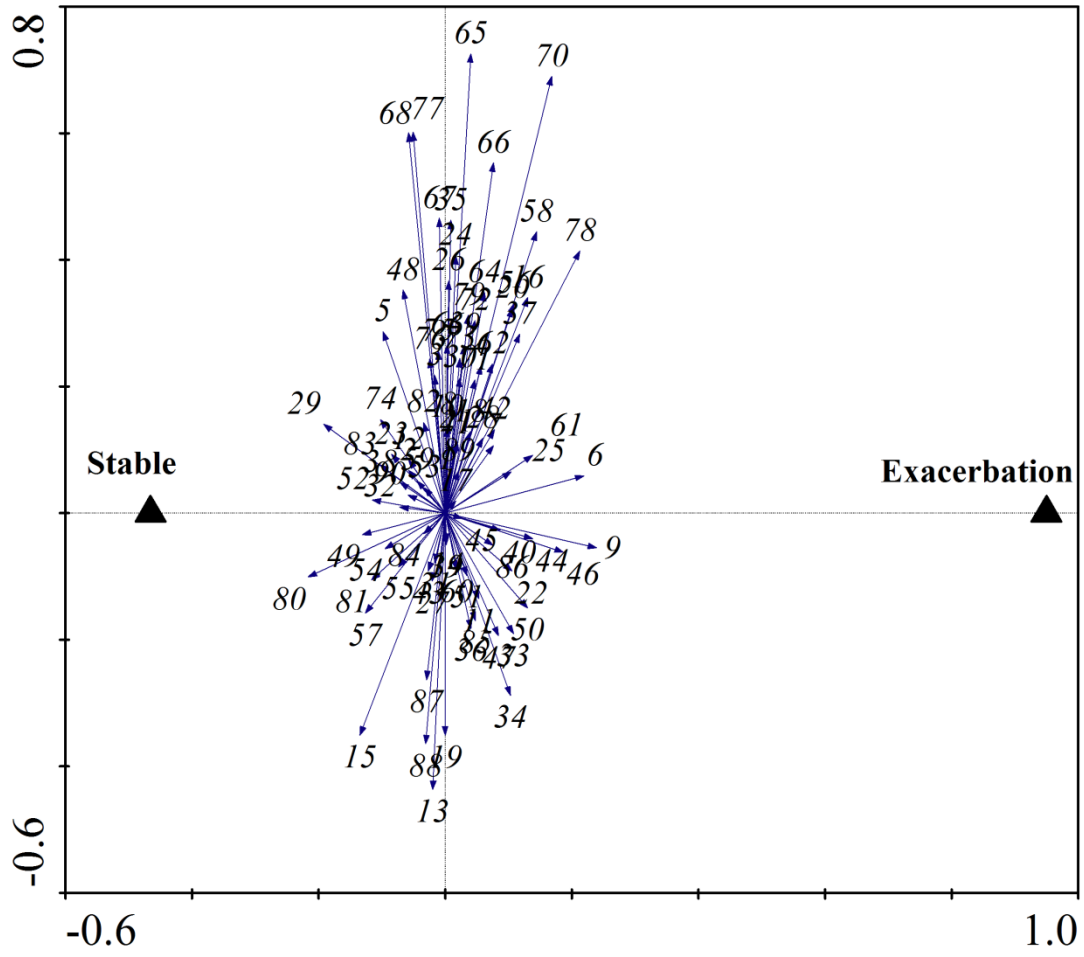


Figure 6.4 RDA showing the relationship of the presence of each bacterial taxa with clinical status

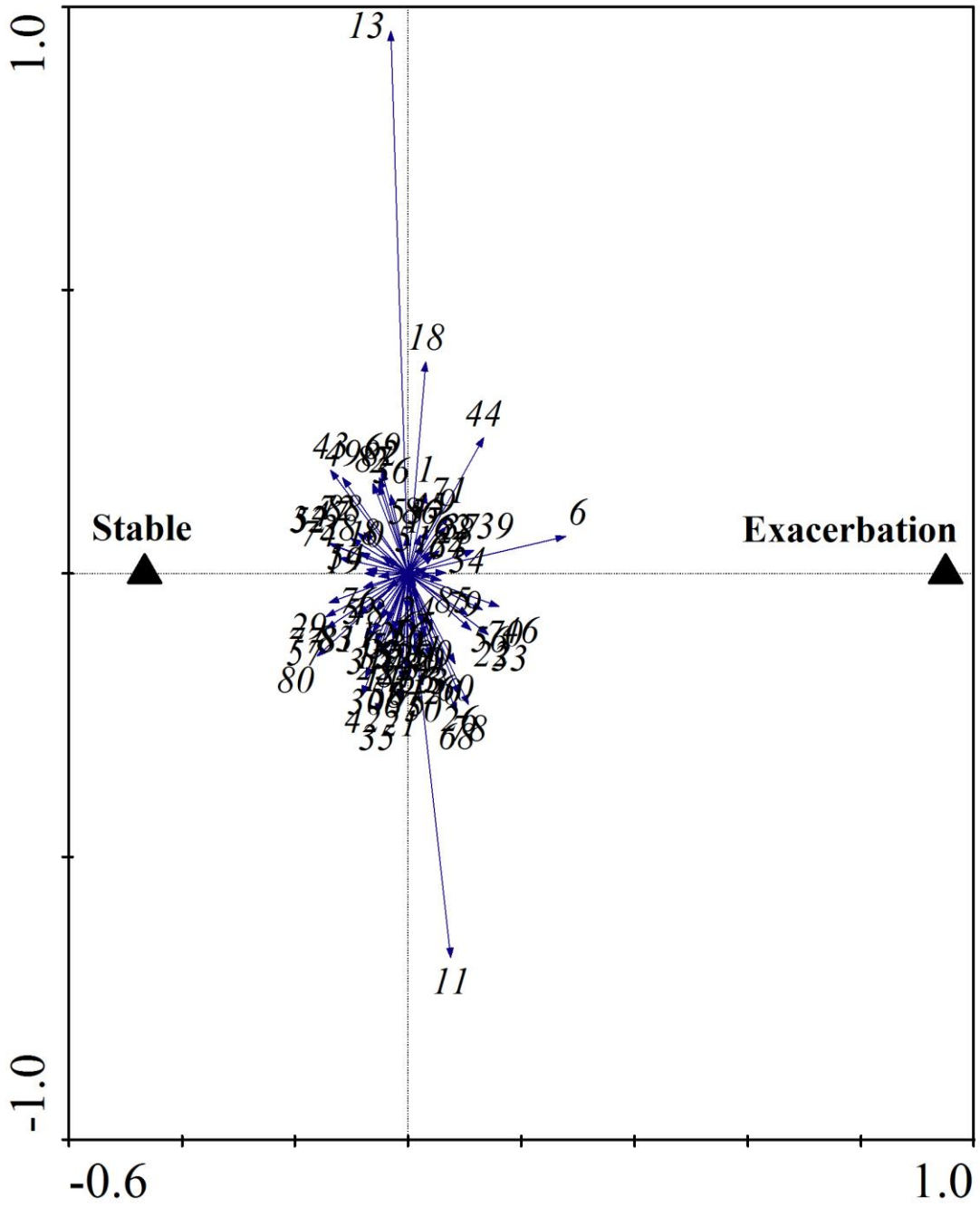


Figure 6.5 RDA showing the relationship of the abundance of each bacterial taxa with clinical status

clinical status, yielding P values of 0.192 for the binary band matrix and 0.236 for the abundance band matrix. Biplots of species against clinical status were created to see if individual bands in either profile displayed a strong correlation with clinical status. The biplots show that there were no significant correlation present between individual taxa and clinical status when examining the entire cohort (Fig 6.4 & 6.5 respectively). In order to determine if there was a change in any aspects of the community, Shannon diversity index, bacterial species richness and bacterial community evenness were calculated for the abundance profiles and analysed against clinical status using one-way ANOVA. None of these factors were significantly different between samples obtained when the patients were stable or having an exacerbation (P = 0.774, 0.285 and 0.675 respectively).

The binary and abundance DGGE profiles for individual patients against clinical status were then analysed using RDA to see if the overall variance was masking changes in individual patients. Only patients who had greater than one exacerbation were included in this analysis to prevent casual trends being linked to exacerbations. These analyses found that none of the patients had significantly different bacterial communities in the stable and exacerbation samples (Table 6.4).

Biplots were created using the RDA data for the binary band matrix of each patient to see if the presence of particular bacterial taxa were linked to clinical status. The biplot from patient 16 shows that two taxa that were strongly correlated with an exacerbation (Fig 6.6). Further investigation found that bands 21 and 76 were present in all of the samples obtained when the patient presented with an exacerbation (n = 3) and none of the samples obtained when the patient was stable (n = 4). The RDA analysis was repeated for the abundance band matrix for each patient who had

Table 6.4 Bacterial community binary and abundance RDA P-Values comparing stable and exacerbated bacterial communities

	Presence band matrix	**Abundance band matrix**
Patient	**P-value**	**P-value**
P1	0.888	0.570
P2	0.098	0.320
P3	0.948	0.546
P5	0.652	0.446
P6	0.514	0.304
P7	0.450	0.776
P9	0.918	0.404
P10	0.516	0.492
P11	0.892	0.724
P12	0.860	0.600
P13	0.904	0.720
P16	0.096	0.104
P17	0.700	0.902

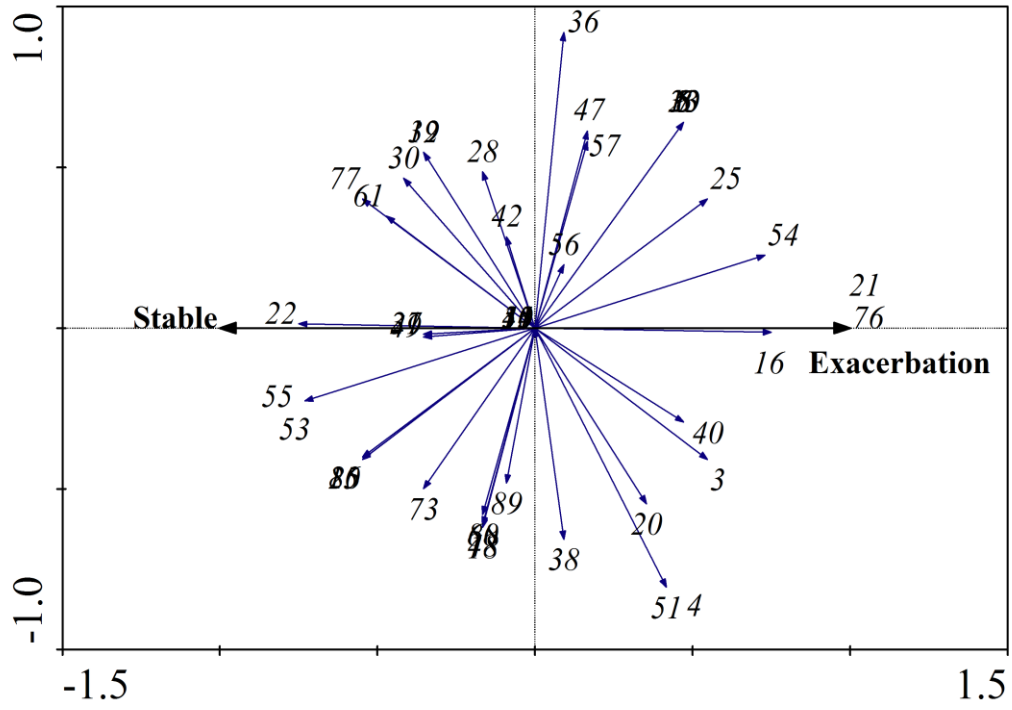


Figure 6.6 RDA analysis of the binary band matrix for patient 16

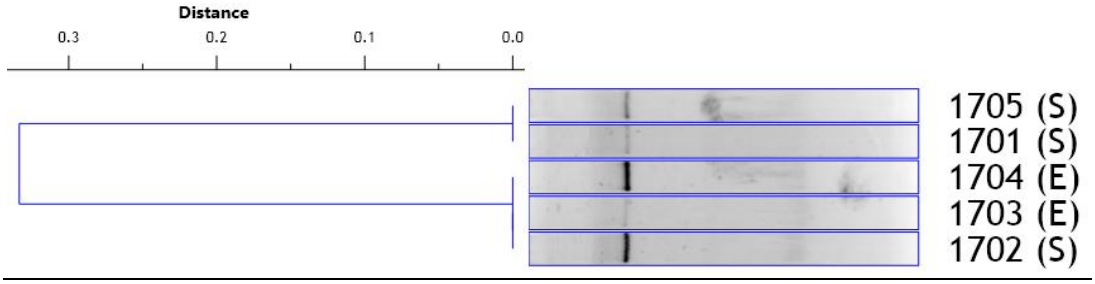
greater than one exacerbation and found that none of the cohort had the increased abundance of any taxa associated with an exacerbation.

6.3.4 Fungal community analysis

From the cohort, 94% (17/18) of the patients had a fungal isolate detected from at least one of the samples during the study period. Of the 149 samples collected, 42% (63/149) were positive for fungal species. A total of 89 bands were identified in the positive samples. Analysis of the fungal communities by DGGE found that all but one patient had fungal colonisation on at least one sampling date; however, this patient was receiving antifungal therapy for part of the study period. The Dice index could not be calculated for fungal communities due to at least one sample being negative for fungal species in the majority of patients. Therefore, cluster analysis was performed and qualitative analysis of the gels was used to examine fungal colonisation patterns. Cluster analysis found that patient 17 was colonised by the same fungal species for the entire sampling period (Fig 6.7a). Similarly, patient 13 was colonised by fungi from the second sample date and this taxa persisted for the remaining samples collected (Fig 6.7b). The cluster analysis from the remainder patients revealed that particular taxa were present in the patient on at least one sampling date but then disappeared. Interestingly, in eight of the patients, several bands went through periods of presence and absence throughout the sampling period.

The Pearson product moment correlation of fungal species richness against the continuous variables (described above) found that there were no significant correlations present. One-way ANOVA analysis found that fungal species richness

(a)



(b)

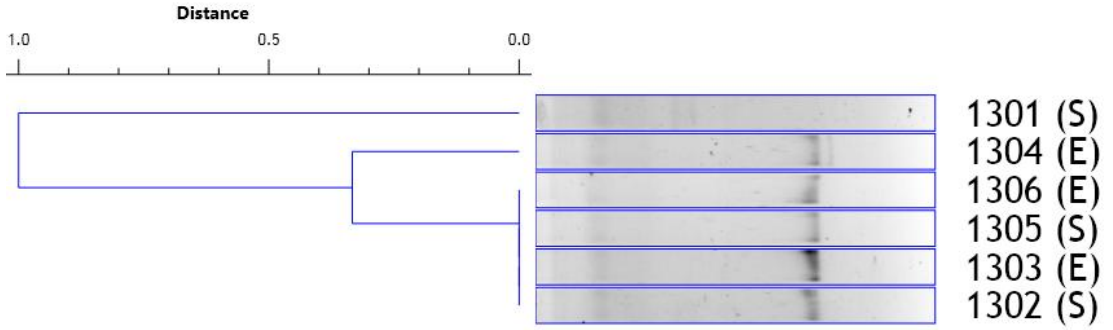


Figure 6.7 Cluster analysis for (a) patient 17 who was chronically colonised for the duration of the sampling period and (b) patient 13 who acquired a fungal pathogen on the second sampling date

was significantly greater in patients who were homozygous F508del compared to heterozygotes (P = 0.016). The presence and richness of fungal species was used to determine whether fungi were a cause of exacerbations in CF. The analysis found that fungal presence and richness were not the cause of exacerbation in CF (P = 0.802 and 0.795 respectively).

6.4 Discussion

The progression of CF lung disease is known to be a constant decline in lung function towards respiratory failure due to inflammation and infection. Recent evidence has found that exacerbations in CF contribute to lung function decline (Amadori *et al.* 2009; Sanders *et al.* 2010a). A great deal of research has been aimed at finding the cause of exacerbation, with bacterial and viral agents being implicated. However, a definitive conclusion has yet to be reached. A recent study has demonstrated that the bacterial community richness does not change significantly after an exacerbation, despite a decrease in bacterial load (Tunney et al. 2011). However, the data from this study demonstrates that, although species richness remains stable, the community composition is subject to change. Further work examining the temporal dynamics of CF microbial communities may provide useful information on the susceptibility of these communities.

Statistical analysis of the entire cohort using diversity measures against continuous variables found that FEV_1 % predicted, Shannon diversity index and the bacterial community richness displayed a significant negative correlation with age (P < 0.0001, P = 0.004, 0.001 respectively). The decline of FEV_1 with age confirms the data derived from our cross sectional study and other studies examining lung

function in CF (Kerem *et al.* 1992; Corey *et al.* 1997). Similarly, the decline of bacterial diversity and bacterial community richness has also been identified in a recent study (Cox *et al.* 2010). Our data suggests that, as has been previously reported, DGGE analysis is capable of detecting major changes in bacterial communities (0.1 – 1 %) which are normally due to the more prevalent taxa (Muyzer et al. 1993). However, the methodology used by Cox *et al.* (2010) does have a number of advantages over DGGE. Firstly, microarray analysis can detect bacterial taxa that represent as little as 0.01% of the total community with an increased sensitivity of up to two orders of magnitude compared to DGGE (Cox *et al.* 2010). The ability to detect the rarer species in a community can be important, especially when looking at perturbations (Flanagan et al. 2007; Szabo et al. 2007). Szabo *et al.* (2007) found in a study examining lake water microcosms that certain more abundant species in the community were unable to cope with the perturbation because they lacked a particular functional gene. As a consequence, rarer members of the community with the gene were able to take advantage of the new resource and flourish (Szabo et al. 2007). Similarly, in a study examining the microbial communities of patients on mechanical ventilation, microarray analysis identified *Klebsiella* as a minor component in the starting community composition that eventually became dominant after antibiotic administration. This observation was missed by clone library analysis, adding weight to the findings that under the right conditions rare taxa can become dominant under specific conditions and more robust techniques are required to detect these changes (Flanagan et al. 2007). An additional advantage of microarray analysis is that it identifies the bacterial taxa present in the lung without the need for further laboratory work, whereas, DGGE analysis requires

multiple rounds of band excision and re-amplification prior to DNA sequence analysis of the band.

Current ecological thinking suggests that niche (deterministic) community assembly will lead to a single stable equilibrium as species sorting occurs along the gradients present in the environment. In contrast, a neutral (stochastic) community assembly model predicts multiple stable equilibria as species randomly arrive, with each species being regarded as ecologically equivalent, resulting in divergent community assembly (Fargione et al. 2003). In reality, microbial communities are likely to assemble by neither strictly niche or neutral mechanisms. A study by van der Gast *et al.* (2008) demonstrated that environmental selective pressure switches community assembly from stochastic to more deterministic assembly. Therefore, the addition of selective pressure to the CF lung microbiota may select for bacterial taxa that possess the fitness traits to survive. Cox *et al.* (2010) found that bacterial communities in the CF lung became more diverse until patients reached adolescence which was followed by a decrease in bacterial diversity, richness and evenness. This finding suggests that the decreased diversity in bacterial communities between older CF patients may be driven by selective pressures towards a deterministic community assembly model. We also found that bacterial diversity ($P = 0.004$) and community richness ($P = 0.001$) decreased with age but we did not find a significant relationship between bacterial evenness and age when examining the whole cohort ($P = 0.173$). Furthermore, Cox *et al.* (2010) found that as patients age the bacterial community composition becomes more closely related to those of other patients than between younger individuals. This data suggests that bacterial community assembly in younger patients obeys a stochastic assembly rule, whereas in older patients a more deterministic community assembly is observed. However, the selective pressures that

cause the community assembly to shift from a stochastic to a more deterministic community assembly remain to be elucidated.

6.4.1 Temporal dynamics of the CF lung microbiota

Although we have demonstrated that diversity and richness displayed a significant reduction as patients age, the temporal dynamics within patients has yet to be elucidated. We monitored bacterial diversity, species richness and community evenness in each patient throughout the study period. We detected a reduction in diversity and richness between patients, however, the within patient diversity, richness and evenness did not change significantly over the study period in most cases. We found that only patient 9 had a significant reduction in sputum bacterial diversity over the study period (Table 6.2). The mean Bacterial diversity, richness and evenness were close to zero. However, it was evident from visual analysis of the DGGE profiles that immigration and extinction of bacterial taxa was occurring. Therefore, the Dice pair-wise similarity between consecutive sample points within individual patients was used to measure the stability of the bacterial community profiles. The Dice similarities observed in the patient cohort had a mean of 72.0% (Range 60.9 – 92.2%) within individual patients. This is greater than the similarity between patients observed in our cross sectional study where the bacterial communities displayed only 41% similarity (Page 90). The greater variation seen between patients compared to within patients has been demonstrated in other human body sites. For example, the mean stability of the bacterial communities in the oral cavity was approximately 88% within patients and only 66% between patients

(Rasiah et al. 2005). Our data adds weight to the claim that microbial communities are patient specific in CF.

We also found that in all patients, certain taxa persist throughout the sampling period regardless of the number of perturbations. This finding is in concordance with a recent study that identified a core CF microbiome (van der Gast *et al.* 2010). Furthermore, evidence from the CF Foundation found that *P. aeruginosa* had been isolated from up to 80% of CF adults suggesting that these organisms have a selective advantage when colonising the CF lung (Cystic Fibrosis Foundation 2009). Using the aligned PCR-DGGE banding pattern for bacteria alongside the ladder of known organisms we attempted to define the frequency of *P. aeruginosa* across the entire patient cohort. There are some caveats for identifying bacteria by DGGE using a ladder of known organisms. The first is that single species of bacteria can carry several heterogeneous copies of the 16S rRNA gene (Dahllöf et al. 2000) Furthermore, the predominant band from several organisms can have the same migration distance through the gel resulting in one band possibly relating to several bacterial taxa (Jackson et al. 2000). The most predominant organism identified was *P. aeruginosa* which was present in 91% of the samples. However; bands relating to *P. aeruginosa* have a similar Rf to other *Pseudomonas* spp, such as *P. fluorescens* (Fig 4.1). In contrast, recent work suggests that other members of the genus are not regularly colonising CF patients (Spilker et al. 2004). One reason that *P. aeruginosa* and other organisms may be able to persist in the CF lung is the formation of biofilms which increase resistance to the immune response and antibiotics (Stewart & Costerton 2001; Leid et al. 2005). Furthermore, recent evidence suggests that persister cells may also contribute to the maintence of *P. aeruginosa* (Mulcahy et al. 2010). Persister cells are a phenotypic variant of *P. aeruginosa* that display reduced

levels of metabolic activity and, therefore, increased tolerance to antimicrobials (Mulcahy et al. 2010). The two mechanisms may contribute to the maintenance of bacterial diversity seen in our patient cohort in-spite of repeated antimicrobial perturbation.

A study on the bacterial communities of the gut has found that administration of antibiotics reduces the similarity between sampling points within patients by removing susceptible bacterial species (Donskey et al. 2003). Further research on the effect of antimicrobials on the gut microbiome has revealed that the majority of the bacterial taxa in the pre-treatment sample recovered within 4 weeks but some taxa failed to repopulate the gut during the study period (Dethlefsen et al. 2008). Therefore, the use of oral antibiotics used to manage the bacterial communities of the CF lung coupled with the sporadic administration of I.V. antibiotics may cause a decrease in similarity between sampling points as seen in the bacterial communities of the gut. The similarity of the gut community has been previously examined using PCR-DGGE and the mean within patient similarity was between 88 and 91% for samples collected over an eight month period (Donskey et al. 2003). However, when the patients received ciprofloxacin and clindamycin the consecutive similarity dropped to 73% and 11% respectively. The mean similarity seen for our CF cohort was 72% which suggests that routine antibiotics may contribute to species turnover in the CF lung as indicated by the reduced similarity between samples but the relative stability of bacterial diversity across the study period. As all of the patients were receiving at least one oral antibiotic, the number of I.V. antibiotic therapies was used to see if the administration of I.V. antibiotics increased bacterial species turnover. We compared the mean similarity from each patient to the number of perturbations by I.V. antibiotic therapies received during the study period. We found

a significant negative correlation between the normalised number of I.V. antibiotic therapies and the mean percentage similarity between the sampling dates from the patients (P = 0.02). However, the lowest mean pair-wise similarity was 60.9% which suggests that the bacterial communities of the CF lung are not as susceptible to perturbation as those of the gut. Tunney *et al.* (2011) compared the bacterial richness pre and post exacerbation and this did not change significantly even after I.V. therapy. Their data suggests that the hypothesis of I.V. antibiotic administration reducing bacterial diversity and thus, increase the risk of invasion may not be accurate. However, we have shown here that, although diversity, richness and evenness remained fairly constant over the study period that the composition of the community was subject to change and that this was affected by the number of I.V. antibiotic treatments received. Therefore, it would also be interesting to study the effects of different combinations of I.V. antibiotics on community composition within individual patients (Tunney et al. 2011). We have some anecdotal evidence surrounding this relationship within individual patients. However, a major caveat in examining this relationship in our study is the length of time between receiving I.V. antibiotic treatment and the next sampling date. Therefore, a more controlled sampling regimen would be required to analyse this relationship.

One relationship that remains to be examined is the community dynamics regarding exacerbations in CF. It may be the case that the rate of change in the community is indicative of an exacerbation, where communities with an increased turnover are more susceptible to exacerbation. However, as the patients received I.V. antibiotics after presenting with an exacerbation, and we have shown that this affects species turnover, the relationship between species turnover and exacerbations could not be examined in this study.

In the cross sectional study, we found the fungal communities of CF sputum to be more diverse than is suggested by culture. Here, we aimed to follow patients longitudinally to determine if fungi are persistent or transient colonisers of the CF lung. We demonstrated that fungal bands were identified in at least one sample by DGGE in 94% (17/18) of patients. The patient who did not give a sample that was positive for fungi was receiving the antifungal agent itraconazole for part of the sampling period and was the only patient who received antifungal therapy at any point during the study. Fungal species were identified in 42% (63/149) of the samples collected from the entire patient cohort. From the samples positive for fungi, a total of 10 distinct fungal species were identified. In total, 89 fungal species were identified from the positive samples. We used cluster analysis to determine the similarity between fungal communities within individual patients (Fig 6.7). We found that patient 17 had chronic fungal colonisation that persisted for the entire sampling period. Furthermore, we found that patient 13 was positive for fungi on the second sampling date and this persisted in the samples collected subsequently. The band that persisted throughout the sampling period in patient 17 related to *C. parapsilosis* and the band that colonised and persisted in patient 13 related to *C. albicans.* This data suggests that fungal species can chronically colonise the LRT of CF patients. However, the other patients enrolled in the study only sporadically presented with fungal positive specimens. Interestingly, in eight of the patients who sporadically presented with fungal species, the same band seemed to re-colonise the lung across the sampling period. These bands related to *C. albicans* in 3 of the patients, *C. dubliniensis* in 3 patients and *C. parapsilosis* in 2 of the patients. Patient 4 was sporadically colonised by *C. dubliniensis* and *C. albicans* across the study period. These data taken together suggest that fungal species have the ability to

chronically colonise the CF lung but that they do not always persist. Furthermore, the same fungal species seem to cycle through periods of presence and absence during the sampling period that we tested. These data may suggest that these patients are being re-exposed to the same pathogen repeatedly, which may implicate the oral cavity as a reservoir for fungal, as well as bacterial pathogens. The fungal microbiome of the oral cavity of CF patients has not been assessed regarding fungal pathogens. However, a recent study on the fungal species present in the oral cavity of healthy subjects found that common CF pathogens were present (Ghannoum et al. 2010). These included members of the genus Aspergillus, although not *A. fumigatus*, *C. albicans*, *C. paprapsilosis* which we identified in our cross sectional study as well as *C. tropicalis* that has been identified from CF sputum in previous studies (Bouchara et al. 2009) and *S. cerevisiae* which we also identified in the cross sectional study (Ghannoum et al. 2010).

6.4.2 Exacerbations in CF

It has been shown that exacerbations cause not only a short bout of ill health, but also over time, they contribute to lung function decline (Amadori *et al.* 2009; Sanders *et al.* 2010a). Pre-emptive treatment of patients thought to be in the early stages of an exacerbation without severe symptoms has been vindicated in adults and children (Sanders 2010a; Sanders *et al.* 2010b). Current theories regarding exacerbations include acquisition of bacterial or viral pathogens and an increase in the bacterial load. In our study, an exacerbation was defined as deterioration in their clinical status with an increase in cough, sputum or chest symptoms, and/ or a fall in

FEV_1. Therefore, we wanted to ensure that clinical symptoms, indicated by a decrease in FEV_1, were the main prognostic indicator in the study. In order to achieve this aim, we compared the FEV_1 when patients were stable to when they presented with an exacerbation to see if there was a significant difference in lung function. We found that there was a significantly lower FEV_1 in patients when they presented with an exacerbation compared to when they were stable ($P = 0.006$; Fig 6.1).

6.4.3 Diagnostic bacterial community

Evidence from bacterial vaginosis suggests that a shift in environmental conditions can cause a pathologic community to assemble which causes disease (Oakley et al. 2008). Anecdotal evidence from a study in CF found that a shift in bacterial community structure during an exacerbation was not evident in CF (Sibley *et al.* 2008b). Recently, Tunney *et al.* (2011) examined the relationship between Bacterial diversity and load during times of pulmonary exacerbation. They found that, as expected, I.V. antibiotics reduced the numbers of viable aerobic and anaerobic bacteria present in the lung. However, very little change in the overall community composition was observed between exacerbation, post-I.V. antibiotics and stable bacterial communities using T-RFLP. In order to examine this relationship further, we examined the binary band matrix for the entire cohort using cluster analysis to see if samples obtained during an exacerbation clustered separately from the stable samples (Fig 6.3). However, this analysis did not demonstrate separate clustering of stable and exacerbation samples. In order to confirm that a diagnostic bacterial

community was not responsible for exacerbations we analysed the binary band matrix against clinical status using RDA to see if a significant difference was observed. This analysis confirmed that the samples obtained when the patients presented with an exacerbation were not significantly different from the stable samples across the entire cohort (P = 0.192). In order to investigate if there was a change in any aspects of the community Shannon diversity index, bacterial species richness and bacterial community evenness were calculated for the abundance profiles and analysed against clinical status using one-way ANOVA. None of these factors were significantly different between samples obtained when the patients were stable or having an exacerbation (P = 0.774, 0.285 and 0.675 respectively).

However, evidence from our previous work and from other studies suggests that patients have their own characteristic bacterial community (Rogers et al. 2004; Rogers et al. 2005; Harris et al. 2007). Therefore, we decided to examine the binary band matrix of each patient individually to see if a patient specific bacterial community assembled to cause an exacerbation using RDA with clinical status as the environmental variable (Table 6.4). The data from the cohort suggests that a shift in bacterial community composition, at least in terms of the major taxa that can be detected using the methods employed in the study, is most likely not the cause of exacerbations in CF which confirms the findings of previous studies (Sibley *et al.* 2008b; Tunney *et al.* 2011). A potential reason for this may be the sampling strategies used in this study to sample the microbial communities. Davis *et al.* (2007) found that inflammation in the lung is confined to a small area during exacerbations. Therefore, sampling whole sputum may be inadequate to examine effects that are being confined to a localised area. However, to circumvent this problem and analyse the appropriate region of the lung would require utilising computerised tomography

scans of the patients when they present with an exacerbation, followed by a BALF sample to sample the appropriate lobe. Although invasive, this approach does demonstrate the heterogeneity of bacterial distribution throughout the CF lung (Gutierrez *et al.* 2001; Davis *et al.* 2007).

6.4.4 Acquisition of new taxa

The acquisition of new bacterial taxa has been suggested as a possible cause for exacerbation in CF (Sibley *et al.* 2008b). Sibley *et al.* (2008) found that members of the SMG were only isolated when patients presented with an exacerbation. Furthermore, they found that treatment directed at these organisms resolved the exacerbation. The binary band matrix for the whole cohort was analysed using RDA to see if individual taxa, defined as bands in the DGGE profile, displayed a strong correlation with clinical status. This analysis found that there were no significant correlation present between individual taxa and clinical status (Fig 6.4). We then repeated this analysis for the binary matrix for individual patients. Only patients who had greater than one exacerbation were included in this analysis because any species acquired in patients with only a single exacerbation would display a strong correlation when this could be merely due to species turnover. Biplots were created for each patient to see if the presence of particular bacterial taxa were linked to clinical status. This analysis found strong correlations in only one patient (Fig 6.6). Further investigation found that bands 21 and 76 were present in all of the samples obtained when the patient presented with an exacerbation (n = 3) and none of the samples obtained when the patient was stable (n = 4). Unfortunately, neither of these bands corresponded with the ladder of common CF isolates that were included on the DGGE gel so they could not be identified. It may be the case that this phenomenon is

present in other patients included in the study but the bands are being obscured by co-migration of the 16S rRNA fragments with those from other taxa. Using a more robust methodology such as microarray will give a more in-depth understanding of bacterial community dynamics during exacerbations.

6.4.5 Increased bacterial burden

Treatment strategies for CF exacerbations are targeted at reducing the load of pathogenic bacteria, namely *P. aeruginosa* (Regelmann et al. 1990). To determine whether bacterial load was related to exacerbations one-way ANOVA analysis was carried out on the entire cohort. This analysis found that the total bacterial load was not significantly different between samples obtained when the patients were stable or having an exacerbation ($P = 0.580$). This analysis was repeated for each individual to examine whether changes in bacterial load were being masked by the variation between patients. The bacterial load for individual patients was not significantly different between samples obtained when the patients were stable or presenting with an exacerbation (Table 6.3). We then went on to examine whether the abundance of individual bacterial taxa was the cause of exacerbation in CF. The abundance band matrix for the bacterial profiles from the whole cohort was analysed using RDA to see if there was a significant difference between stable and exacerbation samples. This analysis found no significant differences between the stable and exacerbation samples ($P = 0.236$). From the RDA analysis, a biplot of species against clinical status was created to see if the abundance of individual bacterial taxa was strongly correlated with an exacerbation. However, this analysis found no strong correlation between bacterial abundance and clinical status (Fig. 6.5). We then repeated this

analysis for patients who had greater than one exacerbation and also found no strong correlations between abundance and clinical status.

6.4.6 Fungal acquisition

Traditionally in CF, only *A. fumigatus* and *C. albicans* were identified as fungal pathogens. However, it is only recently that these species are being linked with CF exacerbations (Amin et al. 2010; Chotirmall et al. 2010). Amin *et al.* (2010) found that patients colonised with *A. fumigatus* were at an increased risk of hospitalisation but that *A. fumigatus* infection was not significantly associated with an increased risk of exacerbations. However, recent work on *C. albicans* has found that both chronic and intermittent infection is significantly associated with an exacerbation (Chotirmall et al. 2010). Therefore, we decided to examine the relationship between fungi and clinical status in CF. The presence and richness of fungal species was used to determine whether fungi were a cause of exacerbations in CF. The analysis found that fungal presence and richness were not significantly different between stable and exacerbation samples ($P = 0.802$ and 0.795 respectively). Therefore, the diversity of fungi is not significantly different between stable and exacerbation samples. Suggesting, the presence of fungi is unlikely to be the cause of exacerbations in CF. However, particular species, such as *C. albicans*, may predispose patients to exacerbations.

6.5 Conclusions

In this study we have followed 18 adult CF patients over a 20 month period to assess the dynamics of the bacterial and fungal communities. We have found, by examining between patients, that the bacterial diversity and community richness is significantly lower in older patients with CF, which is concordance with a previous study (Cox *et al.* 2010). However, we found that a reduction in diversity, richness and evenness was not seen within patients over the course of the study period. We have also demonstrated that, within patients, the bacterial communities fluctuate between sampling dates but that certain taxa persist throughout the study period. Furthermore, the degree of variance between the samples is increased in patients who received a greater number of I.V. antibiotic interventions during the study. However, we could not assess whether the dynamics of the bacterial communities where indicative of an exacerbation due to the administration of I.V. antibiotics. It would be interesting to investigate whether an increased rate of species turnover in the bacterial communities was correlated with an increase in the number of exacerbations. However, I.V. antibiotics are an important intervention which alleviate the symptoms of exacerbations and improve patient quality of life. Therefore, any study examining this relationship would have to be investigated using an appropriate animal or *in-vitro* model.

Recent studies have found that a pathogenic bacterial community assembles to cause bacterial vaginosis which is thought to be initiated by changes in environmental conditions (Oakley et al. 2008). Therefore, a shift in environmental conditions may cause the assembly of a characteristic community and cause exacerbations in CF. However, we have demonstrated that a diagnostic bacterial community does not assemble to cause exacerbations in CF which supports the work from previous studies (Sibley *et al.* 2008b; Tunney *et al.* 2011). Furthermore, we have examined

the link between acquisition and abundance of key taxa using PCR-DGGE and found no evidence that these factors are linked with exacerbations in CF. However, DGGE analysis can only identify the most dominant taxa in the community and further work using more sensitive techniques will be required to make any solid conclusions.

Traditionally, only *C. albicans* and *A. fumigatus* were considered as pathogens in CF respiratory infection (Amin et al. 2010; Chotirmall et al. 2010). However, recent work has found that many more fungal taxa are capable of colonising the CF lung (Cimon et al. 2005; Bouchara et al. 2009). Therefore, it is important to assess the prevalence and pathology caused by these organisms. We have found that fungal species were found in at least one sample from all but one patient in this study. Furthermore, we found that fungal species could persist in the CF lung, although some were only transient colonisers. The factors that affect fungal colonisation in CF need to be investigated to achieve a better understanding of their role in the community. However, research suggests that colonisation with particular fungal taxa can reduce lung function and increase the risk of exacerbations in CF patients (Cimon et al. 2005; Bouchara et al. 2009). Our data shows that the presence and richness of fungal taxa is not the cause of exacerbations in CF.

7. Concluding Remarks

The lower respiratory tract is a diverse environment harbouring complex microbial communities. In previous studies, it has been demonstrated that bacterial, fungal and viral pathogens cause lung function decline and ultimately result in death due to respiratory failure. However, how these communities interact and the factors that cause particular organisms to frequently colonise this environment remains to be elucidated. In this study we aimed to characterise the intrinsic and extrinsic factors which have an effect on bacterial and fungal communities present in the CF lung in a cohort of patients who were homo- or heterozygous for the F508del CFTR allele. A further objective was to follow these patients longitudinally, to determine the stability of the CF lung microbiota, the effects of antibiotic therapy, and to assess if any changes occurred in the CF lung during times of pulmonary exacerbation which could be identified as the causative agent.

7.1 Assessment of sample handling practices on microbial activity in sputum samples from cystic fibrosis patients

The aim of element of the project was to examine the effects of sample storage on molecular microbiological analysis of the communities in CF sputum. We found that storage of samples at room temperature caused a shift in the bacterial communities present in the CF sputum. This shift was characterised by an over estimation of the bacterial load and the numbers of *Pseudomonas* spp. and a reduction in the numbers of *H. influenzae*. Furthermore, the bacterial communities detected by DGGE demonstrated an increased similarity when stored at room temperature whereas the

refrigerated samples appeared to be less similar. This phenomenon may be due to the overgrowth of particular bacterial taxa, such as *Pseudomonas* spp., masking the variability between the lung microbiome of different CF patients.

However, sample storage appeared to have less of an effect on the fungal communities present in CF sputum samples. The PCR-DGGE analysis of these communities demonstrated that the bands found in the samples stored at room temperature were less intense than those that had been refrigerated immediately.

7.2 Bacterial and fungal communities present in the lungs of cystic fibrosis patients with a severe genotype are affected by genetic and environmental factors

In this study we aimed to characterise the intrinsic and extrinsic factors which have an effect on bacterial and fungal communities present in the CF lung in a cohort of patients who were homo- or heterozygous for the F508del CFTR allele. Additionally, we investigated whether microbial community assembly in the CF lung is stochastic or deterministic. We found that sex was a significant factor in the assembly of bacterial communities, due to a reduction in bacterial diversity and community evenness. Previous studies have found that less diverse bacterial communities are associated with a more severe disease and this may be the reason for female CF patients having a poorer prognosis than males. Furthermore, we identified that *P. aeruginosa* colonisation affected bacterial community composition. We have yet to identify a reason for this but changes in oxygen availability after *P. aeruginosa* colonisation may be the cause. We have also identified that bacterial community assembly in the CF lung appears to be predominantly stochastic. However, our data also shows that sex and *P. aeruginosa* colonisation affect

assembly suggesting that some deterministic factors play a role in community assembly.

Fungal communities consisting of up to five different taxa were identified using molecular techniques. This data suggests that fungal communities are more diverse than is currently recognised. We have also identified that patients who are homozygous for the F508del CFTR mutation harbour more rich fungal communities than patients who are heterozygous. Therefore, further analysis of the interactions with other species and their role in the pathogenesis of CF lung disease is required to see if more in depth routine screening for fungal pathogens is required.

7.3 Temporal dynamics of the microbial communities in the Cystic Fibrosis lung

The aim of this study was to assess the diversity and stability of both bacterial and fungal communities in individual CF patients. To determine whether changes in the bacterial and fungal community structure and diversity have a demonstrable link to pulmonary exacerbations in CF and whether fungal species are chronic colonisers of the CF lung. We demonstrated that the bacterial communities in older CF patients are less diverse and rich than those of younger patients. However, we did not observe this relationship over the study period within individual patients. We did observe that the bacterial communities within individual patients were not in a steady state and that immigration and extinction of species was occurring between consecutive sampling dates. However, we also found that some of the species were present throughout the sampling period, suggesting that these species may be chronic colonisers of the CF lung. To assess the factors that affect species turnover we

compared the stability of the bacterial communities with the number of I.V. antibiotic treatments received during the study and found that patients who had received a greater number of treatments had less stable communities than patients who received fewer treatments.

We also examined whether fungal species were capable of chronic colonisation of the CF lung. We found that in most cases that fungal colonisation was transient, although there was evidence in two patients that suggests that fungi can chronically colonise the CF lung. We also observed that patients appeared to be repeatedly colonised with the same species of fungi, suggesting that they are being repeatedly exposed to the source of infection.

The relationship between bacterial communities and exacerbations in CF was also examined. A significant relationship between exacerbations and the bacterial communities present was not identified in this study. However, in one patient we found that bacterial taxa were present when the patient presented with an exacerbation but was absent when the patient was stable, suggesting that acquisition of a new bacterial taxa can potentially cause an exacerbation. We also found that an increase in bacterial load was not the cause of exacerbations in our cohort. Furthermore, the presence and abundance of fungal species was found not to be the cause of exacerbations.

7.4 Future work

In this study we have demonstrated that the bacterial communities in the CF lung are affected by patient specific factors. However, we have not examined the relationship

between these factors and bacterial taxa. Using primers that focus on particular groups of bacteria, for example γ-proteobacteria, we could examine whether particular groups of CF patients are more prone to infection by particular taxa. Furthermore, using more specific primer sets may reveal taxa that were previously below the detection threshold when examining the entire bacterial population. Furthermore, specific primers sets could be used to assess whether particular groups of bacterial taxa are more likely to be chronic colonisers of the CF lung than others. Similarly, the efficacy of antibiotics against these organisms could be assessed more effectively when examining single groups of taxa compared to the community as a whole.

In this study we have used PCR-DGGE to assess the fungal communities present in the CF lung. We have found that this was an effective technique at identifying diverse fungal communities. However, the ability of the primer set to detect a wide range of CF fungal pathogens has not been assessed. In order to fully determine whether these primer sets could be used routinely to differentiate fungal pathogens a wide range of known and emerging fungal pathogens is required as well as the assessment of similar sets of primers.

8. References

Abe, S. et al., 2005. Professional oral care reduces influenza infection in elderly. *Archives of gerontology and geriatrics*, 43(2), pp.157-64.

Acostamartinez, V. et al., 2008. Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. *Soil Biology and Biochemistry*, 40(11), pp.2762-2770.

Alfonso-Sánchez, M.A. et al., 2010. An evolutionary approach to the high frequency of the Delta F508 CFTR mutation in European populations. *Medical hypotheses*, 74(6), pp.989-92.

Amadori, A. et al., 2009. Recurrent exacerbations affect FEV(1) decline in adult patients with cystic fibrosis. *Respiratory medicine*, 103(3), pp.407-13.

Amin, R. et al., 2010. The effect of chronic infection with Aspergillus fumigatus on lung function and hospitalization in patients with cystic fibrosis. *Chest*, 137(1), pp.171-6.

Armougom, F. et al., 2009. Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 28(9), pp.1151-4.

Armstrong, D. et al., 1998. Severe viral respiratory infections in infants with cystic fibrosis. *Pediatric pulmonology*, 26(6), pp.371-9.

Arora, S.K. et al., 1996. Cloning and characterization of Pseudomonas aeruginosa fliF, necessary for flagellar assembly and bacterial adherence to mucin. *Infection and immunity*, 64(6), pp.2130-6.

Arora, S.K. et al., 1998. The Pseudomonas aeruginosa flagellar cap protein, FliD, is responsible for mucin adhesion. *Infection and immunity*, 66(3), pp.1000-7.

Atkinson, T.J., 2008. Cystic fibrosis, vector-mediated gene therapy, and relevance of toll-like receptors: a review of problems, progress, and possibilities. *Current gene therapy*, 8(3), pp.201-7.

Bakare, N. et al., 2003. Prevalence of Aspergillus fumigatus and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses*, 46(1-2), pp.19-23.

Baldwin, A. et al., 2005. Multilocus sequence typing scheme that provides both species and strain differentiation for the Burkholderia cepacia complex. *Journal of clinical microbiology*, 43(9), pp.4665-73.

Balough, K. et al., 1995. The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis. *Pediatric pulmonology*, 20(2), pp.63-70.

Baltimore, R.S. et al., 1982. Occurrence of nonfermentative gram-negative rods other than Pseudomonas aeruginosa in the respiratory tract of children with cystic fibrosis. *Helvetica paediatrica acta*, 37(6), pp.547-54.

Barasch, J. et al., 1991. Defective acidification of intracellular organelles in cystic fibrosis. *Nature*, 352(6330), pp.70-3.

Bargon, J. et al., 1999. Prophylactic antibiotic therapy is associated with an increased prevalence of Aspergillus colonization in adult cystic fibrosis patients. *Respiratory medicine*, 93(11), pp.835-8.

Barrio, M.I. et al., 2008. Isolation of Nocardia species in patients with cystic fibrosis. *Archivos de bronconeumología*, 44(2), pp.109-12.

Baxter, J. & Cummings, S.P., 2008. The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. *Journal of applied microbiology*, 104(6), pp.1605-16.

Belkum, A. van et al., 2000. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. *FEMS immunology and medical microbiology*, 27(1), pp.51-7.

Bittar, F. et al., 2008. Inquilinus limosus and cystic fibrosis. *Emerging infectious diseases*, 14(6), pp.993-5.

Bittar, F. et al., 2008. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PloS One*, 3(8), p.e2908.

Blackburn, L. et al., 2004. "Cepacia syndrome" with Burkholderia multivorans, 9 years after initial colonization. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 3(2), pp.133-4.

Block, J.K. et al., 2006. Predictors of pulmonary exacerbations in patients with cystic fibrosis infected with multi-resistant bacteria. *Thorax*, 61(11), pp.969-74.

Borowitz, D. et al., 2005. Gastrointestinal outcomes and confounders in cystic fibrosis. *Journal of pediatric gastroenterology and nutrition*, 41(3), pp.273-85.

Bouchara, J.P. et al., 2009. Development of an oligonucleotide array for direct detection of fungi in sputum samples from patients with cystic fibrosis. *J. Clin. Microbiol*, 47(1), pp.142-52.

Boucher, R.C., 2007. Evidence for airway surface dehydration as the initiating event in CF airway disease. *Journal of internal medicine*, 261(1), pp.5-16.

Brinkhoff, T. & Hannen, E.J. van, 2001. Use of silicone grease to avoid "smiling effect" in denaturing gradient gel electrophoresis. *Journal of rapid methods and automation in microbiology*, 9(4), pp.259-261.

Bruzzese, E. et al., 2004. Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. *Alimentary pharmacology & therapeutics*, 20(7), pp.813-9.

Bruzzese, E. et al., 2007. Effect of Lactobacillus GG supplementation on pulmonary exacerbations in patients with cystic fibrosis: a pilot study. *Clinical Nutrition* , 26(3), pp.322-8.

Burns, J.L. et al., 1998. Microbiology of sputum from patients at cystic fibrosis centers in the United States. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 27(1), pp.158-63.

Chandra, J. et al., 2001. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. *Journal of bacteriology*, 183(18), pp.5385-94.

Chattoraj, S.S. et al., 2011. Rhinovirus infection liberates planktonic bacteria from biofilm and increases chemokine responses in cystic fibrosis airway epithelial cells. *Thorax*, 66(4), pp.333-9.

Chotirmall, S.H. et al., 2010. Sputum Candida albicans presages FEV1 decline and hospital-treated exacerbations in cystic fibrosis. *Chest*, 138(5), pp.1186-95.

Cigana, C. et al., 2009. Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PloS one*, 4(12), p.e8439.

Cimon, B. et al., 2005. Airway colonization by Acrophialophora fusispora in patients with cystic fibrosis. *Journal of clinical microbiology*, 43(3), pp.1484-7.

Collinson, J. et al., 1996. Effects of upper respiratory tract infections in patients with cystic fibrosis. *Thorax*, 51(11), pp.1115-22.

Corey, M. et al., 1997. Longitudinal analysis of pulmonary function decline in patients with cystic fibrosis. *The Journal of pediatrics*, 131(6), pp.809-14.

Cox, M.J. et al., 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PloS One*, 5(6), p.e11044.

Cugini, C. et al., 2007. Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa. *Molecular microbiology*, 65(4), pp.896-906.

Cystic Fibrosis Foundation, 2009. *Registry Report: Annual data report*, Bethesda, Maryland.

Dahllöf, I., Baillie, H. & Kjelleberg, S., 2000. rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and environmental microbiology*, 66(8), pp.3376-80.

Dakin, C. et al., 2001. Defining an exacerbation of pulmonary disease in cystic fibrosis. *Pediatric pulmonology*, 31(6), pp.436-42.

Dalwai, F., Spratt, D.A. & Pratten, J., 2007. Use of quantitative PCR and culture methods to characterize ecological flux in bacterial biofilms. *Journal of clinical microbiology*, 45(9), pp.3072-6.

Davies, J. et al., 1995. Binding of Haemophilus influenzae to purified mucins from the human respiratory tract. *Infection and immunity*, 63(7), pp.2485-92.

Davis, S.D. et al., 2007. Computed tomography reflects lower airway inflammation and tracks changes in early cystic fibrosis. *American journal of respiratory and critical care medicine*, 175(9), pp.943-50.

De Lisle, R.C., 2007. Altered transit and bacterial overgrowth in the cystic fibrosis mouse small intestine. *American journal of physiology. Gastrointestinal and liver physiology*, 293(1), pp.G104-11.

De Lisle, R.C., Roach, E. & Jansson, K., 2007. Effects of laxative and N-acetylcysteine on mucus accumulation, bacterial load, transit, and inflammation in the cystic fibrosis mouse small intestine. *American journal of physiology. Gastrointestinal and liver physiology*, 293(3), pp.G577-84.

DeSantis, T.Z. et al., 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microbial ecology*, 53(3), pp.371-83.

Dethlefsen, L. et al., 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS biology*, 6(11), p.e280.

Di, A. et al., 2006. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nature cell biology*, 8(9), pp.933-44.

Donskey, C.J. et al., 2003. Use of denaturing gradient gel electrophoresis for analysis of the stool microbiota of hospitalized patients. *Journal of microbiological methods*, 54(2), pp.249-56.

Dorfman, R. et al., 2008. Cystic Fibrosis Mutation database. Available at: http://www.genet.sickkids.on.ca/StatisticsPage.html.

Drevinek, P. et al., 2010. Direct culture-independent Strain typing of Burkholderia cepacia complex in sputum samples from patients with cystic fibrosis. *Journal of clinical microbiology*, 48(5), pp.1888-91.

Duan, K. et al., 2003a. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. *Molecular microbiology*, 50(5), pp.1477-91.

Duan, K. et al., 2003b. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. *Molecular microbiology*, 50(5), pp.1477-91.

Duan, K. et al., 2009. Chemical interactions between organisms in microbial communities. *Contributions to microbiology*, 16, pp.1-17.

Dunbar, J. et al., 2002. Empirical and theoretical bacterial diversity in four Arizona soils. *Applied and environmental microbiology*, 68(6), pp.3035-45.

Döring, G., Parameswaran, I.G. & Murphy, T.F., 2011. Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. *FEMS microbiology reviews*, 35(1), pp.124-46.

Emerson, J. et al., 2010. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. *Pediatric pulmonology*, 45(4), pp.363-70.

Fargione, J., Brown, C.S. & Tilman, D., 2003. Community assembly and invasion: an experimental test of neutral versus niche processes. *Proceedings of the National Academy of Sciences of the United States of America*, 100(15), pp.8916-20.

Farrell, P.M. et al., 2008. Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. *The Journal of pediatrics*, 153(2), p.S4-S14.

Flanagan, J.L. et al., 2007. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with Pseudomonas aeruginosa. *Journal of clinical microbiology*, 45(6), pp.1954-62.

Flotte, T.R. & Carter, B.J., 1997. In vivo gene therapy with adeno-associated virus vectors for cystic fibrosis. *Advances in pharmacology*, 40, pp.85-101.

Foweraker, J.E. et al., 2005. Phenotypic variability of Pseudomonas aeruginosa in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *The Journal of antimicrobial chemotherapy*, 55(6), pp.921-7.

Fridge, J.L. et al., 2007. Risk factors for small bowel bacterial overgrowth in cystic fibrosis. *Journal of pediatric gastroenterology and nutrition*, 44(2), pp.212-8.

Gardner, A., West, S A & Buckling, A., 2004. Bacteriocins, spite and virulence. *Proceedings of The Royal Society. Biological sciences* , 271(1547), pp.1529-35.

Ghannoum, M.A. et al., 2010. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS pathogens*, 6(1), p.e1000713.

Giraud, S. et al., 2010. Geosmithia argillacea: an emerging pathogen in patients with cystic fibrosis. *Journal of clinical microbiology*, 48(7), pp.2381-6.

Girvan, M.S. et al., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental microbiology*, 7(3), pp.301-13.

Goss, C.H. & Burns, J.L., 2007. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax*, 62(4), pp.360-7.

Gould, F.K. et al., 1996. Does storage of sputum specimens adversely affect culture results? *Journal of clinical pathology*, 49(8), pp.684-6.

Gray, R.D. et al., 2010. Sputum and serum calprotectin are useful biomarkers during CF exacerbation. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 9(3), pp.193-8.

Guilbault, C. et al., 2002. Influence of gender and interleukin-10 deficiency on the inflammatory response during lung infection with Pseudomonas aeruginosa in mice. *Immunology*, 107(3), pp.297-305.

Guss, A.M. et al., 2011. Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. *The ISME journal*, 5(1), pp.20-9.

Gutierrez, J.P. et al., 2001. Interlobar differences in bronchoalveolar lavage fluid from children with cystic fibrosis. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 17(2), pp.281-6.

Haase, G. et al., Long-term fungal cultures from sputum of patients with cystic fibrosis. *Mycoses*, 34(9-10), pp.373-6.

Hamilton, W.D., 1964. The genetical evolution of social behaviour. I. *Journal of theoretical biology*, 7(1), pp.1-16.

Hammer, Ø., Harper D A T & P D Ryan, 2001. PAST: Palaeontological statistics software package for education and data analysis. *Palaeontologia Electronica* , 4(1), p.9.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. *Journal of molecular biology*, 166(4), pp.557-80.

Harris, J.K. et al., 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proc Natl Acad Sci U S A*, 104(51), pp.20529-33.

Health protection agency, 2009. *Investigation of bronchoalveolar lavage, sputum and associated specimens*,

Helbich, T.H. et al., 1999. Cystic fibrosis: CT assessment of lung involvement in children and adults. *Radiology*, 213(2), pp.537-44.

Henry, R.L., Mellis, C.M. & Petrovic, L., 1992. Mucoid Pseudomonas aeruginosa is a marker of poor survival in cystic fibrosis. *Pediatric pulmonology*, 12(3), pp.158-61.

Hiatt, P.W. et al., 1999. Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. *Pediatrics*, 103(3), pp.619-26.

Hogan, D.A., Vik, A. & Kolter, R., 2004. A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology. *Molecular microbiology*, 54(5), pp.1212-23.

Huletsky, A. et al., 2004. New real-time PCR assay for rapid detection of methicillin-resistant Staphylococcus aureus directly from specimens containing a mixture of staphylococci. *Journal of clinical microbiology*, 42(5), pp.1875-84.

Hyde, S.C. et al., 2000. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene therapy*, 7(13), pp.1156-65.

Inglis, R.F. et al., 2009. Spite and virulence in the bacterium Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, 106(14), pp.5703-7.

Isles, A. et al., 1984. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. *The Journal of pediatrics*, 104(2), pp.206-10.

Jackson, C.R., Roden, E.E. & Churchill, P.F., 2000. Denaturing gradient gel electrophoresis can fail to separate 16S rDNA fragments with multiple different bases. *Molecular biology today*, 1(2), pp.49-51.

Janse, I., Bok, J. & Zwart, G., 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *Journal of microbiological methods*, 57(2), pp.279-81.

Jones, A.M. et al., 2004. Burkholderia cenocepacia and Burkholderia multivorans: influence on survival in cystic fibrosis. *Thorax*, 59(11), pp.948-51.

Jorde, L.B. & Lathrop, G.M., 1988. A test of the heterozygote-advantage hypothesis in cystic fibrosis carriers. *American journal of human genetics*, 42(6), pp.808-15.

Jurcisek, J.A. & Bakaletz, L.O., 2007. Biofilms formed by nontypeable Haemophilus influenzae in vivo contain both double-stranded DNA and type IV pilin protein. *Journal of bacteriology*, 189(10), pp.3868-75.

Kalish, L.A. et al., 2006. Impact of Burkholderia dolosa on lung function and survival in cystic fibrosis. *American journal of respiratory and critical care medicine*, 173(4), pp.421-5.

Keays, T. et al., 2009. A retrospective analysis of biofilm antibiotic susceptibility testing: a better predictor of clinical response in cystic fibrosis exacerbations. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 8(2), pp.122-7.

Kerem, B. et al., 1989. Identification of the cystic fibrosis gene: genetic analysis. *Science* , 245(4922), pp.1073-80.

Kerem, E. et al., 1992. Prediction of mortality in patients with cystic fibrosis. *The New England journal of medicine*, 326(18), pp.1187-91.

Kim, E.J., Sabra, W. & Zeng, A.P., 2003. Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of Pseudomonas aeruginosa PAO1. *Microbiology* , 149(9), pp.2627-34.

Klepac-Ceraj, V. et al., 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. *Environmental microbiology*, 12(5), pp.1293-303.

Kulich, M. et al., 2003. Improved survival among young patients with cystic fibrosis. *The Journal of pediatrics*, 142(6), pp.631-6.

Kutty, P.K. et al., 2007. Multistate outbreak of Burkholderia cenocepacia colonization and infection associated with the use of intrinsically contaminated alcohol-free mouthwash. *Chest*, 132(6), pp.1825-31.

Landry, R.M. et al., 2006. Mucin-Pseudomonas aeruginosa interactions promote biofilm formation and antibiotic resistance. *Molecular microbiology*, 59(1), pp.142-51.

Larsen, G.Y., Stull, T.L. & Burns, J.L., 1993. Marked phenotypic variability in Pseudomonas cepacia isolated from a patient with cystic fibrosis. *Journal of clinical microbiology*, 31(4), pp.788-92.

Leid, J.G. et al., 2005. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing. *Journal of immunology* , 175(11), pp.7512-8.

Levy, H. et al., 2008. Predictors of mucoid Pseudomonas colonization in cystic fibrosis patients. *Pediatric pulmonology*, 43(5), pp.463-71.

Lewindon, P.J. et al., 1998. Bowel dysfunction in cystic fibrosis: importance of breath testing. *Journal of paediatrics and child health*, 34(1), pp.79-82.

LiPuma, J.J. et al., 1990. Person-to-person transmission of Pseudomonas cepacia between patients with cystic fibrosis. *Lancet*, 336(8723), pp.1094-6.

Littlewood, J.M., 1992. Cystic fibrosis: gastrointestinal complications. *British medical bulletin*, 48(4), pp.847-59.

Liu, W.T. et al., 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and environmental microbiology*, 63(11), pp.4516-22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=168770&tool=pmcentrez&rendertype=abstract.

Lucke, K. et al., 2006. Prevalence of Bacteroides and Prevotella spp. in ulcerative colitis. *Journal of medical microbiology*, 55(5), pp.617-24.

Ma, L. et al., 2009. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. *PLoS pathogens*, 5(3), p.e1000354.

MacArthur, R.H. & Wilson, E.O., 1963. An equilibrium theory of insular zoogeography. *Evolution*, 17, pp.373-387.

MacArthur, R.H. & Wilson, E.O., 1967. *The Theory of Island Biogeography*, Pinceton, NJ: Pinceton University Press.

Magurran, A.E., 2007. Species abundance distributions over time. *Ecology letters*, 10(5), pp.347-54.

Magurran, A.E. & Henderson, P.A., 2003. Explaining the excess of rare species in natural species abundance distributions. *Nature*, 422(6933), pp.714-6.

Marshall, B.C., 2004. Pulmonary exacerbations in cystic fibrosis: it's time to be explicit! *American journal of respiratory and critical care medicine*, 169(7), pp.781-2.

Marshall, B.C. et al., 2005. Epidemiology of cystic fibrosis-related diabetes. *The Journal of pediatrics*, 146(5), pp.681-7.

Mashburn, L.M. et al., 2005. Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. *Journal of bacteriology*, 187(2), pp.554-66.

Matsui, H. et al., 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*, 95(7), pp.1005-15.

Matsui, H. et al., 2005. Reduced three-dimensional motility in dehydrated airway mucus prevents neutrophil capture and killing bacteria on airway epithelial surfaces. *Journal of immunology* , 175(2), pp.1090-9.

May, J.R. & Delves, D.M., 1964. The Survival of H. influenzae and Pnumococci in specimens of sputum sent to the laboratory by post. *Journal of clinical pathology*, 17, pp.254-6.

McKone, E.F., Goss, C.H. & Aitken, M.L., 2006. CFTR genotype as a predictor of prognosis in cystic fibrosis. *Chest*, 130(5), pp.1441-7.

McMenamin, J.D. et al., 2000. Misidentification of Burkholderia cepacia in US cystic fibrosis treatment centers: an analysis of 1,051 recent sputum isolates. *Chest*, 117(6), pp.1661-5.

Mulcahy, L.R. et al., 2010. Emergence of Pseudomonas aeruginosa Strains Producing High Levels of Persister Cells in Patients with Cystic Fibrosis. *Journal of bacteriology*, 192(23), pp.6191-9.

Murphy, T.F. & Kirkham, C., 2002. Biofilm formation by nontypeable Haemophilus influenzae: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiology*, 15(2), p.7.

Muyzer, G., Waal, E.C. de & Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology*, 59(3), pp.695-700.

Nielsen, A.T. et al., 2000. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environmental microbiology*, 2(1), pp.59-68.

Norkina, O., Burnett, T.G. & De Lisle, R.C., 2004. Bacterial overgrowth in the cystic fibrosis transmembrane conductance regulator null mouse small intestine. *Infection and immunity*, 72(10), pp.6040-9.

Oakley, B.B. et al., 2008. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Applied and environmental microbiology*, 74(15), pp.4898-909.

Ong, E.L. et al., 1989. Infective respiratory exacerbations in young adults with cystic fibrosis: role of viruses and atypical microorganisms. *Thorax*, 44(9), pp.739-42.

Palmer, K.L., Aye, L.M. & Whiteley, M., 2007. Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. *Journal of bacteriology*, 189(22), pp.8079-87.

Peltroche-Llacsahuanga, H., Döhmen, H. & Haase, G., 2002. Recovery of Candida dubliniensis from sputum of cystic fibrosis patients. *Mycoses*, 45(1-2), pp.15-8.

Pier, G.B., 2000. Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to Pseudomonas aeruginosa infections. *Proceedings of the National Academy of Sciences of the United States of America*, 97(16), pp.8822-8.

Pier, G.B. et al., 1998. Salmonella typhi uses CFTR to enter intestinal epithelial cells. *Nature*, 393(6680), pp.79-82.

Pribble, C.G. et al., 1990. Clinical manifestations of exacerbations of cystic fibrosis associated with nonbacterial infections. *The Journal of pediatrics*, 117(2), pp.200-4.

Prosser, J.I. et al., 2007. The role of ecological theory in microbial ecology. *Nature reviews. Microbiology*, 5(5), pp.384-92.

Pugin, J. et al., 1991. Oropharyngeal decontamination decreases incidence of ventilator-associated pneumonia. A randomized, placebo-controlled, double-blind clinical trial. *JAMA : the journal of the American Medical Association*, 265(20), pp.2704-10.

Pye, A. et al., 2008. Effect of storage and postage on recovery and quantitation of bacteria in sputum samples. *Journal of clinical pathology*, 61(3), pp.352-4.

Quinton, P.M., 1990. Cystic fibrosis: a disease in electrolyte transport. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 4(10), pp.2709-17.

Rabin, H.R. et al., 2004. Pulmonary exacerbations in cystic fibrosis. *Pediatric pulmonology*, 37(5), pp.400-6.

Rapaka, R.R. & Kolls, J.K., 2009. Pathogenesis of allergic bronchopulmonary aspergillosis in cystic fibrosis: current understanding and future directions. *Medical mycology : official publication of the International Society for Human and Animal Mycology*, 47 Suppl 1, pp.S331-7.

Rasiah, I.A. et al., 2005. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. *Archives of oral biology*, 50(9), pp.779-87.

Raskin, S. et al., 2008. Incidence of cystic fibrosis in five different states of Brazil as determined by screening of p.F508del, mutation at the CFTR gene in newborns and patients. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 7(1), pp.15-22.

Ratjen, F. et al., 2001. Effect of continuous antistaphylococcal therapy on the rate of P. aeruginosa acquisition in patients with cystic fibrosis. *Pediatric pulmonology*, 31(1), pp.13-6.

Regelmann, W.E. et al., 1990. Reduction of sputum Pseudomonas aeruginosa density by antibiotics improves lung function in cystic fibrosis more than do bronchodilators and chest physiotherapy alone. *The American review of respiratory disease*, 141(4), pp.914-21.

Reid, D.W. & Kirov, S.M., 2004. Iron, Pseudomonas aeruginosa and cystic fibrosis. *Microbiology* , 150(3), pp.516-8.

Reid, D.W. et al., 2002. Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic Pseudomonas aeruginosa infection. *Chest*, 121(1), pp.48-54.

Riordan, J.R. et al., 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science ,* 245(4922), pp.1066-73.

Rishmawi, N. et al., 2007. Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. *Journal of clinical microbiology*, 45(4), pp.1278-83.

Rogan, M.P. et al., 2004. Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. *The Journal of infectious diseases,* 190(7), pp.1245-53.

Rogers, G.B. et al., 2004. characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. *Journal of clinical microbiology*, 42(11), pp.5176-83.

Rogers, G.B. et al., 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. *Journal of clinical microbiology*, 44(7), pp.2601-4.

Rogers, G.B. et al., 2005. Bacterial activity in cystic fibrosis lung infections. *Respiratory research*, 6, p.49.

Rogers, G.B. et al., 2009. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC pulmonary medicine*, 9, p.14.

Rogers, G.B. et al., 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *Journal of clinical microbiology*, 41(8), pp.3548-58.

Rogers, G.B. et al., 2010. Determining cystic fibrosis-affected lung microbiology: comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. *Journal of clinical microbiology*, 48(1), pp.78-86.

Rogers, G.B. et al., 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. *Diagnostic microbiology and infectious disease*, 62(2), pp.133-41.

Ronaghi, M., 2001. Pyrosequencing sheds light on DNA sequencing. *Genome research*, 11(1), pp.3-11.

Rosenfeld, M., 2001. Defining a pulmonary exacerbation in cystic fibrosis. *The Journal of Pediatrics*, 139(3), pp.359-365.

Rosenfeld, M. et al., 1997. Gender gap in cystic fibrosis mortality. *American journal of epidemiology*, 145(9), pp.794-803.

Ryan, R.P. et al., 2008. Interspecies signalling via the Stenotrophomonas maltophilia diffusible signal factor influences biofilm formation and polymyxin tolerance in Pseudomonas aeruginosa. *Molecular microbiology*, 68(1), pp.75-86.

Sagel, S.D. et al., 2009. Impact of Pseudomonas and Staphylococcus infection on inflammation and clinical status in young children with cystic fibrosis. *The Journal of pediatrics*, 154(2), pp.183-8.

Saiman, L. et al., 1992. Comparison of adherence of Pseudomonas aeruginosa to respiratory epithelial cells from cystic fibrosis patients and healthy subjects. *Infection and immunity*, 60(7), pp.2808-14.

Sanders, D.B. et al., 2010. Pulmonary exacerbations are associated with subsequent FEV(1) decline in both adults and children with cystic fibrosis. *Pediatric pulmonology*, 46(4), pp.393-400.

Sanders, D.B. et al., 2010. Failure to recover to baseline pulmonary function after cystic fibrosis pulmonary exacerbation. *American journal of respiratory and critical care medicine*, 182(5), pp.627-32.

Sandhu, G.S. et al., 1995. Molecular probes for diagnosis of fungal infections. *Journal of clinical microbiology*, 33(11), pp.2913-9.

Seidler, M.J., Salvenmoser, S. & Müller, F.M.C., 2008. Aspergillus fumigatus forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrobial agents and chemotherapy*, 52(11), pp.4130-6.

Sibley, C.D. et al., 2008. Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. *PLoS pathogens*, 4(10), p.e1000184.

Sibley, C.D. et al., 2008. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America*, 105(39), pp.15070-5.

Smith, E.E. et al., 2006. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America*, 103(22), pp.8487-92.

Spasenovski, T. et al., 2010. Modelling the bacterial communities associated with cystic fibrosis lung infections. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 29(3), pp.319-28.

Spilker, T. et al., 2004. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. *Journal of clinical microbiology*, 42(5), pp.2074-9.

Sriramulu, D.D. et al., 2005. Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *Journal of medical microbiology*, 54(7), pp.667-76.

Starner, T.D. et al., 2006. Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis. *American journal of respiratory and critical care medicine*, 174(2), pp.213-20.

Stewart, P.S. & Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. *Lancet*, 358(9276), pp.135-8.

Stressmann, F.A. et al., 2011. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. *Journal of clinical microbiology*, 49(1), pp.281-91.

Stutts, M.J. et al., 1995. CFTR as a cAMP-dependent regulator of sodium channels. *Science* , 269(5225), pp.847-50.

Sudfeld, C.R. et al., 2010. Prevalence and risk factors for recovery of filamentous fungi in individuals with cystic fibrosis. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 9(2), pp.110-6.

Szabo, K.E. et al., 2007. Importance of rare and abundant populations for the structure and functional potential of freshwater bacterial communities. *Aquatic microbial ecology*, 47(1), pp.1-10.

Tarran, R. et al., 2001. The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. *The Journal of general physiology*, 118(2), pp.223-36.

Tawfik, O.W. et al., 1989. Saccharomyces cerevisiae pneumonia in a patient with acquired immune deficiency syndrome. *Journal of clinical microbiology*, 27(7), pp.1689-91.

Teichgräber, V. et al., 2008. Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nature medicine*, 14(4), pp.382-91.

Tejedor, C., Foulds, J. & Zasloff, M., 1982. Bacteriophages in sputum of patients with bronchopulmonary Pseudomonas infections. *Infection and immunity*, 36(1), pp.440-1.

Toivanen, P., Vaahtovuo, J. & Eerola, E., 2001. Influence of major histocompatibility complex on bacterial composition of fecal flora. *Infection and immunity*, 69(4), pp.2372-7.

Tomashefski, J.F. et al., 1996. Nontuberculous mycobacteria in cystic fibrosis. An autopsy study. *American journal of respiratory and critical care medicine*, 154(2 Pt 1), pp.523-8.

Tourlomousis, P. et al., 2010. PCR-Denaturing Gradient Gel Electrophoresis of Complex Microbial Communities: A Two-Step Approach to Address the Effect of Gel-to-Gel Variation and Allow Valid Comparisons Across a Large Dataset. *Microbial ecology*, 59(4), pp.776-86.

Trosvik, P., Stenseth, N.C. & Rudi, K., 2010. Convergent temporal dynamics of the human infant gut microbiota. *ISME J*, 4(2), pp.151-8.

Tunney, M.M. et al., 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *American journal of respiratory and critical care medicine*, 177(9), pp.995-1001.

Tunney, M.M. et al., 2011. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax*.

Van Der Gast, C.J., Ager, D. & Lilley, A.K., 2008. Temporal scaling of bacterial taxa is influenced by both stochastic and deterministic ecological factors. *Environmental microbiology*, 10(6), pp.1411-8.

Van Der Gast, C.J. et al., 2006. Bacterial diversity is determined by volume in membrane bioreactors. *Environ Microbiol*, 8(6), pp.1048-55.

Van Der Gast, C.J. et al., 2010. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *The ISME journal*, p.doi:10.103.

Walters, S. & Mehta, A., 2007. *Cystic Fibrosis* 3rd ed. M. Hodson, A. Bush, & D. Geddes, eds., Hodder Arnold.

Wang, Y. et al., 2010. Estrogen aggravates inflammation in Pseudomonas aeruginosa pneumonia in cystic fibrosis mice. *Respiratory research*, 11, p.166.

Welsh, M.J. & Smith, A.E., 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*, 73(7), pp.1251-4.

West, S A et al., 2006. Social evolution theory for microorganisms. *Nature reviews. Microbiology*, 4(8), pp.597-607.

West, S A. et al., 2007. The Social Lives of Microbes. *Annual Review of Ecology, Evolution, and Systematics*, 38(1), pp.53-77.

Willner, D. et al., 2009. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PloS one*, 4(10), p.e7370.

Wittebolle, L. et al., 2009. Initial community evenness favours functionality under selective stress. *Nature*, 458(7238), pp.623-6.

Woodcock, S. et al., 2007. Neutral assembly of bacterial communities. *FEMS Microbiol Ecol*, 62(2), pp.171-80.

World Health Organisation, 2002. *The molecular genetic epidemiology of cystic fibrosis*, Genoa.

Worlitzsch, D. et al., 2009. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 15(5), pp.454-60.

Worlitzsch, D. et al., 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *The Journal of clinical investigation*, 109(3), pp.317-25.

Yamamoto, Y. et al., 2002. Extrinsic allergic alveolitis induced by the yeast Debaryomyces hansenii. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 20(5), pp.1351-3.

Yoon, S.S. et al., 2002. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Developmental cell*, 3(4), pp.593-603.

Zeng, A.P. & Kim, E.J., 2004. Iron availability, oxygen limitation, Pseudomonas aeruginosa and cystic fibrosis . *Microbiology*, 150, pp.516-518.

Ziegler, M.M., 1994. Meconium ileus. *Current problems in surgery*, 31(9), pp.731-77.

Appendices

Appendix 1 - NADsens agar

NADsens agar was prepared at the Freeman hospital microbiology department as part of their routine media consortia by a member of staff. The media is based upon isosensitest agar which is used in antibiotic disk diffusion assays and is comprised of;

Step 1;

Hydrolysed casein

Peptones

Glucose

Sodium chloride

Starch

Disodium hydrogen phosphate

Sodium acetate

Magnesium glycerophosphate

Calcium gluconate

Cobaltous sulphate

Cupric sulphate

Zinc sulphate

Ferrous sulphate

Manganous chloride

Menadione

Cyanocobalamin

L-cysteine hydrochloride

L-tryptophan

Pyridoxine

Pantothenate

Nicotinamide

Biotin

Thiamine

Adenine

Guanine

Xanthine

Uracil

Agar

Autoclave at 121°C for 60 minutes

Step 2;

Cool to 50°C and supplement with 5% whole horse blood and 20mg/l NAD

NB For NADsens broth omit agar from step 1.

Appendix 2 – Luria-Bertani media

Basic recipe (per litre)

Tryptone	10g
Yeast Extract	5g
Sodium Cholride	5g
Agar	15g

Autoclave at 121°C for 45 minutes, cool to 50°C and pour 20ml in to each Petri dish

N.B. For broth omit agar from the recipe

JM109 LB plates

Once the media from the basic recipe has cooled to 50°C supplement with Ampicillin (100µg/ml) and X-gal (400µg/ml) and pour as described above.

Antibiotic selection broth

Omit agar from the basic recipe and then proceed as described above. When the media has cooled to 50°C supplement with Ampicillin (100µg/ml) and dispense in to 5ml aliquots.

Appendix 3 – TAE Buffer

TAE buffer was prepared at 50x concentrate then diluted as required.

Step 1 – 100ml EDTA pH 8.0;

18.61g EDTA

100ml dH_2O

The beaker containing the EDTA and 50ml dH_2O was placed on to a magnetic stirrer and the pH was measured throughout. Sodium hydroxide pellets were added to the solution until the solution was at pH 8.0. Addition dH_2O was added as required to achieve a final volume of 100ml.

Step 2

242g Tris base ultrapure

57.1ml Glacial acetic acid

100ml EDTA pH 8.0

dH_2O to 1L

The Tris base was weighed and placed into a 1L Duran bottle along with the glacial acetic acid and the EDTA which was prepared fresh as described above. The buffer was then made up to 1L with dH_2O.

To make 1x TAE dilute 1 part 50x TAE in 49 parts dH_2O.

Appendix 4 – Dyes

Bromophenol blue (6x concentrate)

Bromophenol blue was prepared at 6x concentrate and diluted appropriately with the sample as required. The solution was prepared as follows;

0.025g Bromophenol blue

4.0g Sucrose

dH_2O up to 1L

DCode Dye

DCode dye was added to the 'high' denaturing solution so that the efficacy of mixing between the low and high solutions when pouring the gradient for the DGGE gel could be established. The solution was prepared as follows;

0.05g Bromophenol blue

0.05g Xylene cyanol

1x TAE up to 10 ml

DGGE loading dye (2x concentrate)

DGGE loading dye was prepared at 2x concentrate and diluted appropriately with the sample as required. The solution was prepared as follows;

Step 1

A 2% (w/v) solution of bromophenol blue and a 2% (w/v) solution of xylene cyanol were prepared by dissolving 0.002g of each solid in 1ml dH2O.

Step 2

The solution was prepared as follows;

0.25ml 2% (w/v) bromophenol blue

0.25ml 2% (w/v) xylene cyanol

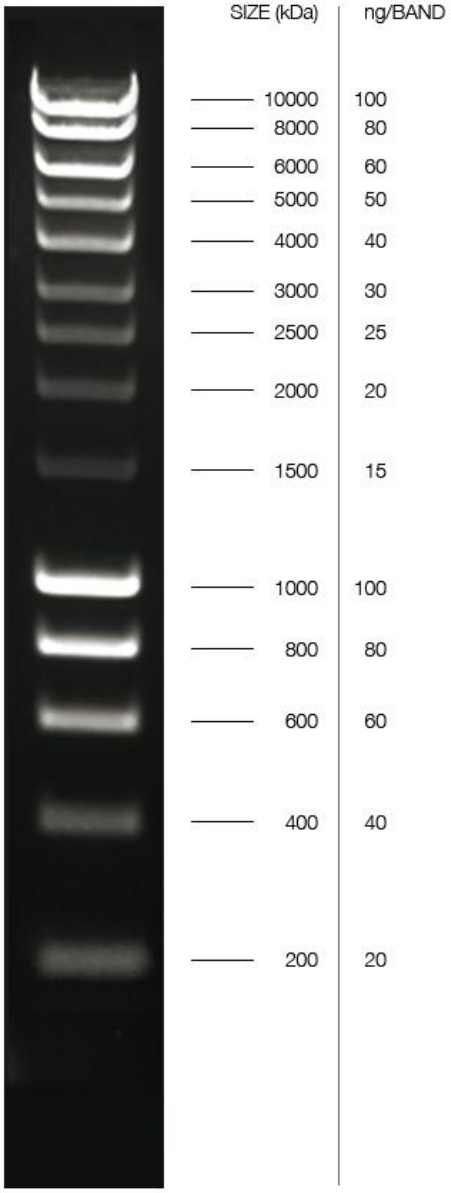
7.0ml 100% glycerol

2.5ml dH_2O

Appendix 5 – Denaturing solutions

Reagent	30%	35%	55%	60%	65%
40%(v/v) acrylamide (37.5:1 acrylamide:bisacrylamide)	30ml	30ml	30ml	30ml	30ml
50x TAE	2ml	2ml	2ml	2ml	2ml
Deionised formamide	12ml	14ml	22ml	24ml	26
Urea (electrophoresis grade)	12.6g	14.7g	23.1g	25.2	27.3
dH_2O	To 100ml	To 100ml	To 100ml	To 100ml	To 100ml

Appendix 6 – Hyperladder I concentrations



HyperLadder I
1% Agarose gel
5µl per lane

Appendix 7 – M9 minimal media

Step 1

Prepare 5x M9 salt solution as described below

Reagent	Amount
Sodium phosphate	64g
Potassium phosphate	15g
Sodium chloride	2.5g
Ammonium chloride	5g
18.2Ω H_20	1L
Autoclave	

The salts can then be used to make M9 minimal media with Vitamin B1 as described below.#

Step 2

Reagent	Amount
Bacteriological agar No. 1 (OXOID)	15g
18.2Ω H_20	750ml
Autoclave at 121˚C	

5x M9 salts	200ml
1M magnesium sulphate (filter sterilise)	2ml
20% (v/v) Glucose (filter sterilise)	20ml
1M Calcium chloride (filter sterilise)	0.1ml
10mg/ml Thiamine hydrochloride (filter sterilise)	0.1ml

Add 20ml of molten M9 minimal agar with Vitamin B1 to a Petri dish and allow cooling at room temperature. Dry the plates and store at 4ºC (up to 1 month).

Appendix 8 – FSB solution

Reagent	Amount
1mM Potassium acetate	0.009814g
45mM Manganese chloride	0.89055g
10mM Calcium chloride	0.1470g
100mM Potassium chloride	0.7455g
3mM Hexamine cobalt chloride	0.08024g
10% (v/v) Glycerol	10ml
18.2Ω H_20	90ml
Filter sterilise (0.22µm) and store at 4ºC	

Appendix 9 – SOC media

Make the following solutions;

1M NaCl

0.5844g NaCl

dH_2O to 10ml

1M KCl

0.7455g KCl

dH_2O to 10ml

2M Mg^{2+} stock

2.330g $MgCl_2 \cdot 6H_2O$

2.465g MgSO4 • $7H_2O$

dH_2O to 10ml filter sterilise with a 0.22µM filter

2M glucose

3.603g Glucose

dH_2O to 10ml filter sterilise with a 0.22µM filter

To make the media add;

2.0g Tryptone

0.5g Yeast extract

1ml 1M NaCl

1ml 1M KCl

dH_2O to 100ml

Autoclave and allow to cool to room temperature. Then add;

1ml 2M Mg2+

1ml 2M Glucose

Check the pH is 7.0, adjust accordingly if it is not.

Appendix 10 - Assessment of sample handling practices on microbial activity in sputum samples from cystic fibrosis patients

Bacterial DGGE band matrix (Part 1)

		Band Number									
Patient	**Treatment**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**Fridge**	0.00082	0	0.000581	0.000561	0	0.001785	0.008444	0.004374	0	0
2	**Fridge**	0.001035	0	0	0	0	0	0.008205	0.000745	0	0
3	**Fridge**	0.000252	0.000188	0	0	0.000218	0	0.003543	0.00094	0	0.000621
4	**Fridge**	0.000491	0.000632	0	0.00038	0	0	0.008118	0.002949	0	0
5	**Fridge**	0.000377	0	0	0	0.000439	0	0.003677	0	0.001701	0
1	**Room Temp.**	0.000481	0	0	0	0	0.000497	0.001715	0	0	0
2	**Room Temp.**	0.000701	0	0	0	0	0	0.001262	0	0	0
3	**Room Temp.**	0.00052	0	0	0	0	0	0.001102	0	0	0
4	**Room Temp.**	0.000451	0	0	0	0	0.000556	0.002968	0	0	0
5	**Room Temp.**	0.000517	0	0	0.000349	0	0	0.000892	0	0.001154	0

Bacterial DGGE Band matrix continued (Part 2)

		Band Number									
Patient	**Treatment**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	**Fridge**	2.1E-05	0	1.49E-05	1.44E-05	0	4.58E-05	0.000217	0.000112	0	0
2	**Fridge**	2.93E-05	0	0	0	0	0	0.000232	2.11E-05	0	0
3	**Fridge**	4.5E-06	3.36E-06	0	0	3.9E-06	0	6.33E-05	1.68E-05	0	1.11E-05
4	**Fridge**	1.21E-05	1.56E-05	0	9.37E-06	0	0	0.0002	7.26E-05	0	0
5	**Fridge**	7.33E-06	0	0	0	8.54E-06	0	7.15E-05	0	3.31E-05	0
1	**Room Temp.**	8.57E-06	0	0	0	0	8.85E-06	3.05E-05	0	0	0
2	**Room Temp.**	1.41E-05	0	0	0	0	0	2.54E-05	0	0	0
3	**Room Temp.**	9.82E-06	0	0	0	0	0	2.08E-05	0	0	0
4	**Room Temp.**	8.17E-06	0	0	0	0	1.01E-05	5.38E-05	0	0	0
5	**Room Temp.**	9.61E-06	0	0	6.49E-06	0	0	1.66E-05	0	2.14E-05	0

Bacterial DGGE band matrix Continued (Part 3)

		Band Number									
Patient	**Treatment**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
1	**Fridge**	5.4E-07	0	3.82E-07	3.69E-07	0	1.17E-06	5.56E-06	2.88E-06	0	0
2	**Fridge**	8.31E-07	0	0	0	0	0	6.58E-06	5.98E-07	0	0
3	**Fridge**	8.05E-08	6.01E-08	0	0	6.97E-08	0	1.13E-06	3E-07	0	1.98E-07
4	**Fridge**	2.98E-07	3.83E-07	0	2.31E-07	0	0	4.92E-06	1.79E-06	0	0
5	**Fridge**	1.43E-07	0	0	0	1.66E-07	0	1.39E-06	0	6.43E-07	0
1	**Room Temp.**	1.53E-07	0	0	0	0	1.58E-07	5.44E-07	0	0	0
2	**Room Temp.**	2.84E-07	0	0	0	0	0	5.12E-07	0	0	0
3	**Room Temp.**	1.85E-07	0	0	0	0	0	3.93E-07	0	0	0
4	**Room Temp.**	1.48E-07	0	0	0	0	1.83E-07	9.76E-07	0	0	0
5	**Room Temp.**	1.78E-07	0	0	1.21E-07	0	0	3.08E-07	0	3.98E-07	0

Bacterial DGGE Band matrix continued (Part 4)

		Band Number						
Patient	**Treatment**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
1	**Fridge**	0.538669	0.56432	0.589971	0.615621	0.641272	0.666923	0.692574
2	**Fridge**	1.53E-08	0	1.08E-08	1.05E-08	0	3.33E-08	1.57E-07
3	**Fridge**	1.48E-08	0	0	0	0	0	1.18E-07
4	**Fridge**	1.98E-09	1.48E-09	0	0	1.72E-09	0	2.79E-08
5	**Fridge**	5.79E-09	7.45E-09	0	4.48E-09	0	0	9.57E-08
1	**Room Temp.**	2.54E-09	0	0	0	2.96E-09	0	2.48E-08
2	**Room Temp.**	3.07E-09	0	0	0	0	3.17E-09	1.1E-08
3	**Room Temp.**	5.37E-09	0	0	0	0	0	9.66E-09
4	**Room Temp.**	3.36E-09	0	0	0	0	0	7.13E-09
5	**Room Temp.**	2.75E-09	0	0	0	0	3.4E-09	1.81E-08

Fungal DGGE Band matrix (Part 1)

		Band number							
Patient	**Treatment**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	**Fridge**	0	0.24697	0	0	0	0	0	0
2	**Fridge**	0.21001	0.11968	0.18197	0.17614	0	0	0	0
3	**Fridge**	0.06862	0.08944	0.18387	0	0	0	0	0
4	**Fridge**	0	0	0	0	0.33311	0	0	0.29873
5	**Fridge**	0.0954	0.10143	0.1412	0	0	0.11696	0.10299	0
1	**Room Temp.**	0	0	0.32857	0	0	0	0	0
2	**Room Temp.**	0	0	0.50416	0	0	0	0	0
3	**Room Temp.**	0	0	0.32326	0.18203	0	0	0	0
4	**Room Temp.**	0	0	0	0	0.20531	0	0	0.2697
5	**Room Temp.**	0.12715	0	0	0	0	0.25983	0.20344	0

Fungal DGGE band matrix continued (Part 2)

		Band number								
Patient	**Treatment**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	**Fridge**	0	0	0.22216	0	0	0	0	0.22576	0.30095
2	**Fridge**	0	0.05672	0.14321	0	0	0	0	0.05456	0.05771
3	**Fridge**	0	0.16746	0.15303	0	0	0	0	0.24977	0.0878
4	**Fridge**	0	0	0	0.04633	0.05239	0.06091	0.12699	0.04158	0.03997
5	**Fridge**	0.0883	0.0544	0.14835	0	0	0	0	0.05731	0.09367
1	**Room Temp.**	0	0	0	0	0	0	0	0.28236	0.38907
2	**Room Temp.**	0	0	0	0	0	0	0	0.20771	0.28814
3	**Room Temp.**	0	0	0	0	0	0	0	0.21351	0.2812
4	**Room Temp.**	0	0	0	0.06281	0.17639	0	0.11887	0.07319	0.09372
5	**Room Temp.**	0.14462	0	0	0	0	0	0	0.10822	0.15673

Eubacterial qPCR data

Patient	Fridge Mean calculated concentration (Copies/ml)	Room Temperature Mean calculated concentration (Copies/ml)	Sum of the mean calculated concentration (copies/ml)	Fridge normalised value	Room Temperature normalised value
1	4.10E+09	7.17E+09	1.13E+10	0.36	0.64
2	2.57E+08	5.73E+08	8.29E+08	0.31	0.69
3	8.62E+07	2.98E+08	3.84E+08	0.22	0.78
4	5.37E+08	7.97E+08	1.33E+09	0.40	0.60
5	2.42E+07	3.52E+07	5.94E+07	0.41	0.59
			Mean	0.34	0.66
			Standard error	0.02	

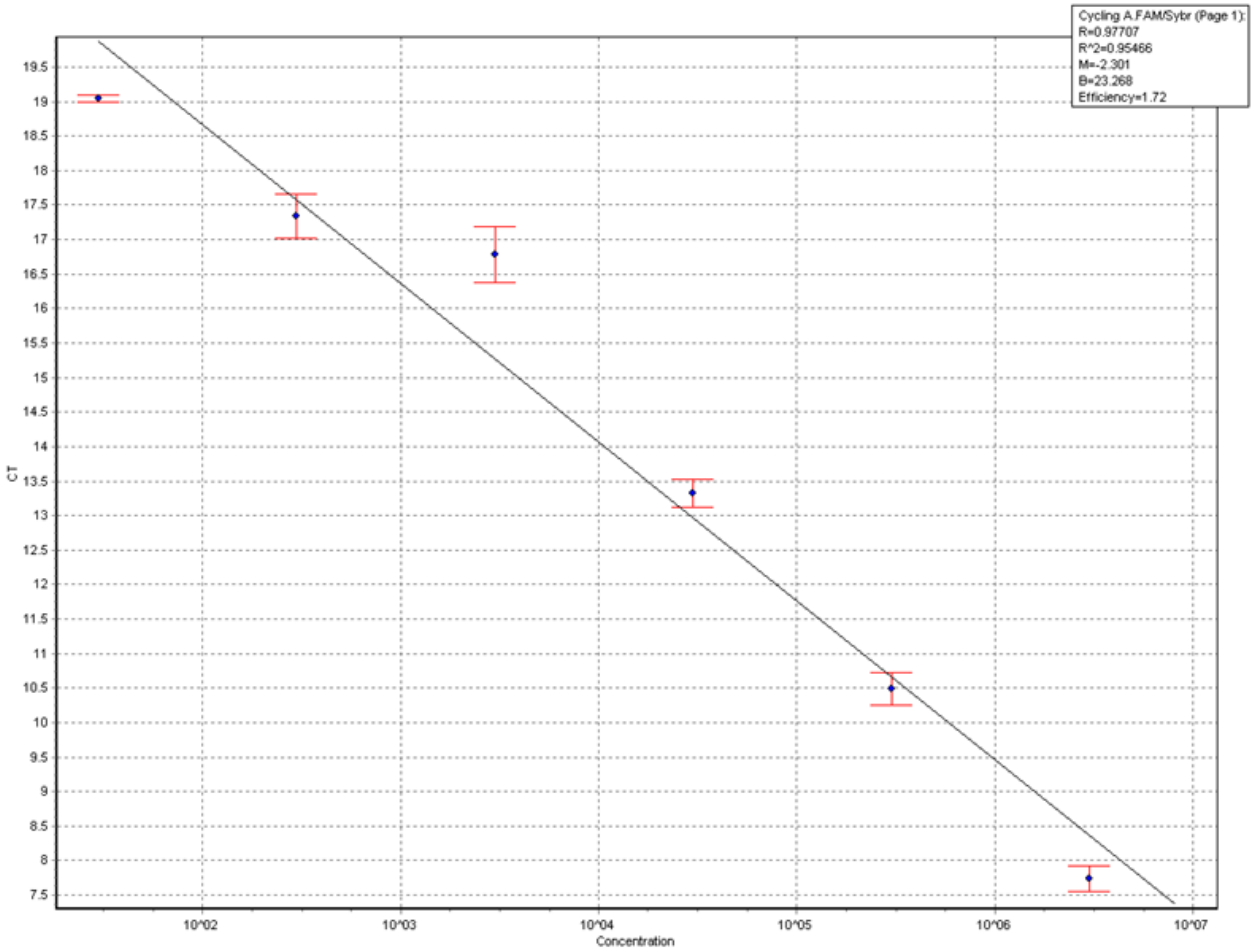
Pseudomonas Spp. qPCR data

Patient	Fridge Mean calculated concentration (Copies/ml)	Room Temperature Mean calculated concentration (Copies/ml)	Sum of the mean calculated concentration (copies/ml)	Fridge normalised value	Room Temperature normalised value
1	5.66E+07	5.25E+07	1.09E+08	0.52	0.48
2	1.20E+06	6.34E+06	7.54E+06	0.16	0.84
3	8.74E+05	3.57E+06	4.44E+06	0.20	0.80
4	4.60E+06	9.86E+06	1.45E+07	0.32	0.68
5	3.73E+05	6.19E+05	9.92E+05	0.38	0.62
			Mean	0.31	0.69
			Standard error	0.03	

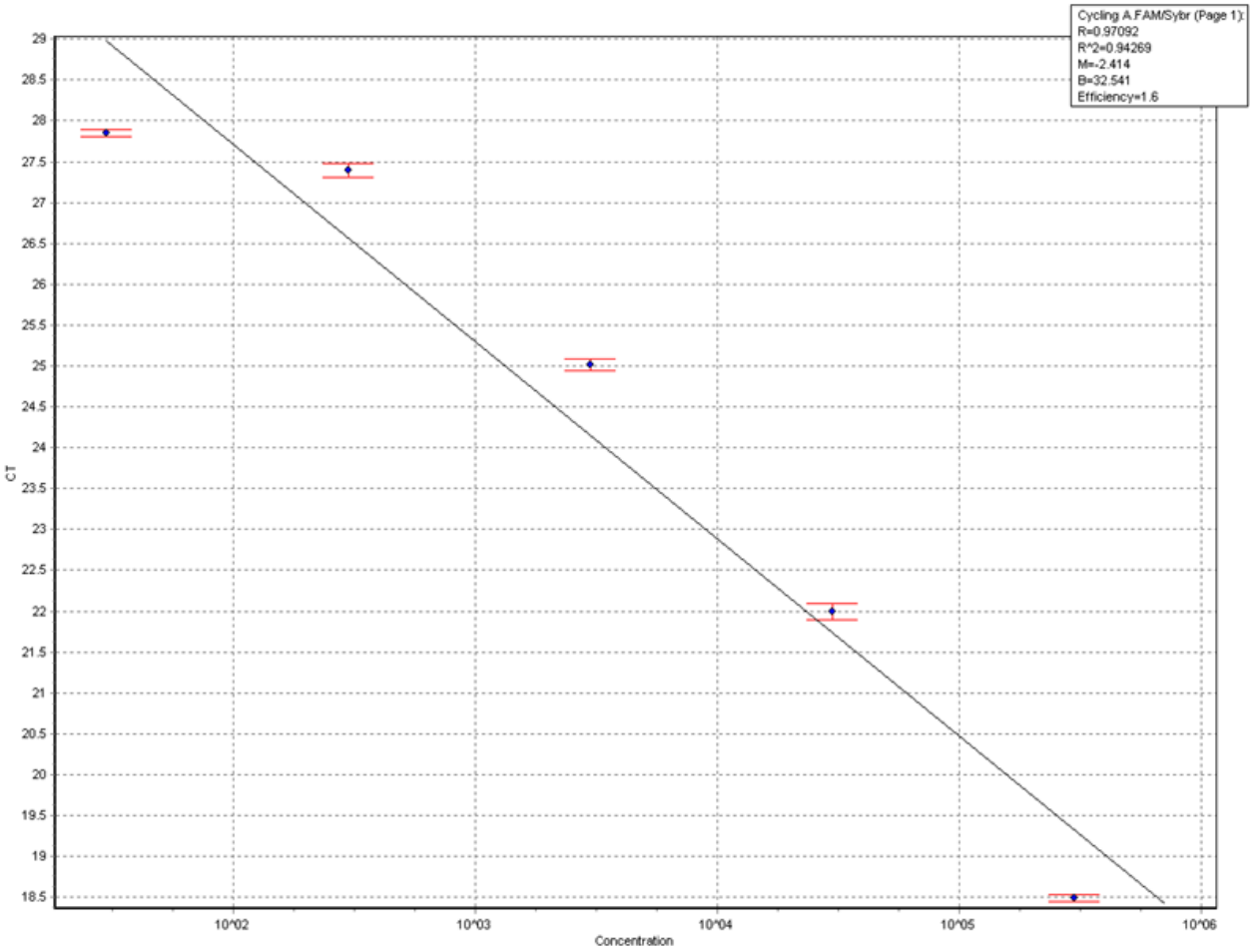
H. influenzae qPCR data

Patient	Fridge Mean calculated concentration (Copies/ml)	Room Temperature Mean calculated concentration (Copies/ml)	Sum of the mean calculated concentration (copies/ml)	Fridge normalised value	Room Temperature normalised value
P1	4.13E+03	2.00E+03	6.13E+03	0.67	0.33
P2	6.22E+03	2.25E+03	8.47E+03	0.73	0.27
P3	6.76E+03	4.17E+03	1.09E+04	0.62	0.38
P4	7.49E+03	3.75E+03	1.12E+04	0.67	0.33
P5	1.22E+04	5.15E+03	1.74E+04	0.70	0.30
			Mean	0.68	0.32
			Standard error	0.01	

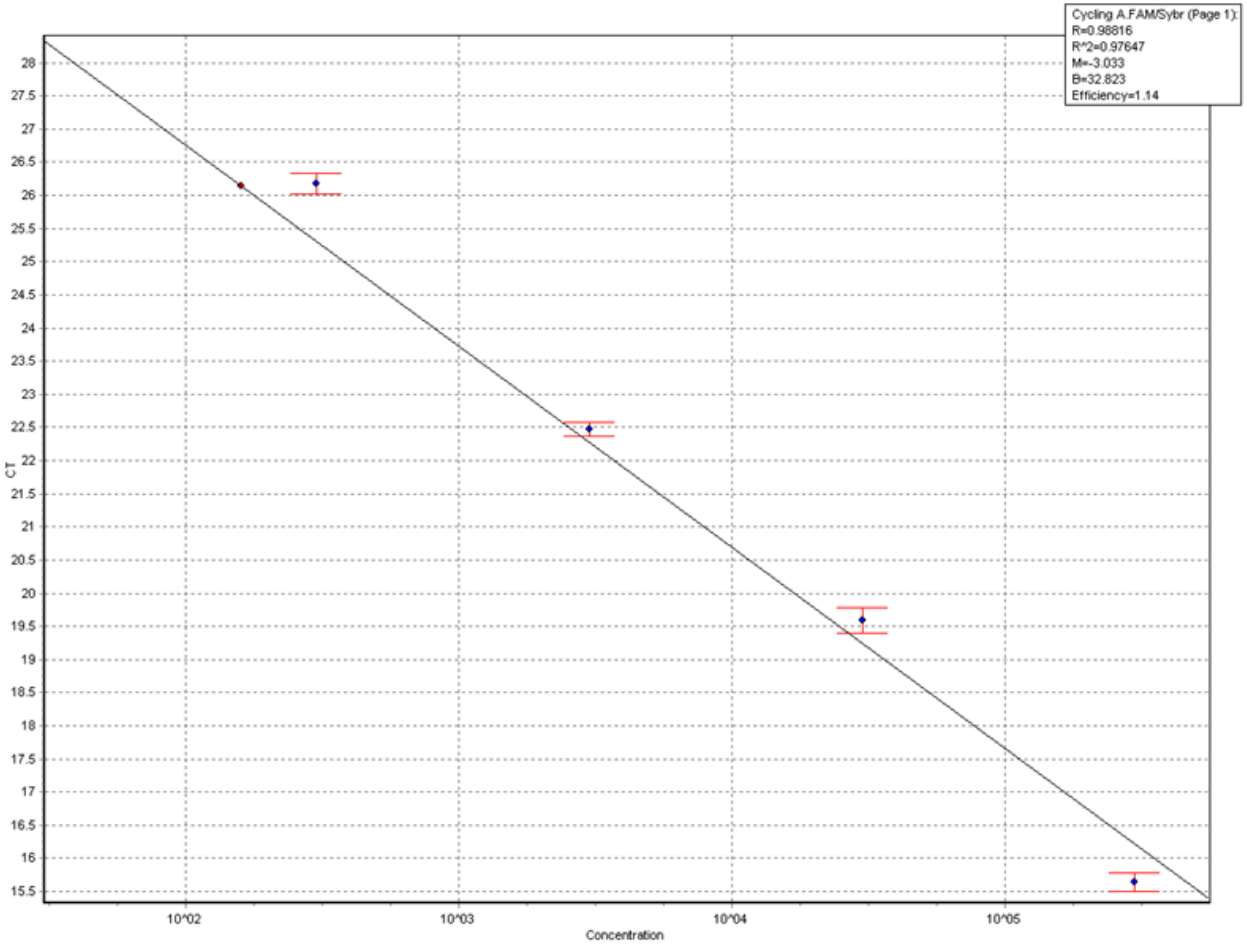
Eubacteria qPCR standard curve



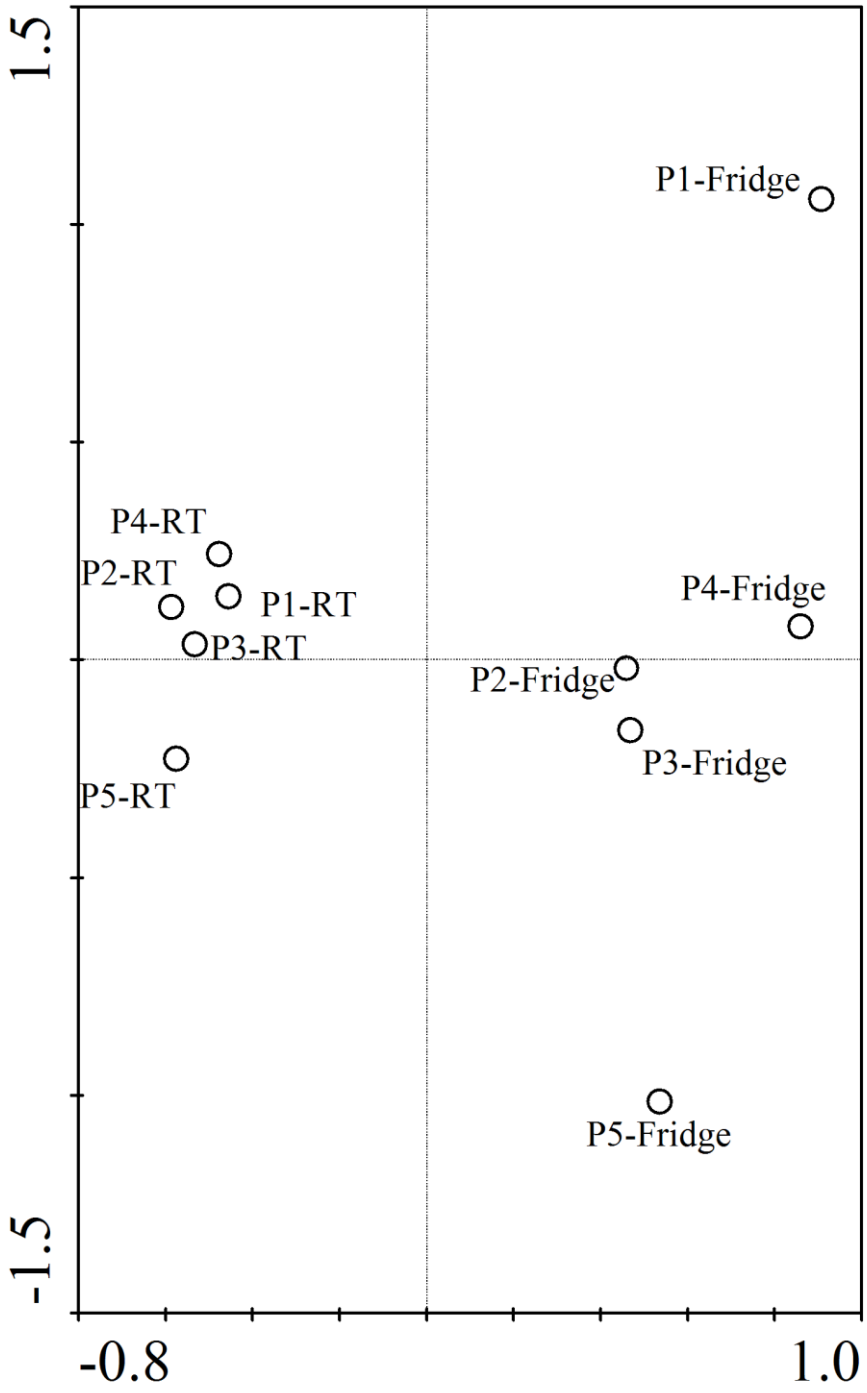
Pseudomonas Spp. qPCR standard curve



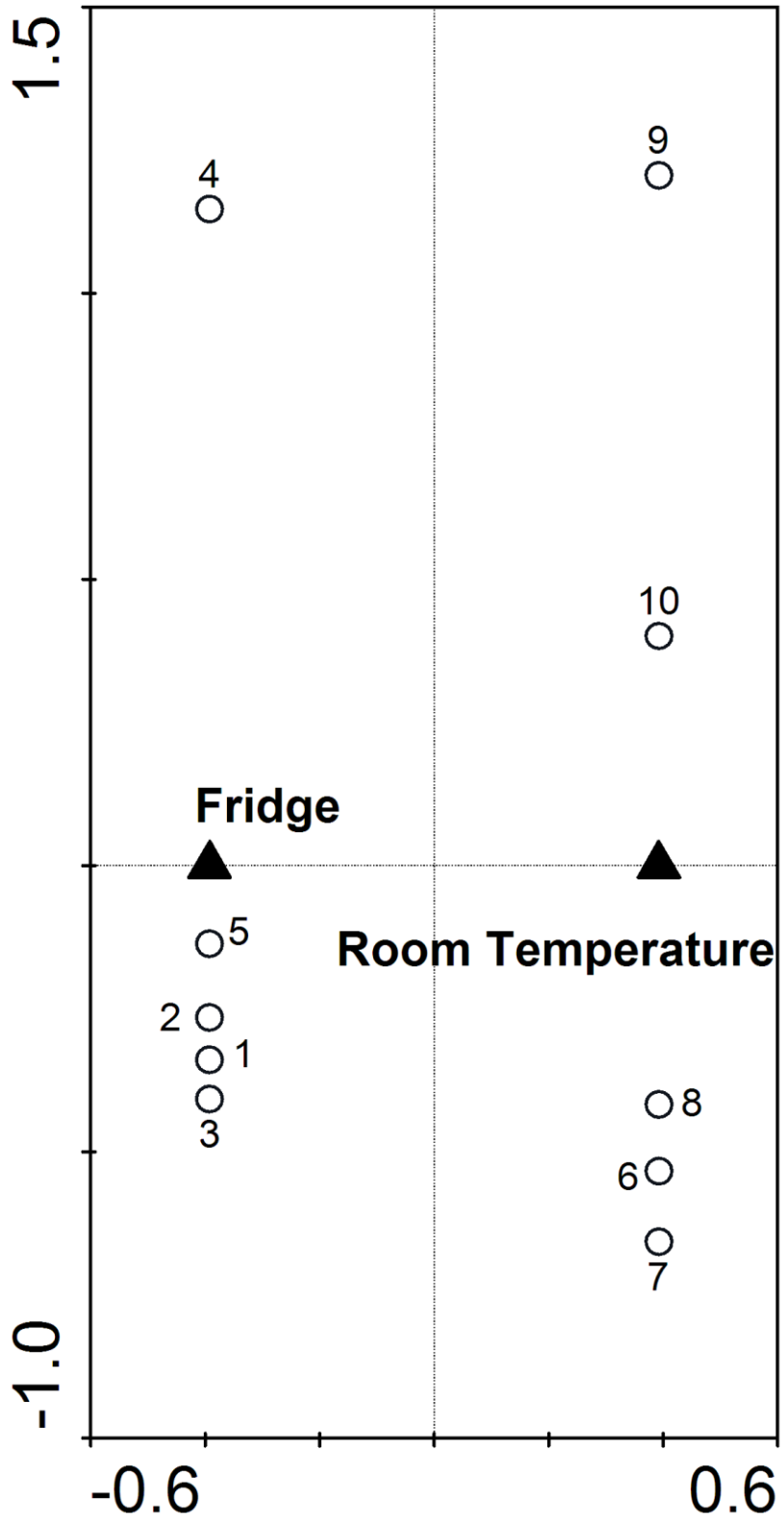
H. influenzae qPCR standard curve



Bacterial correspondence analysis



Fungal CCA



Sequence from Fungal DGGE excised bands

Band	Accession number	Sequence
F1	GU001640	TACCGGGCCAGCATCGGTTTGGAGCGGTAGGATAAATGGCGGGGGAATGTGGCACGACTTTGGTTGTGTGTTATAGCCTCTGACGATACTGCCAGCCTAGACCGAGGACTGCGGTTTTTACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAAACACGGACCAAGGAGTCCCCCCCGTGCCCCCGCCCCG
F2	GU065334	GGATAATGGCGGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGCCTCTGACGATACTGCCAGCCTAGACCGAGGACTGCGGTTTTTACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAAACACGGACCAAGGAGTCCCCCCCGTGCCCC
F3	GU065335	AATGTGGCACTGCTTCGGTAGTGTGTTATAGTCTTTGTCGATACTGCCAGCTTAGACTGAGGACTGCGGCTTCGGCCTAGGAGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAAACACGGACCAAGGAGTCACCCCCGTGCCCCCGCCCCGCT
F4	GU065336	TCCCCGTGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCCCTCGGAATGTATCACCTCTCGGGGTGTCTTATAGCCGAGGGTGCAATGCGGCCTGCCTGGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCGTAATGGTCGTAAATGACCCGTCTTGAAACACGGACCAAGGA

Appendix 11 - Bacterial and fungal communities present in the lungs of cystic fibrosis patients with a severe genotype are affected by genetic and environmental factors

Patient Demographics (Part 1)

Patient No.	Sex	Age	Genotype	FEV1 (% predicated)	Routine antibiotics	BMI	Recent History
1	F	30	F508del/OM	65	COL, FLU, OT	21.7	*P. aeruginosa* x 3 / MRSA / *Candida spp.*
2	F	33	F508del/F508del	26	AZI, FLU	20.1	*P. aeruginosa* x 2 / *Candida spp.*
3	M	32	F508del/F508del	58	AZI, FLU	22.5	*P. aeruginosa* x 2
4	M	18	F508del/F508del	65	AZI, COL, DOX	21.5	*P. aeruginosa* / *A. fumigatus* / Candida spp.
5	M	27	F508del/F508del	99	AZI, COL FLU	23.0	*P. aeruginosa*
6	F	28	F508del/OM	59	AZI	20.9	*P. aeruginosa* x 2 / *C. albicans*
7	M	20	F508del/F508del	66	AZI, COL	19.6	*P. aeruginosa* x 2 / *C. albicans*
8	F	23	F508del/OM	44	AZI, COL FLU	21.2	*P. aeruginosa* x 2

Patient Demographics continued (Part 2)

Patient No.	**Sex**	**Age**	**Genotype**	**FEV1 (% predicated)**	**Routine antibiotics**	**BMI**	**Recent History**
9	M	34	F508del/OM	59	AZI, COL	22.0	MRSA / Candida sp.
10	M	23	F508del/F508del	62	AZI, COL FLU	16.7	No bacterial pathogens / Candida sp.
11	M	19	F508del/F508del	46	AZI, COL FLU	21.7	No bacterial pathogens / Candida sp.
12	F	19	F508del/F508del	73	AZI, COL FLU	17.9	*P. aeruginosa*
13	M	20	F508del/OM	74	AZI, COL	16.7	*P. aeruginosa* / MRSA
14	M	29	F508del/F508del	89	AZI	22.0	*P. aeruginosa*
15	M	22	F508del/OM	92	COL, FLU	20.0	*P. aeruginosa*
16	M	26	F508del/F508del	43	AZI, CEF, FLU, TOB	18.2	No bacterial pathogens / *A. fumigatus*

Patient Demographics continued (Part 3)

Patient No.	Sex	Age	Genotype	FEV1 (% predicated)	Routine antibiotics	BMI	Recent History
17	M	25	F508del/F508del	18	AZI, COL FLU	24.1	*P. aeruginosa* x 2 / Serratia spp. / Candida spp.
18	F	56	F508del/OM	35	AZI	24.2	MRSA / *Candida spp.*
19	M	26	F508del/F508del	30	AZI, COL FLU	19.1	*P. aeruginosa* x 2
20	M	56	F508del/OM	28	AZI	22.0	*P. aeruginosa* / Mycobacterium spp./ Candida spp.
21	F	28	F508del/OM	31	AZI, COL FLU	19.2	*P. aeruginosa* / *A. xylosoxidans*
22	F	19	F508del/OM	104	AZI, COL FLU	25.9	No bacterial pathogens / Candida spp.

Patient Demographics continued (Part 4)

Patient No.	Sex	Age	Genotype	FEV1 (% predicated)	Routine antibiotics	BMI	Recent History
23	F	28	F508del/F508del	49	COL, FLU	18.9	*P. aeruginosa* x 2 / Candida spp.
24	F	28	F508del/F508del	86	COL, FLU	27.8	*P. aeruginosa* / Serratia spp.
25	F	59	F508del/OM	33	AZI, COL	20.7	*P. aeruginos*a / Candida spp.
26	F	23	F508del/F508del	60	AZI, COL FLU	27.0	*P. aeruginosa* / *S. aureus* / Candida spp.
27	M	28	F508del/OM	60	AZI, COL	28.3	*P. aeruginosa* x 2
28	F	39	F508del/F508del	34	COL, FLU	28.0	*P. aeruginosa*
29	M	39	F508del/F508del	53	AZI, COL FLU	25.3	*P. aeruginosa* / *S. aureus / A. fumigatus*

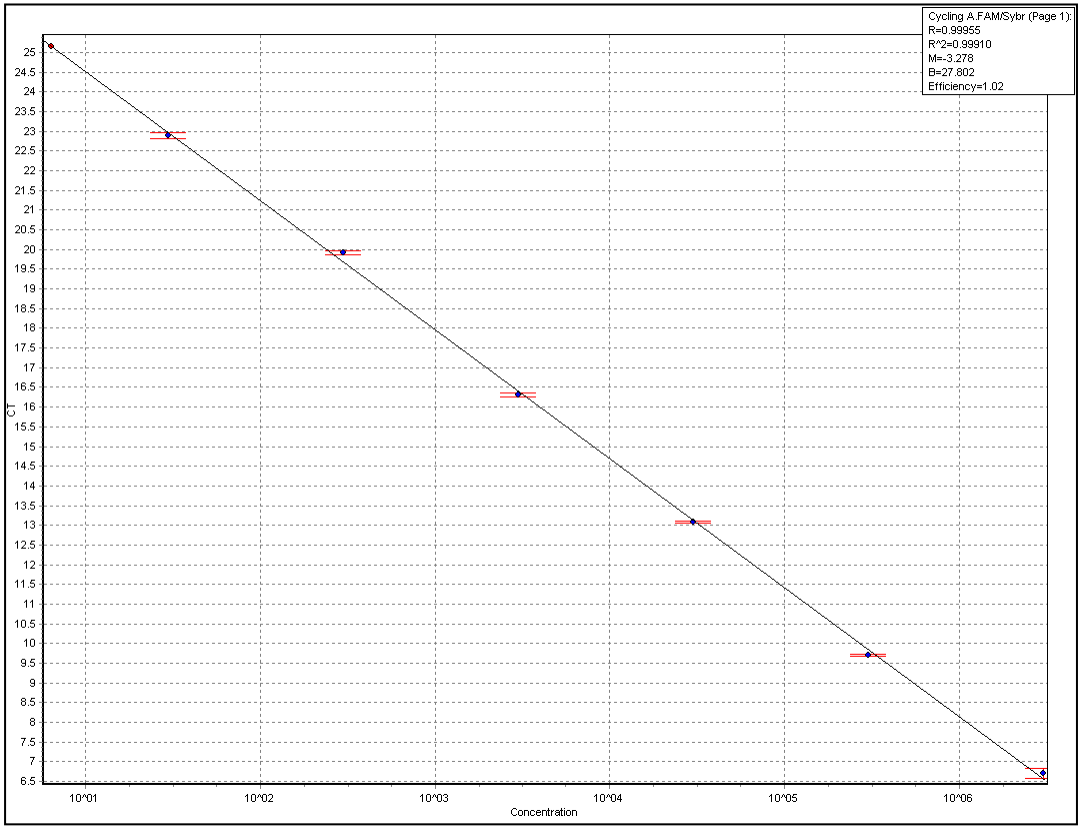
Microbial Community Analysis (Part 1)

Patient	Shannon	Richness	Evenness	Rep. Calc. Conc.	qPCR (copies/rxn)	qPCR (copies/ml)	log qPCR	Fungi Richness
1	1.69	12	0.68	1,339,921.78	133992177.9	6699608893	9.83	1
2	1.84	15	0.68	7,092.78	709277.776	35463889	7.55	1
3	2.39	19	0.81	69,566.18	6956618.308	347830915	8.54	2
4	2.65	22	0.86	65,309.74	6530973.762	326548688	8.51	1
5	2.24	17	0.79	315,439.28	31543928.17	1577196408	9.20	1
6	2.04	15	0.75	109,312.47	10931247.15	546562358	8.74	1
7	1.87	12	0.75	72,912.53	7291252.894	364562645	8.56	5
8	0.91	6	0.51	4,844.48	484448.405	24222420	7.38	0
9	2.30	14	0.87	1,200.26	120026.224	6001311	6.78	0
10	2.00	14	0.76	28,109.97	2810996.79	140549840	8.15	3
11	2.51	16	0.91	9,144.06	914406.099	45720305	7.66	3
12	2.01	14	0.76	14,787.47	1478747.201	73937360	7.87	3
13	2.04	11	0.85	1,399.05	139904.856	6995243	6.84	0
14	1.75	8	0.84	24,042.89	2404288.88	120214444	8.08	1

Microbial Community Analysis continued (Part 2)

Patient	Shannon	Richness	Evenness	Rep. Calc. Conc.	qPCR (copies/rxn)	qPCR (copies/ml)	log qPCR	Fungi Richness
15	2.14	15	0.79	1,162,075.89	116207589.2	5810379461	9.76	2
16	2.24	14	0.85	84,275.54	8427553.788	421377689	8.62	1
17	1.61	9	0.73	14,241.75	1424174.744	71208737	7.85	1
18	2.27	17	0.80	1,759.36	175936.497	8796825	6.94	1
19	1.94	13	0.76	75,103.87	7510386.754	375519338	8.57	0
20	1.53	11	0.64	16,881.96	1688196.398	84409820	7.93	0
21	2.30	15	0.85	3,478.32	347831.529	17391576	7.24	1
22	1.29	6	0.72	2,601,033.31	260103331.5	13005166574	10.11	2
23	1.87	10	0.81	1,409,998.93	1040999893	52049994630	10.72	3
24	1.20	7	0.61	1,571,967.61	157196760.8	7859838038	9.90	0
25	0.78	6	0.44	326,007.58	32600758	1630037900	9.21	0
26	1.53	9	0.69	587,636.43	58763642.92	2938182146	9.47	3
27	1.95	11	0.81	603,779.56	60377955.59	3018897780	9.48	0
28	1.37	7	0.70	5,317.30	531730.377	26586519	7.42	2
29	1.99	10	0.86	482,457.09	48245708.73	2412285436	9.38	3

Example of Eubacterial qPCR standard curve



Appendix 12 - Temporal dynamics of the microbial communities in the Cystic Fibrosis

Longitudinal Patient Demographics (Part 1)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
1	101	F	30	F508del/OM	02/10/2008	Stable	1.65 (59%)	OTC, COL, FLU		*P. aeruginosa* x 3 / MRSA
	102	F	30	F508del/OM	03/11/2008	Exacerbation	1.68 (60%)	OTC, COL, FLU	TAZ/TOB	*P. aeruginosa* x 2 / MRSA
	103	F	30	F508del/OM	22/01/2009	Stable	1.82 (65%)	OTC, COL, FLU		*P. aeruginosa* x 3 / MRSA / Candida sp.
	104	F	30	F508del/OM	18/02/2009	Exacerbation	1.60 (57%)	OTC, COL, FLU	CEF/TOB	*P. aeruginosa* / MRSA / Haemolytic Streptococcus group C
	105	F	30	F508del/OM	01/04/2009	Stable	1.75 (62%)	OTC, COL, FLU		*P. aeruginosa* x 2 / MRSA / Candida sp.
	106	F	30	F508del/OM	24/06/2009	Stable	1.80 (64%)	OTC, COL, FLU		*P. aeruginosa* x 2 / MRSA
	107	F	30	F508del/OM	04/08/2009	Exacerbation	1.56 (56%)	OTC, COL, FLU	CEF/COL	Pseudomonas aeruginosa x 2 / MRSA
	108	F	30	F508del/OM	16/09/2009	Stable	1.76 (63%)	OTC, COL, FLU		Pseudomonas aeruginosa
	109	F	30	F508del/OM	02/11/2009	Stable	1.76 (63%)	OTC, COL, FLU		Pseudomonas aeruginosa x 2 / MRSA
	110	F	30	F508del/OM	09/12/2009	Exacerbation	1.61 (57%)	OTC, COL, FLU	CEF/COL	*P. aeruginosa* x 2 / MRSA / Candida sp.
	111	F	30	F508del/OM	04/01/2010	Routine I.V.	1.71 (61%)	FLU, AZI, COL, MIN	CEF/COL	*P. aeruginosa* x 2 / Candida sp.
	112	F	30	F508del/OM	09/06/2010	Stable	1.85(66%)	FLU, AZI, COL, MIN		*P. aeruginosa* x 3
2	201	F	33	F508del/F508del	02/10/2008	Stable	1.22 (35%)	AZI, FLU		*P. aeruginosa* x 2
	202	F	33	F508del/F508del	08/10/2008	Exacerbation	1.24 (35%)	AZI, FLU	TAZ/TOB	*P. aeruginosa* x 2
	203	F	33	F508del/F508del	05/02/2009	Exacerbation	0.95 (28%)	AZI, FLU	COL/TAZ	*P. aeruginosa* x 2
	204	F	33	F508del/F508del	04/03/2009	Exacerbation	0.94 (28%)	AZI, FLU	COL/MER	*P. aeruginosa* / Candida sp.
	205	F	33	F508del/F508del	15/04/2009	Stable	0.90 (26%)	AZI, FLU		*P. aeruginosa x 2* / Candida sp.
	206	F	33	F508del/F508del	03/06/2009	Stable	1.10 (29%)	AZI		*P. aeruginosa x 2* / Candida sp.
	207	F	33	F508del/F508del	12/08/2009	Exacerbation	1.15 (30%)	AZI	CEF/TOB	*P. aeruginosa*

Longitudinal Patient Demographics continued (Part 2)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
2	208	F	33	F508del/F508del	30/09/2009	Stable	1.28 (37%)	AZI		*P. aeruginosa*
	209	F	33	F508del/F508del	04/11/2009	Stable	1.25 (36%)	AZI		*P. aeruginosa* x 2
	210	F	33	F508del/F508del	02/12/2009	Routine I.V.	1.24 (36%)	AZI	CEF/TOB	*P. aeruginosa x 2* / Candida sp.
	211	F	33	F508del/F508del	20/01/2010	Stable	1.25 (36%)	AZI		*P. aeruginosa*
	212	F	33	F508del/F508del	02/02/2010	Exacerbation	1.17 (34%)	AZI	TAZ/TOB	*P. aeruginosa* x 2
	213	F	33	F508del/F508del	05/03/2010	Exacerbation	1.15 (33%)	AZI	COL/TAZ	*P. aeruginosa* / Candida sp.
	214	F	33	F508del/F508del	10/03/2010	Exacerbation	1.15 (33%)	AZI	COL/TAZ	*P. aeruginosa* x 2
	215	F	33	F508del/F508del	21/04/2010	Exacerbation	1.00 (29%)	AZI	COL/TAZ	*P. aeruginosa x 2* / Candida sp.
3	301	M	32	F508del/F508del	02/10/2008	Stable	2.46 (57%)	AZI, COL FLU		*P. aeruginosa* x 2
	302	M	32	F508del/F508del	31/10/2008	Exacerbation	2.46 (57%)	AZI, COL FLU	MER/TOB	*P. aeruginosa* / Candida sp.
	303	M	32	F508del/F508del	01/04/2009	Stable	2.50 (58%)	AZI, FLU		*P. aeruginosa* x 2
	304	M	32	F508del/F508del	15/07/2009	Exacerbation	2.06 (48%)	AZI, FLU	CEF/TOB	*P. aeruginosa* / *A. fumigatus*
	305	M	32	F508del/F508del	14/10/2009	Routine I.V.	2.30 (54%)	AZI, FLU	CEF/TOB	*P. aeruginosa*
	306	M	32	F508del/F508del	27/01/2010	Stable	1.95 (45%)	AZI, FLU		*P. aeruginosa x 2* / Candida sp.
	307	M	32	F508del/F508del	18/05/2010	Stable	2.00 (47%)	AZI, FLU		*P. aeruginosa x 2* / Candida sp.
4	401	M	27	F508del/F508del	02/10/2008	Stable	3.90 (88%)	AZI, COL FLU		*P. aeruginosa* x 2
	402	M	27	F508del/F508del	20/01/2009	Exacerbation	3.35 (76%)	AZI, COL FLU	TAZ/TOB	*P. aeruginosa* / *S. aureus* / Candida sp.
	403	M	27	F508del/F508del	12/02/2009	Stable	4.30 (99%)	AZI, COL FLU		*P. aeruginosa*
	404	M	27	F508del/F508del	11/11/2009	Routine I.V.	4.00 (92%)	AZI, COL FLU	MER/TOB	*P. aeruginosa*

Longitudinal Patient Demographics continued (Part 3)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
4	405	M	27	F508del/F508del	13/01/2010	Stable	3.80 (88%)	AZI, COL FLU		*P. aeruginosa*
	406	M	27	F508del/F508del	18/03/2010	Stable	3.95(91%)	AZI, COL FLU		*P. aeruginosa* x 2
	407	M	27	F508del/F508del	12/05/2010	Stable	4.15 (96%)	AZI, COL FLU		*P. aeruginosa*
5	501	F	28	F508del/OM	19/02/2009	Stable	1.88 (59%)	AZI		*P. aeruginosa x 2* / *C. albicans*
	502	F	28	F508del/OM	01/04/2009	Exacerbation	1.10 (34%)	AZI	GEN/MER	*P. aeruginosa* x 2
	503	F	28	F508del/OM	08/07/2009	Exacerbation	1.15 (36%)	AZI	CIP (Oral)	*P. aeruginosa* x 2
	504	F	28	F508del/OM	14/10/2009	Stable	1.20 (38%)	AZI		*P. aeruginosa* x 3
	505	F	28	F508del/OM	23/12/2009	Stable	1.20 (38%)	AZI		*P. aeruginosa*
	506	F	28	F508del/OM	07/04/2010	Stable	1.50 (47%)	AZI		*P. aeruginosa* / Pseudomonas sp. / Candida sp.
	507	F	28	F508del/OM	09/06/2010	Exacerbation	1.35(42%)	AZI	CIP (Oral)	*P. aeruginosa x 2* / Candida sp.
6	601	M	34	F508del/OM	12/02/2009	Exacerbation	2.20 (48%)	AZI, COL	MER/TOB	Pseudomonas aeruginosa / MRSA / Candida sp.
	602	M	34	F508del/OM	22/04/2009	Stable	2.32 (51%)	AZI, COL		MRSA / Candida sp.
	603	M	34	F508del/OM	30/07/2009	Stable	2.68 (59%)	AZI, COL		MRSA / Candida sp.
	604	M	34	F508del/OM	09/09/2009	Stable	2.46 (54%)	AZI, COL, TET		Candida sp.
	605	M	34	F508del/OM	23/11/2009	Exacerbation	2.40 (53%)	AZI, COL	MER/TOB	*P. aeruginosa* / MRSA / Candida sp.
	606	M	34	F508del/OM	09/12/2009	Stable	2.25 (49%)	AZI, COL		*P. aeruginosa* / MRSA / Candida sp.
	607	M	34	F508del/OM	13/01/2010	Exacerbation	unable to do	AZI, COL	MER/TOB	*P. aeruginosa* / MRSA
	608	M	34	F508del/OM	10/02/2010	Exacerbation	2.45 (54%)	AZI, COL	MER/TOB/DOX	*P. aeruginosa* / MRSA / Candida sp.
	609	M	34	F508del/OM	28/04/2010	Exacerbation	2.10 (46%)	AZI, COL	MER/TOB	MRSA / Candida sp.
	610	M	34	F508del/OM	27/05/2010	Stable	2.10 (46%)	AZI, COL		MRSA / Candida sp.

Longitudinal Patient Demographics continued (Part 4)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
7	701	M	23	F508del/F508del	13/03/2009	Stable	2.35 (62%)	AZI, COL FLU		No bacterial pathogens / Candida sp.
	702	M	23	F508del/F508del	29/07/2009	Stable	2.40 (63%)	AZI, COL		No bacterial pathogens / Candida sp.
	703	M	23	F508del/F508del	03/09/2009	Exacerbation	2.07 (54%)	AZI, COL	COL/MER	*P. aeruginosa*
	704	M	23	F508del/F508del	04/11/2009	Exacerbation	2.14 (54%)	AZI, COL	CEF/TOB	*P. aeruginosa* / Candida sp.
	705	M	23	F508del/F508del	23/12/2009	Stable	2.08 (54%)	AZI, COL		*P. aeruginosa* / Candida sp.
	706	M	23	F508del/F508del	23/03/2010	Exacerbation	1.95 (51%)	AZI, COL	CEF/COL	*P. aeruginosa* / Candida sp.
	707	M	23	F508del/F508del	12/05/2010	Stable	2.05 (54%)	AZI, COL		*P. aeruginosa*
	708	M	23	F508del/F508del	24/06/2010	Stable	2.0(53%)	AZI, COL		*P. aeruginosa* x 2/ Candida sp.
8	801	M	19	F508del/F508del	13/03/2009	Stable	1.87 (46%)	AZI, COL FLU		No bacterial pathogens / Candida sp.
	802	M	19	F508del/F508del	11/11/2009	Routine I.V.	1.91 (47%)	AZI, COL FLU	CEF/TOB	No bacterial pathogens / Exophiala sp.
	803	M	19	F508del/F508del	30/12/2009	Stable	2.05 (50%)	AZI, COL FLU		*P. aeruginosa*
	804	M	19	F508del/F508del	11/02/2010	Routine I.V.	1.98 (48%)	AZI, COL FLU	CEF/TOB	No bacterial pathogens / Exophiala sp. / Candida sp.
	805	M	19	F508del/F508del	07/04/2010	Stable	2.05 (50%)	AZI, COL FLU		No bacterial pathogens
	806	M	19	F508del/F508del	30/06/2010	stable	1.90 (59%)	AZI, COL FLU		No bacterial pathogens
9	901	F	19	F508del/F508del	05/03/2009	Exacerbation	1.99 (74%)	AZI, COL FLU	CEF/MER	*P. aeruginosa* x 2
	902	F	19	F508del/F508del	01/04/2009	Stable	1.95 (73%)	AZI, COL FLU		*P. aeruginosa*
	903	F	19	F508del/F508del	04/06/2009	Stable	1.75 (66%)	AZI, COL FLU		*P. aeruginosa*
	904	F	19	F508del/F508del	05/08/2009	Routine I.V.	1.81 (68%)	AZI, COL FLU	CEF/COL	*P. aeruginosa*
	905	F	19	F508del/F508del	16/09/2009	Stable	1.75 (66%)	AZI, COL FLU		*P. aeruginosa*
	906	F	19	F508del/F508del	14/10/2009	Routine I.V.	1.71 (64%)	AZI, COL FLU	CEF/COL	*P. aeruginosa*

Longitudinal Patient Demographics continued (Part 5)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
9	907	F	19	F508del/F508del	06/01/2010	Routine I.V.	1.88 (71%)	AZI, COL FLU	CEF/COL	*P. aeruginosa* x 3
	908	F	19	F508del/F508del	24/02/2010	Stable	2.05 (72%)	AZI, COL FLU		*P. aeruginosa*
	909	F	19	F508del/F508del	21/04/2010	Stable	2.00 (72%)	AZI, COL FLU		*P. aeruginosa*
	910	F	19	F508del/F508del	26/05/2010	stable	2.10 (79%)	AZI, COL FLU		*P. aeruginosa*
	911	F	19	F508del/F508del	07/06/2010	Exacerbation	1.90 (71%)	AZI, COL FLU	CEF/COL	*P. aeruginosa*
10	1001	M	20	F508del/OM	11/03/2009	Exacerbation	2.30 (49%)	AZI, CIP, COL	TAZ/TOB	*P. aeruginosa* / MRSA
	1002	M	20	F508del/OM	04/06/2009	Exacerbation	2.40 (52%)	AZI, CIP	CEF/COL	*P. aeruginosa* / MRSA
	1003	M	20	F508del/OM	30/07/2009	Stable	3.24 (74%)	AZI, COL		*P. aeruginosa* / MRSA
	1004	M	20	F508del/OM	09/09/2009	Exacerbation	2.65 (61%)	AZI, COL		*P. aeruginosa* x 2 / Candida sp.
	1005	M	20	F508del/OM	16/11/2009	Exacerbation	Unable to do	AZI, COL	CEF/TOB	*P. aeruginosa* / MRSA / Candida sp.
	1006	M	20	F508del/OM	18/11/2009	Exacerbation	Unable to do	AZI, COL		*P. aeruginosa* / MRSA
	1007	M	20	F508del/OM	13/01/2010	Exacerbation	2.28 (49%)	AZI, COL	CEF/TOB	*P. aeruginosa* / MRSA
	1008	M	20	F508del/OM	08/02/2010	Stable	2.74 (66%)	AZI, COL		*P. aeruginosa*
	1009	M	20	F508del/OM	12/04/2010	Exacerbation	2.45 (52%)	AZI, COL	CEF/COL	*P. aeruginosa* / MRSA / Candida sp.
	1010	M	20	F508del/OM	07/05/2010	Stable	2.75 (66%)	AZI, COL		MRSA
11	1101	M	26	F508del/F508del	15/04/2009	Exacerbation	1.54 (41%)	AZI, FLU	CEF/TOB	*S. aureus* / *B. multivorans*
	1102	M	26	F508del/F508del	14/10/2009	Exacerbation	1.60 (43%)	AZI, FLU	CEF/TOB	*S. aureus* / *H. influenzae*
	1103	M	26	F508del/F508del	26/10/2009	Stable	1.60 (43%)	AZI, FLU, CEF, TOB		No bacterial pathogens / *A. fumigatus*

Longitudinal Patient Demographics continued (Part 6)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
11	1104	M	26	F508del/F508del	02/12/2009	Stable	1.94 (52%)	AZI, FLU, CEF, TOB		No bacterial pathogens / Candida sp.
	1105	M	26	F508del/F508del	18/01/2010	Stable	2.21 (59%)	AZI, FLU		*B. multivorans* / Candida sp.
	1106	M	26	F508del/F508del	01/03/2010	Exacerbation	1.95 (52%)	AZI, FLU	CEF/TOB	*S. aureus* / *B. multivorans* / *Candida sp.*
	1107	M	26	F508del/F508del	12/04/2010	Stable	1.70 (45%)	AZI, FLU		*S. aureus* / *B. multivorans*
	1108	M	26	F508del/F508del	11/05/2010	Routine IV	2.15 (57%)	AZI, FLU	CEF/TOB	No bacterial pathogens
	1109	M	26	F508del/F508del	23/06/2010	Stable	2.16 (57%)	AZI, FLU		*S. aureus*
12	1201	F	56	F508del/OM	15/04/2009	Stable	1.04 (35%)	AZI		MRSA / Candida sp.
	1202	F	56	F508del/OM	03/06/2009	Stable	1.10 (35%)	AZI		MRSA / Candida sp.
	1203	F	56	F508del/OM	02/10/2009	Exacerbation	1.05 (35%)	AZI	CEF/TOB	MRSA / *A. fumigatus*
	1204	F	56	F508del/OM	09/12/2009	Stable	1.10 (35%)	AZI		MRSA / Candida sp.
	1205	F	56	F508del/OM	17/02/2010	Stable	0.94 (31%)	AZI		MRSA / Candida sp.
	1206	F	56	F508del/OM	07/04/2010	Routine I.V.	1.25 (41%)	AZI		MRSA / Candida sp. / *A. fumigatus*
	1207	F	56	F508del/OM	02/06/2010	Stable	1.30 (43%)	AZI	DOX (Oral) I.V. CEF/TOB	No bacterial pathogens
13	1301	M	56	F508del/OM	22/04/2009	Stable	1.01 (28%)	AZI		*P. aeruginosa* / Candida sp. / Mycobacterium sp.
	1302	M	56	F508del/OM	15/07/2009	Routine I.V.	0.90 (25%)	AZI	CEF/TOB	*P. aeruginosa* x 3 / *Ochrobactrum anthropi* / *Mycobacterium chelonae*
	1303	M	56	F508del/OM	24/09/2009	Exacerbation	0.85 (24%)	AZI	CEF	*P. aeruginosa* / *C. albicans* / Mycobacterium sp.

Longitudinal Patient Demographics continued (Part 7)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
13	1304	M	56	F508del/OM	02/12/2009	Exacerbation	0.88 (25%)	AZI	CEF	*P. aeruginosa* x 5 / *C. albicans* / Mycobacterium sp.
	1305	M	56	F508del/OM	31/12/2009	Stable	0.85 (24%)	AZI		*P. aeruginosa* x 3 / *C. albicans* / *Mycobacterium chelonae*
	1306	M	56	F508del/OM	07/01/2010	Exacerbation	0.80 (23%)	AZI	CEF	*P. aeruginosa* x 2 / *C. albicans* / *Mycobacterium chelonae*
14	1401	F	19	F508del/OM	05/05/2009	Stable	4.37 (104%)	AZI, COL FLU		No bacterial pathogens / Candida sp.
	1402	F	19	F508del/OM	06/01/2010	Exacerbation	3.92 (93%)	AZI, COL FLU	CIP (Oral)	*Morganella morganii* / *A. fumigatus*
	1403	F	19	F508del/OM	12/02/2010	Stable	4.58 (109%)	AZI, COL FLU		*P. aeruginosa* / Candida sp. / *A. fumigatus*
	1404	F	19	F508del/OM	27/05/2010	Stable	4.20 (102%)	AZI, COL FLU		No bacterial pathogens / Candida sp.
15	1501	F	59	F508del/OM	13/05/2009	Stable	0.85 (33%)	AZI, COL		*P. aeruginosa* / Candida sp.
	1502	F	59	F508del/OM	12/08/2009	Routine I.V.	0.95 (37%)	AZI, COL	CEF/TOB	*P. aeruginosa*
	1503	F	59	F508del/OM	23/09/2009	Stable	0.85 (33%)	AZI, COL, ITR		*P. aeruginosa*
	1504	F	59	F508del/OM	28/10/2009	Exacerbation	0.86 (33%)	AZI, COL, ITR	CEF/TOB	*P. aeruginosa* / Candida sp.
	1505	F	59	F508del/OM	06/01/2010	Stable	1.00 (39%)	AZI, COL		*P. aeruginosa* / *Morganella morganii* / Candida sp.
	1506	F	59	F508del/OM	17/02/2010	Stable	0.99 (38%)	AZI, COL		*P. aeruginosa* x 2 / Candida sp.
	1507	F	59	F508del/OM	18/03/2010	Routine I.V.	0.96 (37%)	AZI, COL	CEF/TOB	*P. aeruginosa*
	1508	F	59	F508del/OM	24/06/2010	Stable	1.00 (39%)	AZI, COL		*P. aeruginosa* / Candida sp.

Longitudinal Patient Demographics continued (Part 8)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
16	1601	F	23	F508del/F508del	13/05/2009	Stable	1.67 (60%)	AZI, COL FLU		*P. aeruginosa* / *S. aureus* / Candida sp.
	1602	F	23	F508del/F508del	27/05/2009	Exacerbation	0.74 (26%)	AZI, COL FLU	CEF/TOB	*P. aeruginosa* / *S. aureus* / Candida sp.
	1603	F	23	F508del/F508del	15/07/2009	Exacerbation	1.42 (51%)	AZI, COL FLU	CEF/TOB	*P. aeruginosa*
	1604	F	23	F508del/F508del	21/10/2009	Stable	1.25 (45%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1605	F	23	F508del/F508del	21/04/2010	Stable	1.46 (52%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1606	F	23	F508del/F508del	12/05/2010	Exacerbation	1.50 (53%)	AZI, COL FLU	CEF/TOB	*P. aeruginosa* / *A. fumigatus*
	1607	F	23	F508del/F508del	27/05/2010	Stable	1.21 (43%)	AZI, COL FLU		*P. aeruginosa* / Candida sp. / *A. fumigatus*
17	1701	F	39	F508del/F508del	08/04/2009	Stable	1.00 (34%)	COL, FLU		*P. aeruginosa*
	1702	F	39	F508del/F508del	05/08/2009	Stable	0.96 (33%)	COL, FLU		*P. aeruginosa* / Candida sp.
	1703	F	39	F508del/F508del	12/10/2009	Exacerbation	1.05 (36%)	COL, FLU	CEF/TOB	*P. aeruginosa*
	1705	F	39	F508del/F508del	02/12/2009	Exacerbation	0.87 (30%)	AZI, COL	CEF/TOB	*P. aeruginosa* x 2 / Candida sp. / *C. albicans*
	1706	F	39	F508del/F508del	14/04/2010	Stable	0.95 (32%)	AZI, COL		*P. aeruginosa* / Candida sp.
18	1801	M	39	F508del/F508del	20/05/2009	Stable	2.20 (53%)	AZI, COL FLU		*P. aeruginosa* / *S. aureus* / *A. fumigatus*
	1802	M	39	F508del/F508del	15/07/2009	Stable	2.41 (58%)	AZI, COL FLU		*P. aeruginosa* / *S. aureus* / *A. fumigatus*
	1803	M	39	F508del/F508del	16/09/2009	Stable	2.31 (55%)	AZI, COL FLU		*P. aeruginosa* / Candida sp. / *A. fumigatus*
	1804	M	39	F508del/F508del	11/11/2009	Stable	2.22 (53%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*

Longitudinal Patient Demographics continued (Part 9)

DGGE Patient No.	Code	Sex	Age	Genotype	Date collected	Exacerbation	FEV1 (% predicated)	Routine antibiotics	I.V. Antibiotics	Recent History
18	1805	M	39	F508del/F508del	02/12/2009	Routine I.V.	2.18 (52%)	AZI, COL FLU	CEF/TOB	*P. aeruginosa* / Candida sp. / *A. fumigatus*
	1806	M	39	F508del/F508del	20/01/2010	Stable	2.26 (54%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1807	M	39	F508del/F508del	10/03/2010	Stable	1.99 (48%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1808	M	39	F508del/F508del	21/04/2010	Stable	2.25 (54%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1809	M	39	F508del/F508del	03/06/2010	Stable	2.40 (57%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*
	1810	M	39	F508del/F508del	01/07/2010	Stable	2.30 (55%)	AZI, COL FLU		*P. aeruginosa* / *A. fumigatus*

Microbial Community Analysis (Part 1)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
1	101	1.58	16	0.57	0
	102	2.01	19	0.68	1
	103	1.92	17	0.68	1
	104	1.93	17	0.68	1
	105	2.16	21	0.71	1
	106	2.26	18	0.78	0
	107	2.00	19	0.68	0
	108	2.20	18	0.76	0
	109	1.85	15	0.68	1
	110	2.23	18	0.77	1
	111	2.29	19	0.78	1
	112	1.80	11	0.75	0
2	201	2.13	11	0.89	0
	202	2.29	16	0.83	0
	203	2.17	16	0.78	0
	204	2.23	17	0.79	2
	205	2.42	17	0.85	1
	206	1.87	16	0.67	0
	207	2.13	13	0.83	0
	208	1.82	10	0.79	0
	209	1.77	14	0.67	0
	210	1.21	9	0.55	3
	211	1.85	10	0.81	3
	212	2.01	13	0.78	0
	213	2.10	13	0.82	3
	214	1.80	12	0.72	0
	215	2.03	16	0.73	1

Microbial Community Analysis continued (Part 2)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
3	301	2.40	19	0.81	0
	302	2.18	17	0.77	1
	303	2.48	21	0.82	1
	304	2.49	21	0.82	0
	305	2.34	17	0.83	0
	306	2.16	14	0.82	1
	307	1.90	10	0.82	0
4	401	2.11	13	0.82	2
	402	2.20	13	0.86	1
	403	2.45	17	0.86	1
	404	2.04	13	0.79	1
	405	2.01	13	0.78	0
	406	2.28	17	0.81	1
	407	2.09	15	0.77	2
5	501	2.02	19	0.69	1
	502	1.86	15	0.69	0
	503	2.12	17	0.75	0
	504	1.98	17	0.70	0
	505	1.95	21	0.64	0
	506	2.06	22	0.67	0
	507	2.38	20	0.80	0

Microbial Community Analysis continued (Part 3)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
6	601	2.36	17	0.83	0
	602	2.11	18	0.73	1
	603	2.22	17	0.78	0
	604	2.11	18	0.73	0
	605	2.10	20	0.70	0
	606	2.33	19	0.79	0
	607	2.73	27	0.83	0
	608	2.21	17	0.78	0
	609	1.97	16	0.71	0
	610	2.53	17	0.89	0
7	701	1.83	18	0.63	2
	702	2.21	16	0.80	2
	703	1.96	15	0.72	1
	704	1.31	12	0.53	1
	705	1.09	9	0.49	0
	706	0.56	8	0.27	0
	707	1.03	11	0.43	0
	708	1.45	16	0.52	0
8	801	1.87	11	0.78	4
	802	1.89	14	0.71	4
	803	2.21	15	0.82	0
	804	1.98	17	0.70	0
	805	2.18	16	0.79	0
	806	2.01	17	0.71	0

Microbial Community Analysis continued (Part 4)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
9	901	2.58	24	0.81	0
	902	2.44	18	0.84	1
	903	2.63	24	0.83	2
	904	2.59	23	0.82	0
	905	2.59	25	0.80	0
	906	2.42	21	0.79	1
	907	2.50	19	0.85	2
	908	2.23	20	0.74	0
	909	1.85	13	0.72	0
	910	2.38	16	0.86	0
	911	1.70	10	0.74	0
10	1001	2.33	20	0.78	0
	1002	2.42	25	0.75	0
	1003	2.51	31	0.73	0
	1004	2.79	31	0.81	0
	1005	2.39	21	0.79	1
	1006	2.30	25	0.72	0
	1007	2.65	26	0.81	0
	1008	2.46	25	0.76	0
	1009	2.61	25	0.81	1
	1010	2.69	26	0.83	0

Microbial Community Analysis continued (Part 5)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
11	1101	1.84	19	0.62	0
	1102	2.57	23	0.82	0
	1103	2.37	20	0.79	1
	1104	2.78	23	0.89	1
	1105	2.65	23	0.85	0
	1106	2.79	22	0.90	0
	1107	2.90	24	0.91	0
	1108	2.68	25	0.83	0
	1109	2.23	17	0.79	0
12	1201	2.19	17	0.77	1
	1202	2.23	20	0.74	0
	1203	2.22	19	0.75	2
	1204	2.31	19	0.78	1
	1205	2.28	27	0.69	1
	1206	2.26	21	0.74	1
	1207	2.25	21	0.74	1
13	1301	1.82	19	0.62	0
	1302	2.11	22	0.68	1
	1303	2.25	21	0.74	1
	1304	2.26	25	0.70	2
	1305	2.08	19	0.71	1
	1306	2.36	19	0.80	1

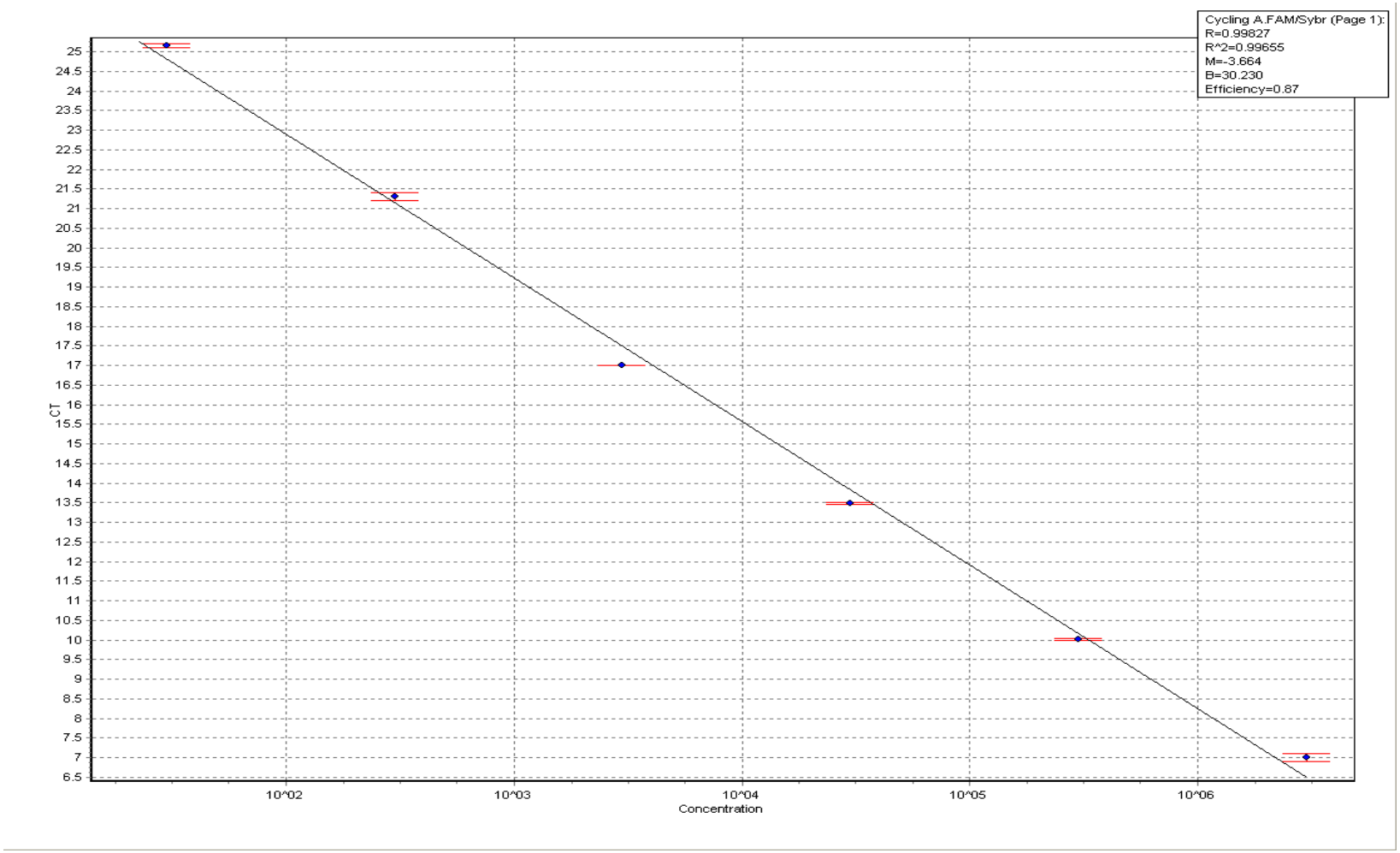
Microbial Community Analysis continued (Part 6)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
14	1401	2.33	19	0.79	0
	1402	2.52	25	0.78	1
	1403	2.60	25	0.81	0
	1404	2.58	25	0.80	1
15	1501	1.65	13	0.64	0
	1502	1.54	11	0.64	0
	1503	2.25	14	0.85	0
	1504	1.92	17	0.68	0
	1505	1.33	7	0.69	0
	1506	2.06	17	0.73	0
	1507	1.55	12	0.63	0
	1508	1.85	14	0.70	0
16	1601	2.40	22	0.78	2
	1602	2.70	27	0.82	2
	1603	2.59	24	0.81	0
	1604	2.37	23	0.76	0
	1605	2.52	27	0.77	0
	1606	2.42	20	0.81	0
	1607	2.22	17	0.78	0
17	1701	2.03	11	0.84	1
	1702	1.74	11	0.73	2
	1703	1.92	13	0.75	2
	1704	1.96	13	0.76	2
	1705	2.04	13	0.80	1

Microbial Community Analysis continued (Part 7)

Patient	Code	Shannon Diversity	Bacterial Richness	Bacterial Evenness	Fungal Richness
18	1801	2.74	22	0.89	0
	1802	2.71	18	0.94	1
	1803	2.61	18	0.90	0
	1804	2.24	15	0.83	1
	1805	2.52	17	0.89	1
	1806	2.64	19	0.90	0
	1807	2.54	19	0.86	0
	1808	2.57	19	0.87	0
	1809	2.66	19	0.90	1
	1810	2.60	18	0.90	1

Example of Eubacterial qPCR standard curve



Appendix 13 - List of accompanying material

Nelson A, De Soyza A, Bourke S J, Perry J D, Cummings S P (2010) Assessment of sample handling practices on microbial activity in sputum from patients with cystic fibrosis Letters in Applied Microbiology 51:272 - 277