Genotyping of *Pseudomonas aeruginosa* isolates from pulmonary infections in non-cystic fibrosis bronchiectasis patients

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Abstract

Non-cystic fibrosis bronchiectasis (NCFBr) is a chronic, progressive respiratory disease characterised by irreversible widening of the airways and thickening of the bronchial walls. There have been large amounts of research into cystic fibrosis (CF) and very little into NCFBr, despite its prevalence in the UK having been predicted to be >10 times that of CF. The leading cause of mortality in CF is chronic bacterial lung infection, particularly with *Pseudomonas aeruginosa*. Transmissible strains of *P. aeruginosa* are associated with increased morbidity and mortality in CF. Chronic *P. aeruginosa* infections are also known to adversely affect the health of NCFBr patients although very little in-depth research has been performed. This work aims to investigate the diversity of populations of *P. aeruginosa* in NCFBr patients attending bronchiectasis services throughout England and Wales. The maintenance of *P. aeruginosa* strains in long-term infections in the lungs of NCFBr patients were investigated using an array tube genotyping system. The majority (15) of the 20 patients studied were found to maintain the same strain of *P. aeruginosa* throughout the course of infection. The population structure of *P. aeruginosa* isolates from NCFBr patients was investigated using both the array tube system and whole genome sequencing. The population of isolates from NCFBr patients was found to be distributed evenly throughout the general population of *P. aeruginosa*. Whole genome sequencing was also used to investigate the diversity of *P. aeruginosa* isolates from 23 individual patients and it was shown that in the majority of these (16) that all isolates were the same strain and closely related to one another. Only one of the 157 NCFBr patients was found to harbour a transmissible strain of *P. aeruginosa* which is a significantly lower prevalence than in CF. There is still a large disparity between our knowledge regarding CF and NCFBr and so further research into *P. aeruginosa* infections in NCFBr is required.

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Contributions

Provision of *Pseudomonas aeruginosa* isolates from England and Wales
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Provisions of *Pseudomonas aeruginosa* isolates from Liverpool centre
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Polymerase chain reaction assays
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Array tube genotyping
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Core genome extraction
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Construction of phylogenetic tree

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Variant calling

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Abbreviations

AES	Australian epidemic strain
ART	antiretroviral therapy
AT	Array Tube
ATS	American Thoracic Society
BCC	*Burkholderia cepacia* complex
BCOS	COPD-bronchiectasis overlap syndrome
BMI	body mass index
bp	base pair
BSI	bronchiectasis severity index
BTS	British Thoracic Society
CAP	community-acquired pneumonia
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
CRK	CT10 regulator of kinase
CT	computed tomography
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
ERS	European Respiratory Society
FEV_1	forced expiratory volume in 1 second
GATK	Genome Analysis Toolkit
gDNA	genomic DNA

GER	gastro-oesophageal reflux
HIV	human immunodeficiency virus
HRCT	high resolution computed tomography
HSL	homoserine lactone
IPC	International *Pseudomonas aeruginosa* Consortium
LB	Luria broth
LES	Liverpool epidemic strain
MES	Manchester epidemic strain
MGEs	mobile genetic elements
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MLVA	multiple-locus variable number tandem repeat analysis
MRC	Medical Research Council
MRCA	most recent common ancestor
MRSA	methicillin-resistant *Staphylococcus aureus*
NCFBr	non-cystic fibrosis bronchiectasis
NDK	nucleoside diphosphate kinase
NGS	next generation sequencing
NTHi	nontypable *Haemophilus influenzae*
NTM	non-tuberculosis mycobacterium
PCR	polymerase chain reaction
PES	Prairie Epidemic Strain
PFGE	pulsed-field gel electrophoresis
QS	quorum sensing
RAPD-PCR	random amplified polymorphic DNA fingerprinting-PCR

RGP	regions of genomic plasticity
ST	sequence type
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
TB	tuberculosis
TLR	toll-like receptor
UV	ultraviolet
VAP	ventilator associated pneumonia
VNTR	variable number tandem repeat

Chapter One

Introduction

1.1 Bronchiectasis

Bronchiectasis is a chronic and progressive respiratory disease with which patients often suffer from a persistent cough, sputum production, and recurrent microbial infections leading to exacerbations and further lung damage. Bronchiectasis is defined by the British Thoracic Society (BTS) by the irreversible dilation and damage to the bronchi (Pasteur *et al.* 2010) and is often also characterised by thickening of the bronchial walls. Disease progression can occur through damage to the lungs and airways which most often occurs during chronic bacterial infection when the airways are constantly inflamed, but can occur due to other factors such as smoking and particle inhalation. The causes of bronchiectasis are wide ranging, including genetic disorders and environmental factors. The most well characterised and intensively studied cause of bronchiectasis is cystic fibrosis (CF). Other chronic conditions are also associated with bronchiectasis including rheumatoid arthritis, inflammatory bowel disease, and gastro-oesophageal reflux (GER) (De Soyza *et al.* 2013) however the effects of these conditions on the health of bronchiectasis patients is little known due to lack of research into the condition. Bronchiectasis is perceived by many, since the further development of antibiotic treatments, to be a rare and easily managed disease, of little clinical importance (De Soyza *et al.* 2013) although in recent times it is becoming clearer that this is not the case. It is possible that research into bronchiectasis has been neglected due to belief that the disease would disappear in developing countries alongside the decline of diseases such as tuberculosis (Chalmers 2015). Accurate data on the numbers of patients currently undergoing regular treatment for bronchiectasis are sparse; in the USA the prevalence of adult bronchiectasis has been estimated at 52 in 100 000 people, with higher prevalence among women and older individuals (Weycker *et al.* 2005); however in the UK, in just nine centres, there are 5000 patients receiving

regular follow-up treatment for bronchiectasis (De Soyza *et al.* 2013) suggesting that the true prevalence among adult populations may be even higher.

1.1.1 Cystic fibrosis

CF is perhaps the most well-known and best-understood cause of bronchiectasis and is the most common life-threatening hereditary genetic disorder among Caucasians. In 1990 the median survival age for CF patients was 31.9 years but improvements in patient care and treatment have led to an increased median survival age of 49.7 years in the most current five year period (ending in 2012) (Stephenson *et al.* 2015). CF is an autosomal recessive condition in which a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene leads to improper transport of chloride ions. The most prevalent causative mutation is a deletion of three base pairs in the CFTR gene which leads to the loss of a phenylalanine residue at amino acid position 508, known as ΔF508 (Kerem *et al.* 1989; Riordan *et al.* 1989; Rommens *et al.* 1989). Despite chronic airway infections being a hallmark of the condition, CF is defined as a multi-organ disease, affecting the gastrointestinal tract, pancreas, and the reproductive tract as well as the respiratory tract (Pauwels *et al.* 2012). Due to abnormal CFTR protein produced as a result of the mutation in the CFTR gene, the airway mucus becomes thick and sticky and is not easily cleared by cilial beating. Clearing of the mucus is crucial in preventing airway infections and so build-up of mucus provides an ideal environment for microbial pathogens to colonise. Antibiotics are often unable to properly diffuse in the viscous mucus and so these infections are difficult to clear, leading to prolonged inflammatory response by the immune system. This extended immune response leads to damage and widening of the airways, leading to bronchiectasis.

1.1.2 Non-cystic fibrosis bronchiectasis

There are a range of underlying causes that can lead to development of non-cystic fibrosis bronchiectasis (NCFBr), although it is estimated that in 25 – 50% of cases a cause is not identified (http://www.blf.org.uk/page/bronchiectasis); this is known as idiopathic bronchiectasis. Advances in diagnostics mean that patients with CF are often diagnosed shortly after birth and healthcare regimens can be

implemented early on to extend life expectancy. Symptoms of NCFBr often do not manifest until much later in life and are more difficult to definitively diagnose with the average age of many NCFBr cohorts being between 60 and 70 years (Chalmers 2015). Post-infective bronchiectasis is often caused by severe childhood respiratory infections such as pneumonia, whooping cough, and measles; however, vaccination against childhood illnesses is expected to reduce post-infective bronchiectasis in adults in the future (NHS Choices 2015). Immunodeficiency can also leave the lungs vulnerable to damage by the inflammatory response. In the past, there was high mortality in HIV-infected patients who presented with acute pulmonary infections such as bacterial pneumonia, tuberculosis, and acute bronchitis but the arrival of early antiretroviral therapy (ART), widespread prophylactic antibiotic treatment and improvements in life expectancy have seen a significant reduction in the incidence of life-threatening opportunistic infections, resulting in an increase in non-infectious chronic respiratory disease among HIV-positive individuals, including chronic obstructive pulmonary disease (COPD) and NCFBr (Calligaro & Gray 2015). There is also evidence that aspiration of gastric reflux products may play a role in the development on bronchiectasis, although the links between GER and CF have been more extensively studied than the links between NCFBr and GER (Pauwels *et al.* 2012).

Problems often arise in epidemiological studies of NCFBr with acquiring definitive diagnoses of the condition. NCFBr is most commonly diagnosed by the presence of dilated bronchi apparent on a computed tomography (CT) scan, and this is known as radiological bronchiectasis, but studies have shown that dilated bronchi are commonplace among the healthy elderly population. A UK study of patients aged >75 years vs. patients under the age of 55 identified bronchial dilation in 60% of the patients aged 75 and over compared with presence in only 6% of the younger group (Copley *et al.* 2009). This study included both smokers and non-smokers but revealed no relationship between smoking status or history and bronchial dilation (Copley *et al.* 2009). A further study examining CT scans of the lungs of elderly never-smokers with no respiratory disease revealed that 19.1% of over 75's had radiological bronchiectasis (Winter *et al.* 2015). These data have led to the speculation that

bronchiectasis may, in part, be a sign of aging in the lungs; however other symptoms generally expressed by NCFBr patients include chronic cough, sputum production and respiratory tract infections. This has led to some clinicians to use the term "clinically significant bronchiectasis" to refer to patients who present with the symptoms listed above alongside radiological bronchiectasis (Chalmers 2015).

Similarities and overlap between similar pulmonary diseases also hinder conclusive diagnosis of NCFBr. The overlap between bronchiectasis and COPD has not been extensively studied and work carried out so far does not present a conclusive assessment of risk of bronchiectasis and COPD comorbidity (Chalmers 2015). COPD is diagnosed on the basis of mostly-irreversible airflow obstruction which is measured by spirometry and is therefore a physiological diagnosis. Bronchiectasis is diagnosed by imaging (usually CT) which shows permanent airway dilation and thickening of the airway walls which results in a structural diagnosis (Hurst *et al.* 2015). Increasingly, COPD patients are given a CT scan as a part of diagnosis or follow-up care and consequently there is an impact on the detection of structural changes in the airways of COPD patients. The presence of symptoms of both conditions (figure 1.1) has been given the name COPD-bronchiectasis overlap syndrome (BCOS) and recommendations for the treatment of BCOS and improvements in research have recently been made (Hurst *et al.* 2015). A small study previously reported high frequencies of bronchiectasis among those diagnosed with COPD. In a cohort of 201 patients with COPD the frequency of bronchiectasis was reported at 57.6%, with higher mortality among those with bronchiectasis and COPD (Martínez-García *et al.* 2013). However, this high frequency of bronchiectasis has not been corroborated by larger-scale studies. In one study the frequency of bronchiectasis among COPD patients was found to be no higher than that which would be expected among the general population of otherwise-healthy individuals due to the background frequency of bronchiectasis among the elderly (Agusti *et al.* 2010) and this was echoed by later research which found no higher incidence of bronchiectasis in those with COPD than in those with no reported COPD diagnosis; bronchiectasis was reported in 26% of patients with no known diagnosis of pulmonary disease (Jairam *et al.* 2015). Research from the COPDGene study, which

investigated a large cohort of 3636 patients, reported the frequency of bronchiectasis among COPD patients at 20.8% (Stewart *et al.* 2012). A more recent study which focused on patients with high levels of exacerbation found only five cases of bronchiectasis among 96 COPD patients (Uzun *et al.* 2014). These variations in frequencies indicate that the research carried out into BCOS thus far is not robust enough to allow predictions to be made about the frequency of bronchiectasis among COPD patients, although it is probably that it occurs in a maximum of 20% patients (Chalmers 2015).

In the US a research registry was founded in 2008 to give a centralised database of NCFBr patients from clinical institutions across the country. The aim of the registry is to encourage research collaborations and multi-centre clinical trials for the treatment of NCFBr. The demographics of the registry are typical of NCFBr patients; predominantly female (79%), white (91%), with an average age of 65 years (Aksamit *et al.* 2012).

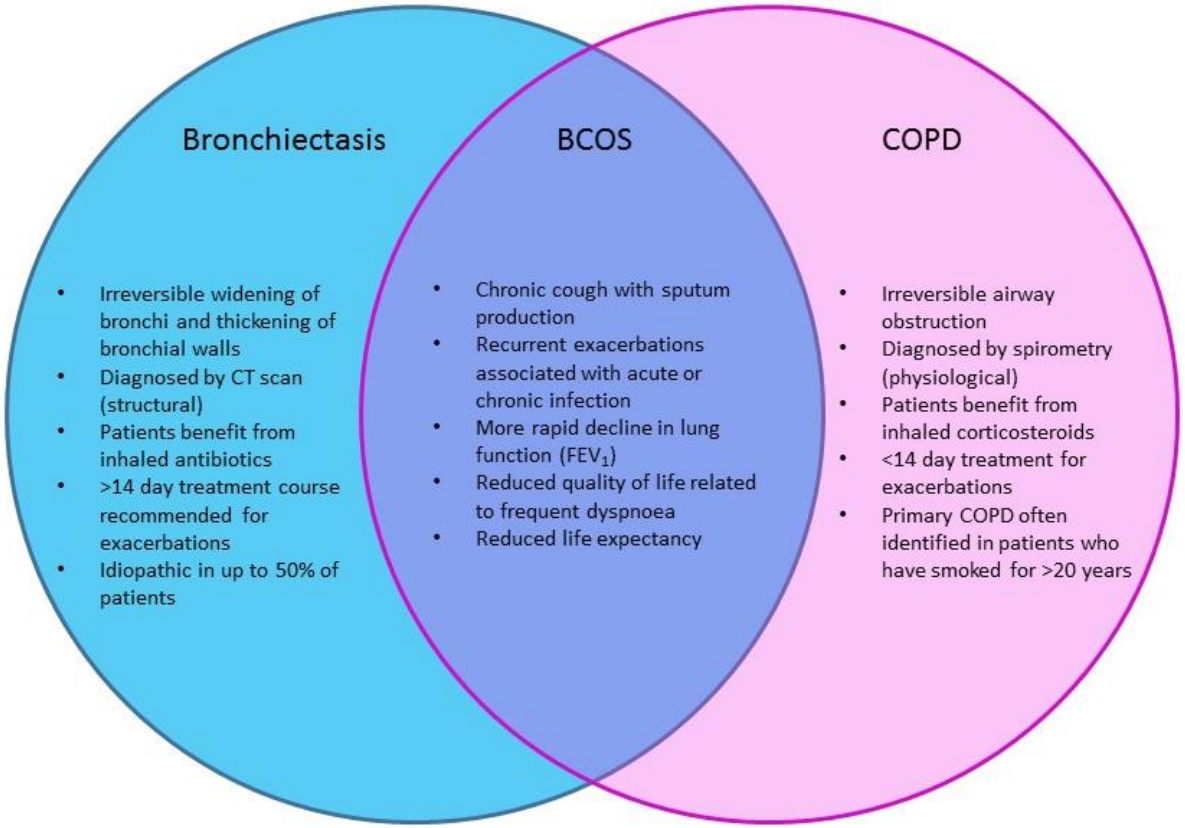


Figure 1.1: Venn diagram illustrating key symptoms and diagnoses of bronchiectasis and COPD, and those found in BCOS.

1.1.3 Lack of research into non-cystic fibrosis bronchiectasis

In the UK prevalence of NCFBr is much higher than that of CF with data suggesting that, of the estimated 150 000 patients living with bronchiectasis (Quint *et al.* 2012), only around 10 000 are due to CF. Despite this, there has been very limited NCFBr research and there are currently no specific licenced drug therapies for the condition (Pasteur *et al.* 2010). The world's first national guidelines for NCFBr diagnosis and treatment were developed in the UK in 2010 by the BTS. Research into NCFBr has been limited partly by lack of funding but also by lack of carefully phenotyped patients. With such a wide range of causes and severities of symptoms, along with other conditions with similar symptoms (e.g. COPD), it can be difficult to strictly categorise NCBFr patients. The BTS Guideline for NCFBr makes recommendations for the diagnosis and treatment of NCFBr in both adults and children (Pasteur *et al.* 2010). The report provides rigorous and detailed testing guidelines for patients exhibiting symptoms of bronchiectasis. The report also identified significant gaps in evidence at discovery (first translational gap) and clinical interfaces (second translational gap) (Pasteur *et al.* 2010). In 2012 the BTS Quality Standards for clinically significant bronchiectasis was published and is recommended to be read alongside the Guideline for NCFBr. The Quality Standards report aims to allow healthcare professionals to make the best decisions regarding diagnosis and treatment based on the latest evidence and best practices. The document also aims to help NCBFr patients and carers understand what services they should expect from their healthcare provider (British Thoracic Society 2012).

In CF patients chronic bacterial infections in the lungs, particularly with *Pseudomonas aeruginosa,* are significant contributors to morbidity and mortality (Fothergill *et al.* 2010a). It has been demonstrated in many studies that there is an association between *P. aeruginosa* infection and mortality, lowered lung function, and worsening quality of life in NCFBr patients (Evans *et al.* 1996; Davies *et al.* 2006; King *et al.* 2007; Loebinger *et al.* 2009). A more rapid decline in lung function associated with *P. aeruginosa* infection was suggested by Evans *et al.* (1996) and Davies *et al.* (2006) although this is not universally acknowledged. None of these

studies have proven, however, that the prognostic effect of *P. aeruginosa* infection is as a direct consequence of infection rather than as a marker of existing disease severity (Chalmers 2015).

1.1.4 Prevalent microbial pathogens

Due to the permanent inflammation and dilation of the airways of NCFBr patients and the associated reduction in host defences, 64 – 79% of NCFBr patients have chronic bacterial airway infections even when the patients are apparently clinically stable (Pasteur *et al.* 2000; Angrill *et al.* 2002; King *et al.* 2007). *Haemophilus influenzae* and *P. aeruginosa* are the most common pathogens affecting NCFBr patients. *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Staphylococcus aureus* are also often isolated from sputum samples, although Pasteur *et al.* (2000) suggested that the presence of *S. aureus* among the lung microbiota may be indicative of undiagnosed CF and advised that clinicians should follow *S. aureus* isolation with genetic and sweat tests. Alongside bacterial pathogens, viruses and fungi also play a pathogenic role in NCFBr patients. Common respiratory viruses (*e.g.* human rhinovirus, respiratory syncytial virus, and influenza A and B) are detected more frequently in patients suffering chronic respiratory diseases, such as COPD and NCFBr, compared to healthy individuals (Mitchell *et al.* 2015) and that the prevalence of viral infections in cases of NCFBr exacerbations is higher than in clinically stable bronchiectasis (Gao *et al.* 2015). It has been shown that patients experiencing exacerbations who tested positive for respiratory viruses by PCR assay also experienced a greater increase in markers of systemic and airway inflammation when compared with virus-negative patients experiencing exacerbations (Gao et al. 2015). Research into the prevalence and effects of fungal infection in NCFBr has been extremely limited and so much of our understanding of the role of fungi in chronic lung conditions is based on work undertaken in the context of CF. *Aspergillus* spp. and *Candida albicans* are known to persist frequently in the lungs of CF patients and so recently the prevalence of these organisms has also been investigated in NCFBr. In work undertaken by Máiz *et al.* (2015) it was shown that there is indeed a high prevalence of persistence of both *Aspergillus* spp. and *C. albicans* in clinically stable

NCFBr patients. The persistence of *Aspergillus* spp. and *C. albicans* were shown to be associated with daily sputum purulence and the administration of long-term antibiotic treatment respectively. Long-term antibiotic treatment is generally only utilised to treat chronic *P. aeruginosa* infection, as opposed to *H. influenzae*, and so persistence of *C. albicans* is found to be associated with *P. aeruginosa* colonisation in NCFBr patients (Máiz *et al.* 2015). *P. aeruginosa* has been shown to form dense biofilms on the hyphae of *C. albicans* without binding to or killing the fungi (Hogan & Kolter 2002). It is likely that the presence of bacteria, fungi, and viruses play a combinatorial role in pathogenesis in NCFBr and so further research into microbial pathogens is necessary.

H. influenzae strains can be categorised into one of two groups: typable and nontypable. Typable isolates have a capsule and generally cause systemic infections, such as bacteraemia and meningitis. Nontypable *H. influenzae* (NTHi) is unencapsulated and often causes non-invasive mucosal infections, such as otitis media, sinusitis, and conjunctivitis (Garmendia *et al.* 2014). In COPD colonisation with NTHi is a marker of disease progression and of severe airflow obstruction and it is for this reason that much of the research carried out into lower airway infections by NHTi has been undertaken with regards to COPD (Garmendia *et al.* 2014). In a study by McDonnell *et al.* (2015) *H. influenzae* was isolated from 89 (57.4%) of 155 COPD patients, although only 51 (32.9%) met colonisation criteria. Of these 51, 14 patients were still colonised at the data capture point. This data suggests that 37 patients (74.5%) had cleared *H. influenzae*, opposed to the 34.0% of patients who cleared *P. aeruginosa* (McDonnell *et al.* 2015).

NCFBr patients are frequently diagnosed with community-acquired pneumonia (CAP), although there are currently no specific clinical recommendations available (Polverino *et al.* 2015). It was found that *S. pneumoniae* was the most common causative pathogen of CAP both among NCFBr patients and among other CAP patients. Whilst these pneumococcal infections usually cause acute disease, they can also be accompanied by chronic infection with *P. aeruginosa* and this was shown to be common among NCFBr patients (Polverino *et al.* 2015).

Comprehensive taxonomic studies have defined several distinct, closely related species of bacteria among those previously designated as "*B. cepacia*" on the basis of phenotype alone (Vandamme *et al.* 1997). These seven distinct organisms (*B. cepacia* genomovars I – VII) are collectively known as the *B. cepacia* complex. Members of the *Burkholderia cepacia* complex (BCC) have been recognised as important opportunistic pathogen among CF patients for many years, with a significant proportion of infected patients succumbing rapidly to progressive necrotising pneumonia (LiPuma *et al.* 2001). In many cases, BCC infection is associated with poor outcomes following lung transplant and so many centres consider BCC infection an absolute contraindication to receive transplant lungs. However many CF patients are either transiently infected with BCC or can remain colonised for years with no apparent adverse clinical effects (LiPuma *et al.* 2001). Although infection with the BCC has been reported in patients with NCFBr (Ledson *et al.* 1998) it is relatively uncommon. In a study by Carraro *et al.* (2014) the BCC was identified in 14 patients of 47 (20 with bronchiectasis and 27 with CF). Of these 14 patients, 13 had CF showing that the BCC is more common among patients with CF than in those with NCFBr (Carraro *et al.* 2014).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a versatile Gram-negative bacterium that can live in a wide range of niche environments and can opportunistically colonise susceptible individuals to cause disease (Winsor *et al.* 2011). As well as being the most prevalent life-threatening pathogen in CF patients (Govan *et al.* 2007), *P. aeruginosa* frequently affects other immunocompromised individuals such as cancer and severe burn patients and is a major cause of bacterial keratitis which can lead to blindness if untreated (Winsor *et al.* 2011). It is notable that infection with *P. aeruginosa* is rare among otherwise healthy individuals and is most likely encountered and rebuffed by the body's immune system on a regular basis due to its abundance in the natural environment (Lyczak *et al.* 2000). *P. aeruginosa* is also a major cause of nosocomial infections worldwide, and is responsible for ~10% of such infection in the European Union (de Bentzmann & Plésiat 2011). The majority of these nosocomial infections

take the form of acute lung infections (pneumonia) in patients in intensive care units (Lyczak *et al.* 2000). The ability of the organism to colonise such a wide variety of environments and to cause a range of human diseases is now widely believed to be due to a multitude of virulence factors which act in a combinatorial fashion to cause injury to the host (Lee *et al.* 2006).

P. aeruginosa can cause both acute and chronic infections and has a wide range of virulence factors which are important in pathogenicity in acute infections and also in establishing chronic infections. Table 1.1 shows some of the most important virulence factors of *P. aeruginosa* and how they act upon host cells to cause damage. Generally, only a small selection of bacterial species are recovered from the wounds of burns victims, including: *S. aureus*, *Klebsiella* spp., *P. aeruginosa*, and *Enterobacter* spp. (Bowen-Jones *et al.* 1990). Despite increasing awareness of the dangers posed by bacterial infection and therefore the necessity for sterility in healthcare environments, *P. aeruginosa* has been found to contaminate floors, bed rails, and sinks in hospitals and this, along with the prevalence of *P. aeruginosa* in the general environment, makes it a threat to the health of burns patients recovering in hospitals (Chitkara & Feierabend 1981). It is quickly apparent that the external defences of the immune system are compromised at the site of a burn wound but due to the limited number of bacterial species which are recovered from infected wounds it is likely that it is necessary for the bacteria to be able to adapt to take advantage of the concerted impairment of many of the host defence mechanisms to colonise a burn, as opposed to the presence of one or a few specific virulence factors (Lyczak *et al.* 2000). *P. aeruginosa* is also one of the leading causes of ulcerative keratitis of the cornea and is associated with users of extended-wear contact lenses. The exact links between contact lens use and bacterial pathogenesis have not been fully elucidated, though work has shown that the ocular mucus, which usually binds bacterial cells and prevents adhesion to the corneal surface, can be altered by the use of contact lenses and may therefore facilitate bacterial adhesion, along with other virulence factors (Versura *et al.* 1987).

Table 1.1: Important virulence factors associated with acute *P. aeruginosa* infections, and establishment of chronic infections.

Virulence factor	**Secretion system**	**Host target**	**Role in pathogenicity**
Type IV pili	-	Cell surface	Major surface adhesin; signal transduction mechanism requires attachment of type IV pili to solid surface, retraction of pilus, and signal transduction through the Chp chemo-sensory system (activates cAMP production and transcription of hundreds of genes, including key virulence factors) (Persat *et al.* 2015).
Type III secretion system (T3SS)	-	Cell surface	Creates a proteinaceous channel through which toxins are transported directly into the host cell cytoplasm; PopB, PopD, and PcrV are required for pore formation but PopB also plays a role in *P. aeruginosa* pathogenicity independently from effector proteins (Galle *et al.* 2012).
ExoS	T3SS	Cell structure	Bifunctional toxin; GTP-ase activating protein activity and ADP ribosyl transferase activity; actin cytoskeleton disruption (associated with cell-rounding) and inhibition of DNA synthesis, vesicular trafficking, and endocytosis; causes irreversible damage to host cell cytoskeletal structure (Galle *et al.* 2012).
ExoT	T3SS	Cell structure	Bifunctional toxin; GTP-ase activation protein activity and ADP ribosyl transferase activity; ribosylates CT10 regulator of kinase (CRK) I and CRKII adaptor proteins (Galle *et al.* 2012).
ExoU	T3SS	Cell structure	Potent phospholipase; causes rapid cell death (Galle *et al.* 2012).
ExoY	T3SS	Cell structure	Secreted adenylyl cyclase; increases concentration of intracellular cAMP in host cell, leads to disruption of actin cytoskeleton and increased endothelial permeability (Galle *et al.* 2012); the role of ExoY in *P. aeruginosa* pathogenicity is unclear.
Nucleoside diphosphate kinase (NDK)	T3SS/ unknown	Macrophages	T3SS may not be involved in NDK secretion, role is unclear; cytotoxicity in host macrophages achieved by disrupting extracellular ATP concentrations; cytotoxicity is not kinase-dependent (Neeld *et al.* 2014).
Flagella	-	Cell surface	Major surface adhesion; elicits strong NFκB-mediated inflammatory response via signalling through toll-like receptor (TLR) 5 and a caspase-1-mediated response through Nod-like receptor, Ipaf; provides bacterium with swimming motility in liquid (Miao *et al.* 2007).
Alkaline protease	T1SS	Complement proteins	Zinc metalloprotease; causes degradation of host complement proteins and fibronectin (Laarman *et al.* 2012); helps interfere with flagellin signalling through host TLR5 by degrading free flagellin monomers and thereby helping *P. aeruginosa* avoid host immune detection (Bardoel *et al.* 2011).

Table 1.1 continued

Virulence factor	**Secretion system**	**Host target**	**Role in pathogenicity**
LasA	T2SS	Matrix proteins	Serine protease; reduced elastolytic properties when compared with LasB but thought to enhance proteolytic activity of LasB (Matsumoto 2004).
LasB	T2SS	Matrix proteins	Major extracellular protease; degrades matrix proteins such as elastin, fibronectin, and vitronectin, as well as a small number of cell receptors (Golovkine *et al.* 2014).
Protease IV	T2SS	Complement proteins	Serine protease; degrades complement proteins, immunoglobulins, and fibrinogen (Gellatly & Hancock 2013); inhibits association of *P. aeruginosa* with alveolar macrophages (Malloy *et al.* 2005).
Pyocyanin	-	Widespread toxicity	Redox-active phenazine; inhibition of host cell respiration, ciliary function, and epidermal cell growth; disrupts calcium homeostasis; induces apoptosis in neutrophils; production partly controlled by oxidative stress response regulator, OxyR, and is thought to play a protective role against phagocytic cells (Lau *et al.* 2004).
Rhamnolipid	-	-	Surfactant; participate in the maintenance of uninhabited channels surrounding biofilm communities which serve to provide nutrients and oxygen to the colonies of bacteria (Davey *et al.* 2003); biofilms can form on implants and on dead or living tissue and are inherently difficult to eradicate with antibiotics due to the inability of antibiotics to penetrate the extracellular matrix.
Alginate	-	-	Extracellular polysaccharide; overproduced in mucoid strains often isolated from chronic CF infections; participates in the production of biofilms; thought to protect bacteria from host response in CF lungs (Gellatly & Hancock 2013); treatment with imipenem induces expression of alginate and leads to thickening of biofilms (Bagge *et al.* 2004).
Pyoverdine	-	Cellular iron	Siderophore; little free iron available in host environment so pyoverdine acts to sequester iron from host depots (Gellatly & Hancock 2013); iron-bound pyoverdine acts as a signalling molecule and interacts with *Pseudomonas* cell receptor FpvA, causing upregulation of exotoxin A, endoprotease, and pyoverdine itself (Jimenez *et al.* 2012).

Whilst presenting the greatest threat in terms of morbidity and mortality to CF patients, *P. aeruginosa* can also cause acute infections within the lungs of patients generally in intensive care units in hospitals. Many of the virulence factors associated with burn wound infections and keratitis are also implicated in acute infections of the lungs. More specifically, the type III secretion system (T3SS), which delivers ExoS, ExoT, and ExoU in mammalian cells, appears to play a major role in acute pathogenicity in the lungs. It has been shown that immunisation against the type III secretion-associated *Pseudomonas* V antigen protects animals from lung damage during acute *P. aeruginosa* infection (Sawa *et al.* 1999).

When chronically infecting the lungs of CF patients *P. aeruginosa* presents with a particular set of phenotypes: alterations in the secretion of mucoid exopolysaccharide, exotoxins, proteases, and siderophores; conversion of lipopolysaccharide from smooth to rough; and a loss of motility (Mahenthiralingam *et al.* 1996). Often isolates from chronic lung infections are found to not produce O antigen, pili, and flagella which are common virulence determinants (Jain *et al.* 2004). These changes are thought to allow the bacterium to thrive in the CF lung environment which is vastly different to the environmental conditions from which unique strains of *P. aeruginosa* often originate.

P. aeruginosa has a large and versatile genome (6 – 7 Mbp) which allows the bacteria to adapt quickly to a wide range of growth conditions. The pangenome of *P. aeruginosa* is made up of a well conserved core genome and a highly variable accessory genome and the organism's ability to thrive in such a wide range of environments is attributed to this adaptable accessory genome. The core genome is defined as the genes always present in strains of *P. aeruginosa*, regardless of origin (laboratory, clinical, or environmental) and that encode metabolic and pathogenic factors observed in all strains of *P. aeruginosa* (Kung *et al.* 2010). Work by Wolfgang *et al.* (2003) showed that between 96.1% and 97.7% of PAO1 genes were detected in 18 strains tested and that 93.4% of the 5549 nonredundant genes making up the PAO1 genome were present in all 18 strains tested. The segments of accessory genome that vary by strain are not randomly scattered throughout the genome and

often cluster around certain loci, which Mathee *et al.* (2008) described as regions of genomic plasticity (RGP). The sequences present in these RGPs are commonly referred to as genomic islets (<10 kbp) or islands (>10 kbp). The definition of what constitutes a genomic island changes as new information comes to light but it is most commonly used to refer to horizontally acquired genetic elements, present in the chromosomes of some strains but absent from others (Kung *et al.* 2010). Analysis also suggests that the genome lacks large amounts of gene replication, with many more distinct gene families than other large bacterial genomes, and therefore that selection for environmental versatility has enabled the expansion of the genome and development of small paralogous gene families which encode specific, discrete functions (Stover *et al.* 2000).

1.2.1 Pathogenicity in chronic lung infections

The morbidity of chronic lung infections is generally measured by recording the decline in lung function of patients at each clinic visit. Lung function is measured frequently by forced expiratory volume in 1 s (FEV_1) according to European Respiratory Society (ERS) and American Thoracic Society (ATS) standards (Brusasco *et al.* 2005). The Medical Research Council (MRC) dyspnoea or breathlessness scale is also used to assess patients' health. The breathlessness scale does not quantify breathlessness itself; it is a short questionnaire answered by the patient which assigns a numerical value (between 1 and 5) to the disability caused by breathlessness experienced. The dyspnoea scale correlates well with other breathlessness scales, lung function measurements (such as FEV_1), and direct measurements of disability (such as walking distance) and is thus regularly used by physicians assessing severity in conditions such as NCFBr and COPD (Stenton 2008). Furthermore, the bronchiectasis severity index (BSI) specifically informs the status of bronchiectasis patients and consists of 8 commonly measured clinical parameters that reflect the age, body mass index (BMI), FEV_1, MRC dyspnoea score, exacerbation frequency, prior hospitalisations, chronic bacterial colonisation status, and the presence of cystic bronchiectasis on high resolution CT (HRCT) (Chalmers *et al.* 2014).

Studies into the effects of *P. aeruginosa* infections in NCFBr patients have been somewhat limited. It has been shown that patients who have, at some point, cultured *P. aeruginosa* from a sputum sample have significantly worse predicted FEV_1% and increased hospital admission rates, although there has been shown to be no significant associated increase in exacerbations or MRC dyspnoea scores (McDonnell *et al.* 2015). Patients colonised with *P. aeruginosa* were up to 4 times more likely to have reduced FEV_1%, up to 10 times more likely to suffer from polymicrobial colonisation, and experienced up to 12 times higher risk of mortality compared to patients who had never cultured *P. aeruginosa* from a sputum sample. Average decline in lung function, measured by FEV_1, for patients with NCFBr is currently understood to be ~50 ml per year (Nicotra *et al.* 1995; King *et al.* 2005; Martínez-García *et al.* 2007) but colonisation with *P. aeruginosa* is shown to be independently associated with accelerated decline in lung function, with an FEV_1 loss of 123.3 ml per year (Martínez-García *et al.* 2007). It was also noted that the levels of antibiotic resistance among isolates were lower in NCFBr patients in comparison to CF patients. From the patients in this study there were no panresistant strains identified and only very few multiresistant strains, furthering support for the separate and distinct disease classification of NCFBr from CF (McDonnell *et al.* 2015). Previously it has been suggested that colonisation of NCFBr patients by *P. aeruginosa* could act as an identifier for patients with an increased decline in lung function, since *P. aeruginosa* selectively colonises those patients with poor lung function (Evans *et al.* 1996) and it was shown by Davies *et al.* (2006) that *P. aeruginosa* colonisation of NCFBr patients was in fact a marker of more severe airflow obstruction. However, McDonnell *et al.* (2015) showed that as well as ~50% of patients with moderate airflow obstruction suffering from *P. aeruginosa* colonisation, there were around 10% of patients with normal FEV_1 volumes who were also colonised, which indicates that *P. aeruginosa* may be an important pathogen across all stages of airflow obstruction and requires careful observation.

1.2.2 Epidemic (transmissible) strains of *Pseudomonas aeruginosa*

Chronic infection with *P. aeruginosa* is well-documented as the leading cause of morbidity and mortality in CF patients and in the past it was widely accepted that the most common source of infection for these patients was by uniquely acquired environmental strains (Fothergill *et al.* 2012). It has been known for some time that CF siblings often share strains of *P. aeruginosa* that are directly related to one another and are not a strain that is abundant in the environment that had been acquired independently (Speert & Campbell 1987; Grothues *et al.* 1988; Renders *et al.* 1997). A wider role for transmissible strains of *P. aeruginosa* among CF patients emerged after a multi-resistant strain of *P. aeruginosa* was identified in a Danish CF centre and was found to be harboured by a number of patients in this centre (Pedersen *et al.* 1986). The number of patients colonised with this particular strain of *P. aeruginosa* continued increasing after its initial identification until a segregation policy, and stricter personal hygiene guidelines, were introduced to the hospital. Thus, this strain of *P. aeruginosa* was identified as an epidemic strain. Further to this, another antibiotic resistant strain of *P. aeruginosa* was found to be highly prevalent among children with CF at a paediatric centre in Liverpool, UK (Cheng *et al.* 1996). This strain was named as the Liverpool Epidemic Strain (LES) and was also later identified among large numbers of patients at an adult CF centre also in Liverpool (Panagea *et al.* 2003). In response to growing concern about the prevalence of transmissible strains of *P. aeruginosa* among CF patients and the increased morbidity and mortality experienced by patients colonised by an epidemic strain (Jones *et al.* 2002; Armstrong *et al.* 2003; Al-Aloul *et al.* 2003) a large scale study was carried out across England and Wales to more fully characterise the epidemic strains present among CF patients (Scott & Pitt 2004).

A nationwide study was carried out analysing 1225 isolates from CF patients at 31 centres across the UK. A total of 849 individual patient isolates were analysed by pulsed-field gel electrophoresis (PFGE) and initially compared against isolates from the same centre in order to ascertain levels of transmission within centres. The number of isolates sharing similar DNA patterns (≥80% similarity) compared with the number of isolates with unique patterns was used to give a ratio which was, in turn,

used as a crude measure of diversity of patient strain populations within each centre. The number of clustered isolates varied greatly between the 31 centres involved in the study. Some hospitals showed a roughly equal number of strains in each category (clustered vs. unique) but in five centres the clustered isolates outnumbered the unique isolates, which indicated a higher occurrence of cross-infection between patients in these centres (Scott & Pitt 2004). All 849 individual patient isolates were compared to investigate transmission of strains between centres. Six clusters containing 233 isolates were identified. The DNA patterns of these clusters were compared with the representatives from previous published outbreaks: Liverpool, Manchester, Melbourne, and clone C. A total of 93 isolates from 15 centres clustered with the representatives of the LES; 11 isolates from three centres clustered with the Manchester epidemic strain (MES); and 15 isolates from eight centres clustered with the clone C genotype (Scott & Pitt 2004). The high incidence and widespread frequency of these epidemic strains of *P. aeruginosa* was not expected, although this and other data has since informed treatment and segregation of patients attending CF clinics in the UK (The UK Cystic Fibrosis Trust Infection Control Group 2004).

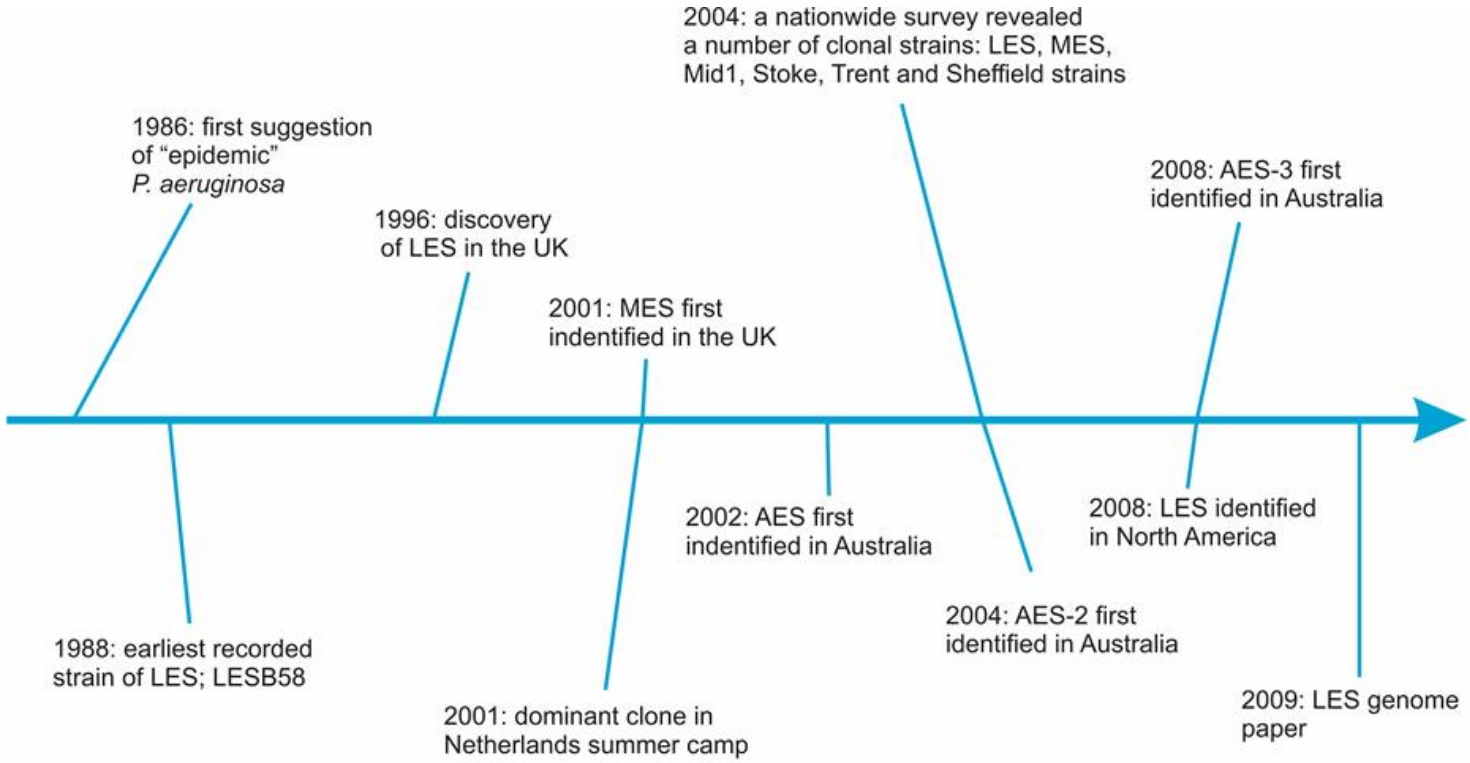


Figure 1.2: Timeline showing key events in the discovery of various transmissible strains of *P. aeruginosa* from the late-1980s to the modern day. Highlighted are key discoveries such as the earliest known strain of LES, work by Scott & Pitt (2004) revealing the presence of multiple epidemic strains present in CF centres in the UK, and the discovery of an Australian epidemic strain (AES). Figure adapted from Fothergill *et al.* (2012).

Three novel clusters were also identified. One of these was named Midlands 1 and contained 86 isolates; 66 of these came from one hospital (in the Midlands) and isolates with this genotype were also found in 8 other centres. Another novel cluster, named cluster 4, contained 12 isolates from four geographically distinct centre. The final novel cluster was formed of ten isolates from one hospital (Trent) and was named Trent. It was found that none of the UK CF *P. aeruginosa* isolates clustered with the Melbourne genotype, now known as the Australian Epidemic Strain-1 (AES-1) (Scott & Pitt 2004). Although a majority of CF patients were found to harbour unique strains of *P. aeruginosa*, around one in five of those sampled were infected by one of two transmissible genotypes. The LES accounted for 11% of the isolates analysed in this study and was found in 48% of the 31 centres. The Midlands 1 strain was found to be the second most common genotype, accounting for 10% of isolates sampled and present in 29% of centres. Despite being found in 14% of 154 patients in the Manchester CF centre (Jones *et al.* 2001) the Manchester genotype was found in only 1% of patients in this study, and clone C was represented by 2% of isolates (Scott & Pitt 2004). The most probable explanation for this distribution of these genotypes among the isolates sampled is cross-infection with *P. aeruginosa* among CF patients both within and between centres. This was an unexpected result, as transmission between CF centres had not previously been reported. The antibiotic susceptibilities of epidemic isolates were also investigated in this study and it was found that there was considerable variation among isolates of the LES genotype: some isolates showed susceptibility to all nine agents (amikacin, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, tazobactam, and colistin) whilst others exhibited resistance to all agents but colistin (Scott & Pitt 2004). There was also variation found among the antibiotic resistance profiles for the Manchester and Midlands 1 strains, one of which showed resistance to colistin (Scott & Pitt 2004). A more recent study has confirmed the LES as the most common clone isolated from UK CF patients (Martin *et al.* 2013).

Outside of the UK there have been numerous reports of other epidemic strains of *P. aeruginosa*. Several distinct epidemic strains have been found in both adult and paediatric CF centres across Australia (figure 1.2). A strain now known as

the Australian epidemic strain-1 (AES-1), previously known as the Melbourne genotype, was initially identified in a paediatric clinic in Melbourne (Armstrong *et al.* 2002). A survey of patients in Sydney found that a large number of patients were infected with the same strain, which was initially named pulsotype-1 (Anthony *et al.* 2002) but was later discovered to also be AES-1 (Armstrong *et al.* 2003). AES-1 was also found to be present in both adult and paediatric CF centres in Brisbane (O'Carroll *et al.* 2004) although a second transmissible strain (AES-2) was found to be more highly prevalent in the Brisbane centres (Syrmis *et al.* 2004). AES-2 is associated with younger CF patients and with lowered spirometry scores and increased antibiotic resistance when compared with other *P. aeruginosa* strains (O'Carroll *et al.* 2004). Another transmissible strains, AES-3, was found to be most common among patients in Tasmania and is associated with increased virulence in patients over 15 years of age (Bradbury *et al.* 2008).

Denmark was the first country to report the presence of an epidemic strain of *P. aeruginosa* among CF patients (Pedersen *et al.* 1986) and there has been further work carried out into prevalent strains present among the population. Genetic analysis of the dominant DK2 clone showed very little diversity among isolates despite the highly variable conditions faced by the bacteria in the CF lung environment (Yang *et al.* 2011), however only a small number of isolates underwent analysis for this work and so further study is almost certainly required to begin to fully understand the changes that occur in populations of *P. aeruginosa* during infection in the CF lung. Evidence for epidemic strains of *P. aeruginosa* in the context of summer camps for CF patients has been provided from Denmark, the Netherlands, and Norway (Hoogkamp-Korstanje *et al.* 1995; Ojeniyi *et al.* 2000; Fluge *et al.* 2001). Indeed, work by Ojeniyi *et al.* (2000) showed a cross-infection rate of 100% when 27 CF patients of mixed infection status attended a one-week winter holiday camp.

The LES was found to be present in patients attending CF clinics in Ontario, Canada along with another, less–prevalent, previously-undescribed epidemic strain of *P. aeruginosa* (Aaron *et al.* 2010). This study was the first to highlight the presence of epidemic strains of *P. aeruginosa* being shared across continents, although there have not been studies into the prevalence of LES, or other epidemic strains, in the

US. It is not possible to determine whether the LES originated in the UK or in Canada, although there is previous evidence of international transmission of bacterial pathogens between CF patients. More recently in Canada a novel clone, named the Prairie Epidemic Strain (PES), with increased antibiotic resistance was found in 31 (29%) of 107 patients attending an adult CF centre in Alberta (Parkins *et al.* 2014). Of these 107 patients there were 66 who had matched isolates from initial and final centre visits. In 90% of these patients (60) a stable infection was maintained for a mean period of 10.8 years, but five patients experienced strain displacement of their unique *P. aeruginosa* strain by the PES within two years of transferring to the adult CF centre (Parkins *et al.* 2014). The PES has been present in this cohort of patients since at least 1987 and is unique to CF patients, having not been isolated from any of the patients in a NCFBr control group. The strain also appears to cause chronic infection during childhood and was not found in any patients who were diagnosed with CF in adulthood (Parkins *et al.* 2014).

Transmissible strains have also been reported for other CF pathogens. Epidemiological studies of *B. cenocepacia* isolates from CF patients in Edinburgh, Scotland; Manchester; and Toronto, Canada in the early 1990s revealed the same infecting strain of *B. cenocepacia* in patients from all 3 centres (LiPuma *et al.* 1990; Govan *et al.* 1993). It was suspected that the index case was a patient from Edinburgh, who acquired the infection in the late 1980s and travelled to Canada for a summer camp with 12 other children from the UK in 1990. Subsequently, 11 of the children from the UK and a number of Canadian children also in attendance at the camp became infected with the same clonal strain of *B. cenocepacia* (Govan *et al.* 1993). Although CF summer camps have since been widely abandoned, it is possible that the LES was transmitted between Canadian and UK patients at one such camp in the late-1980s or early-1990s before the camps were shunned due to infection control concerns. Conversely, a study by Speert *et al.* (2002) undertaken in CF centres in British Columbia, Canada showed that the risk of cross-infection in these centres was very low, and did not warrant segregation of patients beyond the *Pseudomonas*-status based scheme already in place. Ontario and British Columbia are, however,

geographically distinct regions of a large country and what is typical for CF patients in one province is by no means representative of the other.

Transmissibility is also an important issue with regards to infection control in other bacterial species affecting CF patients. A study into non-tuberculosis mycobacterium (NTM) *Mycobacterium abscessus* was prompted by increasing numbers of CF patients becoming infected with the bacterium and concern that person-to-person transmission may occur. Whole genome sequencing and antibiotic susceptibility testing of 168 consecutive isolates from 31 patients attending a UK adult CF centre was undertaken by Bryant *et al.* (2013). It was revealed that there were two clustered outbreaks of near-identical isolates of *M. abscessus* subspecies *massiliense* which differed by fewer than 10 bp. All patients had been exposed to opportunities for interpatient transmission within the hospital and environmental sampling was unable to identify a possible source of environmental acquisition (Bryant *et al.* 2013). Isolates were found to have constitutive resistance to amikacin and clarithromycin and the apparent transmissible nature of the strains means that patients with no previous exposure to long-term macrolides or aminoglycosides were also infected with resistant strains which has serious implications for treatment and future healthcare (Bryant *et al.* 2013).

1.2.2.1 Antibiotic resistance among epidemic strains of *Pseudomonas aeruginosa*

The prevalence of epidemic strains among CF patients in centres across the UK, especially that of the LES, has necessitated further investigation into the levels of antibiotic resistance present in these populations. The LES is particularly aggressive and well-adapted to the CF lung environment and it was hypothesised that these features were likely due to changes in gene expression in LES isolates. This was tested by comparison of two LES isolates (associated with chronic and acute infections) to each other and to PAO1 under two different growth conditions (Salunkhe *et al.* 2005). This study investigated the changes in gene expression for several groups of genes associated with various functions of the cell. In both LES isolates the majority of upregulated genes when grown in Luria broth (LB), compared to PAO1, were found to be those previously reported to be regulated by quorum sensing (QS). The LES

isolate associated with the acute infection of a non-CF parent of a CF patient (LES431) was found to express substantially elevated levels of mRNA transcripts associated with known *P. aeruginosa* virulence genes including those encoding alkaline protease, elastase, LasA protease, pyocyanin and others (Salunkhe *et al.* 2005). Among the genes up-regulated in both LES431 and the chronic CF infection-associated LES (LES400) compared to PAO1 were those associated with antimicrobial susceptibility. Those genes affected included the *ampC* β-lactamase gene, the MexAB-OprM and MexXY efflux pumps, and the pyochelin biosynthesis genes (Salunkhe *et al.* 2005). Both LES400 and LES431 were less sensitive to β-lactams, aminoglycosides, and quinolones although LES431 was more resistant to the β-lactams piperacillin (in combination with the β-lactamase inhibitor tazocin) and imipenem (Salunkhe *et al.* 2005). Table 1.2 summarises the changes to genes and proteins in both LES isolates which were identified through the sequencing of genes of interest.

It is clear from this work that LES isolates have a wide range of defences against antimicrobial agents which, when considered alongside the significantly worse prognosis for LES-positive CF patients (Al-Aloul *et al.* 2003), further necessitates the segregation of patients by LES status as opposed to *Pseudomonas* status only.

To examine the wider effects of antibiotic resistance of LES, the antibiotic resistance profiles of LES and non-LES isolates from an adult CF centre in Liverpool, UK were tested over a period of five years using the disc diffusion method which incorporated tazobactam/piperacillin, meropenem, tobramycin, ciprofloxacin, ceftazidime, and colistin (Ashish *et al.* 2012). Overall, the mean antibiotic resistance of both groups of *P. aeruginosa* was shown to increase between 2004 and 2008 but LES isolates had increased resistance to all antibiotics except colistin when compared to the non-LES group. The non-LES group exhibited increased mean resistance to ciprofloxacin, tobramycin, and colistin but the LES group showed increased mean resistance to all six antibiotics. The relative numbers of resistant and panresistant strains were also found to be significantly higher in the LES group (Ashish *et al.* 2012). Many LES isolates (such as LES431) have been shown to exhibit an unusual QS

phenotype which is characterised by a dysfunctional QS system, leading to overproduction of QS-related factors early in the growth phase, and is termed hypervirulence (Winstanley & Fothergill 2009). The hypervirulence phenotype has been linked in some strains to increased resistance to some antibiotics. Although the mechanism is unknown, this increase may be linked to up-regulation of QS-regulated efflux pumps (Fothergill *et al.* 2007). The hypervirulence phenotype has not been identified in non-LES *P. aeruginosa* isolates and so it is feasible that the change in QS regulation may play a role in the success of LES and the greater morbidity and antibiotic resistance associated with it (Ashish *et al.* 2012).

Table 1.2: Summary of mutations found in LES isolates vs. PAO1 which may impact on antibiotic resistance profiles of clinical isolates (Salunkhe *et al.* 2005).

Protein, gene, or region	**Mutation in LES400 and LES431 vs. PAO1**	**Implications**
AmpR	One amino acid change ($D_{135} \rightarrow G$)	Similar to a previously described mutation which inferred increased β-lactamase activity (Bagge *et al.* 2002).
AmpD	Two amino acid changes	Inactivation of AmpD associated with increased levels of AmpC β-lactamase production (Langaee *et al.* 2000; Bagge *et al.* 2002) but identical mutations also found in isolates with low level basal and inducible AmpC.
MexR	One amino acid change	May affect MexAB-OprM efflux pump but this pump only contributes marginally to β-lactam and fluoroquinolone resistance (Hocquet *et al.* 2003).
mexR - *mexA* intergenic region	Single nucleotide change	May affect MexAB-OprM efflux pump but this pump only contributes marginally to β-lactam and fluoroquinolone resistance (Hocquet *et al.* 2003).
oprD	Expression downregulated	Carbapenem resistance associated with lowered levels of OprD in isolates with MexAB-OprM efflux pump (Pai *et al.* 2001).
mexZ	Stop codon, MexZ protein truncated	Similar truncations have been linked to stable aminoglcoside resistance in clinical CF isolates (Vogne *et al.* 2004).

1.2.3 Hypermutable strains of *Pseudomonas aeruginosa*

Hypermutable strains are most commonly produced by alterations in DNA mismatch repair genes, leaving the bacteria unable to avoid errors in genome

replication and therefore greatly increasing the occurrence of mutations (Miller 1996). In isolates taken from both CF patients and from environmental sources it was found that the most frequently affected gene is *mutS* (Oliver *et al.* 2002). Many mutations acquired by hypermutators prove to be synonymous, offering little or no selective advantage to the organism, or detrimental nonsynonymous mutations which reduce fitness and lead to the death of organisms carrying the mutation. The advantages held by hypermutators are that the increased rate of mutations increases the chance of the organism acquiring a beneficial mutation which allows it to better survive in its current environment. The heterogenous and often-changing environment of the CF lung is therefore ideal for the selection of hypermutator strains. Once adapted to an environment, a hypermutator strain is expected to revert to a nonmutator type to prevent the accumulation of deleterious mutations in the population; however, this is not always observed in CF patients (Oliver 2000). Of 30 CF patients, 11 were shown to harbour mutator strains of *P. aeruginosa* and in most cases the same RAPD-PCR type strain was recovered from each patient over the period of the study, indicating that the mutator strains evolved within the patients (Oliver 2000).

Maciá *et al.* (2005) carried out research into hypermutator strains present in both NCFBr and COPD patients, finding that the prevalence of these strains was high among these patients. Of the isolates collected 53% were found to be hypermutable, and hypermutable strains were found in 17 (57%) of the 30 patients. Eleven of these patients harboured hypermutable strains with a defective *mutS* gene. There was shown to be no evidence of interpatient transmission events; 32 different *P. aeruginosa* clones were identified by PFGE, each present in individual patients. In 28 of the 30 patients a single clone was identified, however the remaining 2 patients were found to be colonised with two different clones. In 10 patients two or more morphologically distinct isolates were recovered but were proven to be of a single clonal type by PFGE analysis. The presence of hypermutable strains in such a high proportion of patients indicates that clonal strains of *P. aeruginosa* are likely to be maintained in NCFBr patients for a number of years once established, as the

increased rate of spontaneous mutation allows near constant adaptation to the variable environment within the lungs of NCFBr patients.

1.2.4 Genomic polymorphism in chronic *Pseudomonas aeruginosa* strains

There are many common mutations that have been shown to occur when *P. aeruginosa* establishes a chronic infection within the lungs of patients with suppurative lung conditions. Much work has been undertaken to investigate the role of mutations in the QS systems of *P. aeruginosa* and their role in chronic infection, particularly in the context of CF patients. QS circuits are complex and rely on multicomponent communication to regulate a network of interactions which affect the destiny of the cell. Communication among a population of bacterial cells occurs via the secretion of the signalling molecules, homoserine lactones (HSLs), into the external environment and, upon reaching a critical concentration, are detected by surrounding bacterial cells which triggers a series of intracellular responses (Winstanley & Fothergill 2009). The QS network of *P. aeruginosa* is of particular interest due to its complexity, involving two interdependent LuxIR-type QS systems: LasIR and RhlIR which interact with a quinolone signal along with numerous regulators and sigma factors. QS regulation provides a method by which bacterial pathogens are able to reduce early detection by the host by delaying the production of virulence factors until the bacterial population reaches such a concentration so as to be able to have a significant effect (Girard & Bloemberg 2008). However other purposes for HSLs have been discovered: 3-O-C_{12}-HSL produced by *P. aeruginosa* can inhibit the filamentation of the fungus *Candida albicans* which can coinfect with *P. aeruginosa* as an opportunistic pathogen (Hogan *et al.* 2004). 3-O-C_{12}-HSL has also been found to act as an antimicrobial agent against Gram-positive organisms (Kaufmann *et al.* 2005) and long-chain HSLs have been found to function as biosurfactants influencing bacterial swarming (Daniels *et al.* 2006). The QS systems have been implicated in many of the classical features of chronic *P. aeruginosa* infection, including that of biofilm formation. The formation of biolfilms is known to contribute significantly to *P. aeruginosa* resistance to even the most aggressive antibiotic therapies (Stewart & Costerton 2001). The direct involvement of the QS

system in biolfilm development has been a contentious issue for some time and studies using *las* or *rhl* mutants do suggest a role for QS in the formation of biofilms but variation between strains, changes in gene expression due to environment, and putative interactions at any of the stages in biofilm formation have all hindered in the definition of a clear role for QS mutations in chronic infection (Winstanley & Fothergill 2009).

As well as common mutations that occur among isolates causing chronic lung infections, there are also "fixed" mutations present in the genomes of many transmissible strains of *P. aeruginosa* that generally offer some competitive advantage to the bacterium in causing long-term lung infections. Whole genome sequencing of seven clinical LES isolates was undertaken by Jeukens *et al.* (2014) in order to compare the genomic variations between transmissible strains of *P. aeruginosa*. Approximately 1/6 of the polymorphisms identified in the genomes of the seven LES isolates were found in regulatory genes, and 29% of them were predicted to have a high functional impact (Jeukens *et al.* 2014). Both *exsA* and *fleR* were identified as frequently variable regulatory genes. ExsA is a transcriptional regulator of the type III secretion system and was found to differ from LESB58 in four isolates, each with a different amino acid substitution (LES431, LESB65, LESlike1, and LESlike4). The LES is notably non-motile with no visible flagellum and so the redundancy of the *fleR* gene, responsible for the expression of flagellum in motile strains, allows for the occurrence of nonsynonymous deleterious mutations which can become fixed in the genome. A nonsense mutation in *fleR* was shared by the three UK LES isolates (LES400, LES431, and LESB65).. These UK isolates also shared a 14 bp deletion in the *gltR* regulatory gene which is necessary for glucose transportation. Fixed mutations have also previously been identified in regulatory genes in the DK2 transmissible lineage of *P. aeruginosa* (Yang *et al.* 2011). Mutations in *lasR*, *rpoN*, and *mucA* (implicated in the mucoid phenotype) were identified in these LES isolates and although they were not fixed mutations they are still likely to have a functional effect on the proteins produced.

1.2.5 Genotyping of *Pseudomonas aeruginosa*

There are a large number of ways to genotype bacterial species, all with varying discriminatory powers (table 1.3). Due to the plasticity of the *P. aeruginosa* accessory genome, which includes prophages and genomic islands, the genomes of individual strains can range between 5.2 and 7 Mbp in size (Tümmler 2006) and this variation means that methods of varying degrees of discrimination need to be utilised to correctly genotype *P. aeruginosa* isolates. Different methods are selected based on the needs of the researcher but some of the most commonly used genotyping methods for *P. aeruginosa* are: variable number tandem repeat (VNTR), PFGE, random amplified polymorphic DNA PCR (RAPD-PCR), multilocus sequence typing (MLST), and the Array Tube (AT).

PFGE was developed by Schwartz & Cantor (1984) as a method of fractionating chromosomal DNA of *Saccharomyces cerevisiae* to produce a molecular karyotype to facilitate the assignment of genes to yeast chromosomes. PFGE has been considered the "gold standard" of identification for many bacterial species and has a high resolution for distinction between strains but cannot be compared between laboratories (Larché *et al.* 2012). RAPD-PCR was developed by Williams *et al.* (1990) as a method by which genetic maps of inheritance could be constructed. RAPD-PCR, as implied by the name, requires no specific knowledge of the sequences to be amplified; the assay is based on the amplification of random DNA segments with single primers with an arbitrary nucleotide sequence (Williams *et al.* 1990). In some cases, multiple genotyping techniques are needed in order to confirm the validity of results, or simply to achieve a greater depth of information. Both RAPD-PCR and PFGE were used to investigate the relationship between the unusual phenotype and genotype of chronic *P. aeruginosa* isolates, collected sequentially from patients. A panel of eight 10-mer primer sequences chosen specifically to identify *P. aeruginosa* were found to give reproducible polymorphisms suitable for strain identification. A small selection of isolates from this study were also subjected to analysis by PFGE after digestion with *Spe*I. This study systematically investigated the relationship between the genotype of an infecting strain of *P. aeruginosa* and the subsequent phenotypic changes observed during chronic infection in CF patients. In

general, the RAPD-PCR profiles of the sequential isolates remained stable, indicating that the phenotypic changes were due to adaptation of the infecting strain to unique conditions of the CF lung, as opposed to strain replacement (Mahenthiralingam *et al.* 1996). Similar work was carried out by Fothergill *et al.* (2010) in an attempt to identify genuine LES isolates. The standard diagnostic PCR for LES consists of two multiplexed sets of primers, PS21 and LESF9 (table 2.1), although there have been several cases in which false positives and negatives have been identified which necessitated the testing of these anomalous isolates. However, Fothergill *et al.* (2010) discovered that PFGE, RAPD-, and BOX-PCR fingerprinting methods were unable to unequivocally resolve which isolates were and which were not genuine LES. The nature of chronic lung infections in CF patients may be partly responsible for the inability of these techniques to fully resolve the identity of isolates. Guidelines on how to interpret PFGE restriction patterns and thus infer relationships between isolates were put forward by Tenover *et al.* (1995) but this method was suggested for use in analysing sets of isolates for epidemiological studies of relatively short-term outbreaks (1 – 3 months) in community or hospital settings. Because of the genomic instability of *P. aeruginosa* in the CF lung, it may be, therefore, that PFGE is not a suitable method for examining relatedness in the context of long-term chronic infections that are typical in CF patients. Further to this, the AT genotyping method was also investigated for its efficacy in discriminating between strains of *P. aeruginosa*. The AT system was developed by Wiehlmann *et al.* (2007) and relies on the amplification of 58 targets for hybridisation with a chip embedded in the base of a 1.5 ml tube. The markers are either single nucleotide polymorphisms (SNPs) or variable genes, the former being used to form a hybridisation profile which is converted into a hexadecimal code and can be directly compared against databases of other *P. aeruginosa* strains. The variable gene markers can be used to investigate the accessory genome of *P. aeruginosa* as the targets include virulence factors and previously reported genomic islands. It has been previously reported that the AT genotyping system is 99.9% accurate in discriminating between strains of *P. aeruginosa* (Morales *et al.* 2004) and it was found by Fothergill *et al.* (2010) to be very useful in resolving anomalous results from other genotyping methods.

VNTR analysis was initially developed by Jeffreys *et al.* (1985) as a method of genetically "fingerprinting" human DNA using minisatellite genetic markers (or VNTRs). Minisatellites were also shown to be present in bacterial genomes and so assays were developed for VNTR typing of bacterial populations. Multiple-locus VNTR analysis (MLVA) is based on a set of polymorphic tandem repeat loci and has been developed for a number of bacterial species (Le Fleche *et al.* 2001; Le Fleche *et al.* 2002). A MLVA scheme was developed for typing of *P. aeruginosa* by Onteniente *et al.* (2003) using seven polymorphic loci identified in the sequenced genome of PAO1. MLVA typing of *P. aeruginosa* was further developed by Vu-Thien *et al.* (2007) to include a further eight loci. The aim of developing MLVA for *P. aeruginosa* was to be able to assign a code to each strain that represented its genetic profile which would allow for the creation of an inter-laboratory database for easy strain comparison. From the 15 alleles selected for MLVA typing there were found to occasionally be problems with amplification of some minisatellites, although amplification of all 15 alleles was reported in most cases (Vu-Thien *et al.* 2007). It was also noted that it was possible to remove certain markers, reducing the number to 14 or even as few as 10, to make an easier and more robust MLVA scheme and still maintain discriminatory powers.

MLST was developed as a portable approach to identifying clones within populations of pathogenic microorganisms by Maiden *et al.* (1998). To develop MLST evaluation for a bacterial species, sequences are determined for fragments of housekeeping genes within the genome and each distinct allele within a locus is assigned an arbitrary number. A major advantage of MLST is that the data is truly transferable between laboratories meaning that data can be easily shared and compared through an online database per species analysed. MLST typing was developed for *P. aeruginosa* by Curran *et al.* (2004) and consists of seven genes identified through the use of the *P. aeruginosa* PAO1 genome database (http://www.pseudomonas.com) (Stover *et al.* 2000) and selected based on a number of factors including biological role, location, and suitability for nested primer design. This work showed that MLST was an effective and suitably discriminatory method for typing *P. aeruginosa* isolates from a wide range of clinical and

Table 1.3: Pros and cons of various genotyping methods commonly used in epidemiological studies of bacterial pathogens.

Genotyping method	Pros	Cons
PFGE	High resolution and discriminatory power	Cannot be readily compared between laboratories
	Can be developed for a wide range of organisms	Time consuming
	Global chromosomal monitoring	Results can be rendered unusable by uneven gel, faulty electrodes, or uneven buffer heights
RAPD-PCR	No prior knowledge of sequences required	Results can vary greatly due to differences in PCR protocol and so cannot accurately be compared between laboratories
	Can be developed for a wide range of organisms	
	Fast and inexpensive	Low resolution; does not provide sequence information
MLVA	Codes assigned to strains can be readily shared between laboratories	High-variable VNTRs not available in all organisms
	High resolution and discriminatory power	Developed protocols may only work to identify specific species and serovars
	Fast and inexpensive	
MLST	Codes assigned to strains can be readily shared between laboratories	Expensive
	Schemes can developed for a wide variety of microbial organisms	Sequence conservation in housekeeping genes can sometimes prevent discrimination between strains
	Large databases of MLST codes available for many organisms (http://pubmlst.org)	
AT	Rapid genotyping	Analysis of hybridisation pattern is largely qualitative
	Provides limited information regarding accessory genome	Some cases of poor hybridisation at markers
	Codes assigned to strains can be readily shared between laboratories	Accessory genome information limited to presence or absence of mobile genetic elements

environmental sources (Curran *et al.* 2004). It was shown that some isolates with identical sequence types (STs) possessed different serotypes and *toxA* types, although this weak association between MLST STs and serotypes and *toxA* types is likely as a result of the effect of recombination in *P. aeruginosa* (Curran *et al.* 2004). Although a large database of MLSTs for *P. aeruginosa* now exists to allow comparison

of isolates from around the world (http://pubmlst.org/paeruginosa/) MLST is often too costly to be carried out, especially in small laboratories, on a regular basis (Curran *et al.* 2004).

1.2.6 Whole genome sequencing of *Pseudomonas aeruginosa*

The large and diverse genomes of *Pseudomonas* spp. play a role in the difficulties experienced when genotyping *P. aeruginosa* isolates and so as the cost, both monetary and timely, of whole genome sequencing has reduced with improvements in accuracy and depth it has become an increasingly common method to investigate aspects of bacterial life. Early sequence-based studies of *P. aeruginosa,* following on from the sequencing of the whole genome of PAO1 (Stover *et al.* 2000), supported the idea of a conserved backbone of sequences interspersed with numerous strain-specific regions in a manner similar to *Escherichia coli* (Kiewitz & Tummler 2000; Spencer *et al.* 2003). It has since been confirmed that mobile genetic elements (MGEs) are commonplace within *Pseudomonas* spp. and have been identified in all *Pseudomonas* species in which they have been sought (Silby *et al.* 2011). It has previously been noted that recombination is a source of genetic diversity among isolates of *P. aeruginosa*. Plasmids are particularly common among the genomes of many *Pseudomonas* species. They are often large and can have a broad host range, even beyond *Pseudomonas* spp. Plasmids frequently encode entire pathways, often virulence related (*i.e.* antibiotic resistance), and the associated regulatory machinery (Spiers *et al.* 2000). More recently it has been shown that recombination among *P. aeruginosa* populations in chronically infected CF patients acts as a driver of genetic and phenotypic diversity (Darch *et al.* 2015). Comparison of *P. aeruginosa* genomes often involves measuring the amount of genetic material in the genome that is different from the core genome of *P. aeruginosa*. The core genome is defined as the conserved sequences of the *P. aeruginosa* genome with the remaining genes comprising the accessory genome, which is defined as the set of genes not present in one or more strain (Mathee *et al.* 2008). Thus, the defined core genome changes and reduces in size every time a new genome sequence is published. Advances in genome sequencing technologies have allowed for the

sequencing of genomes of multiple *P. aeruginosa* isolates for the investigation of virulence mechanisms, including resistance genes and virulence factors (Lee *et al.* 2006; Boyle *et al.* 2012); relatedness of isolates and tracing of transmission events (Snyder *et al.* 2013; Jeukens *et al.* 2014; Quick *et al.* 2014); and evolution of bacterial strains (Wong *et al.* 2012; Dettman *et al.* 2013; Marvig *et al.* 2014). It has been shown that, based on core genome SNP phylogeny, the wider population of *P. aeruginosa* can be separated into two major groups (Stewart *et al.* 2014). The International *Pseudomonas aeruginosa* Consortium (IPC) is aiming to sequence over 1000 genomes and build an analysis pipeline for the comprehensive study of the evolution of the *P. aeruginosa* genome and antibiotic resistance and virulence genes (Freschi *et al.* 2015). The ambition of the IPC is to assemble a large and representative strain collection, with associated genomic data, which will aid research into identification of resistance markers and data mining for new therapeutic targets. The IPC also aims to make improvements in patient care by developing platforms and pipelines to link genomic and clinical data which will allow the identification of prognostic markers by clinicians. Given the importance of *P. aeruginosa* in CF, it is also hoped that the IPC will be able to transform CF diagnostic microbiology and develop tools to enable CF clinicians to better interpret genomic data and thus make informed decisions regarding cross-infection (Freschi *et al.* 2015). Work has also been undertaken to investigate the possibility of next generation sequencing (NGS) of bacterial genomes to provide genotypic information regarding antibiotic resistance to better define nonsusceptible isolates from patient samples. The growing number of novel resistance mechanisms known to exist in various bacterial species has rendered many of the PCR-based methods insufficient (Kos *et al.* 2015). Analysis of the genomes and antibiotic resistance profiles of 390 *P. aeruginosa* isolates from diverse locations and clinical infections was used to resolve the ability of identification of resistance genes to predict phenotypic changes in antibiotic resistance. In the case of meropenem and levofloxacin it was shown that the genome-based resistome was in good agreement with the susceptibility data, which suggested that the identification of some resistance mechanisms can be readily achieved through sequence analysis (Kos *et al.* 2015). Difficulty was experienced reconciling the

susceptibility data for amikacin with resistome data and this may be due to the role played by efflux pumps and their relative expression in aminoglycoside resistance. One of the downfalls of whole genome sequencing in this context is its inability to measure gene expression which is known to play a large role in antibiotic resistance (Kos *et al.* 2015). Nonetheless this research highlights one of the many potential uses of whole genome sequencing and could, in the future, be combined with techniques such as transcriptome sequencing (RNA-Seq) to better predict resistance profiles from genetic information (Kos *et al.* 2015).

Alongside the potential uses of whole genome sequencing in investigating bacterial isolates in depth it has also been suggested that it could be used in future as a diagnostic tool, allowing the rapid and accurate identification of bacterial species and associated risk factors (*i.e.* antibiotic resistance) to allow the best possible decision to be made with regards to patient health. As technological advances are made and sequencing platforms become more compact and user-friendly it is likely that increasing number of diagnostic laboratories will have access to sequencing platforms, and that the technical requirements for their use will become comparable to those required for diagnostic PCR (Török & Peacock 2012). At present, the depth of information provided by whole genome sequencing is not warranted for use in the majority of work undertaken in diagnostic microbiology, although there have already been cases in which the benefits of sequencing could be beneficial to public health; in particular, the outbreak of *E. coli* 0104:H4 in Germany in 2011. Investigators sequenced four outbreak strains and two historical reference strains to create optical maps of the strains within 62 h, demonstrating real-time use of diagnostic sequencing to investigate an ongoing outbreak of disease (Mellmann *et al.* 2011). In this context, sequencing may be useful for the rapid identification of multidrug-resistant Gram-negative bacteria, such as *P. aeruginosa*, in nosocomial outbreaks. Sequence data would allow diagnostic laboratories to identify an outbreak strain and analyse the presence of antibiotic resistance genes present in the genome in order to present the patient with an effective treatment for infection (Török & Peacock 2012). At present the greatest obstacle to rapid diagnostic sequencing is the lack of automated interpretation software that can accurately translate sequence data to

provide the required information in a format that is accessible to microbiologists, as opposed to bioinformaticians, and can be applied rapidly to a clinical situation (Török & Peacock 2012).

1.3 Aims

The overall aim of this work is to further understanding of the importance of *P. aeruginosa* infection in patients with NCFBr. The disease itself is grossly understudied and it is only in recent years that the burden of NCFBr both on patients and on healthcare systems has been fully realised. Research into *P. aeruginosa* infection in CF patients has been extensive, though is by no means complete, and by comparison the available information regarding chronic *P. aeruginosa* infections in NCFBr patients is incredibly sparse. Very little is known about the retention of strains or the presence of transmissible strains of *P. aeruginosa* among NCFBr patient populations and this work aims to begin to resolve this. An understanding of the prevalence of transmissible strains of *P. aeruginosa* among NCFBr patients is important in terms of healthcare standards and is necessary to inform segregation policies in bronchiectasis centres. It is also hoped that this work will act as a foundation for others to continue research into *P. aeruginosa* infections in NCFBr patients, particularly with regards to the long-term effects of chronic infection on both patients and bacterial populations. It has been shown that antibiotic resistance among *P. aeruginosa* isolates increases over the course of chronic infection in CF patients (Ashish *et al.* 2012) and so future research into similar outcomes from NCFBr patients should be undertaken given the current burden placed upon public healthcare providers by increasing levels of antibiotic resistance.The specific aims of this work are to:

1. **Use AT genotyping to investigate the maintenance of *P. aeruginosa* strains in chronic lung infections in NCFBr patients over an extended period of time;**

2. **Compare the four digit AT codes to those in databases from previous studies to place NCFBr *P. aeruginosa* isolates in the context of the wider *P. aeruginosa* population;**
3. **Use whole genome sequencing to investigate the diversity and population structure of *P. aeruginosa* isolates from NCFBr patients;**
4. **Use whole genome sequencing to investigate the heterogeneity of *P. aeruginosa* populations within chronically infected individuals with NCFBr.**

Chapter Two

Methods and Materials

2.1 Collection and storage of isolates

The collection of UK-wide isolates were provided by Dr Juliet Foweraker (Papworth Hospital) and isolates from Liverpool were provided by Paul Roberts (Royal Liverpool University Hospital). A total of 408 isolates (appendix, table A1) were collected from 16 adult bronchiectasis centres in the UK. The patients were adults with confirmed bronchiectasis, with no known cystic fibrosis (CF), who regularly attend clinics at the centres. Isolates were catalogued with the information provided by the centre and stored at -80°C in 1 ml 5% (V/V) glycerol Luria broth (LB) (table 2.2). In preparation for storage, isolates provided from frozen bead stocks at Royal Liverpool and Broadgreen University Hospitals (RLBUHT) and from slopes from other centres were plated on to Columbia agar (Oxoid) (table 2.2) and grown overnight at 37°C. Following overnight growth a sweep of each organism was taken with a 5 µl inoculation loop and resuspended in 1 ml 5% (V/V) glycerol LB in a 1.5 ml Eppendorf tube. Each tube was vortexed to homogenize the mixture before being labelled and stored at -80°C.

2.2 PCR amplification screening

Polymerase chain reaction (PCR) screening was performed on all isolates in order to confirm their identity as *P. aeruginosa*; screen for Liverpool Epidemic Strain (LES), Midlands 1 and Manchester Epidemic Strain (MES) (Fothergill *et al.* 2008). To prepare a crude DNA extract a sweep of colonies from overnight growth on Columbia agar (Oxoid) at 37°C was suspended in 100 µl sterile distilled water and heated at 99°C for 5 min. Following centrifugation, 1 µl of supernatant was used in 25 µl volumes containing 5 µl GoTaq buffer, 0.5 µl dNTPs (10 mM), 300 nM forward primer per primer set, 300 nM reverse primer per primer set, 2 µl $MgCl_2$ (25 mM), 0.1 µl GoTaq 2G polymerase (5 u μl^{-1}), and sterile distilled water to final volume of 25 µl.

Following initial denaturation by heating at 94°C for 2 min, amplification was performed for 30 cycles consisting of 20 s at 94°C, 20 s at annealing temperature (table 2.1) and 40 s at 72°C, with a 2 min extension time of 72°C following completion of the cycles. 5 µl of PCR product was loaded into a 1% (W/V) agarose gel (0.5 x TBE buffer (W/V); table 2.2) and subjected to electrophoresis at 100 V for approximately 40 min. The products were visualised under UV light using GeneSnap software with a 1 kb+ DNA ladder (Invitrogen) for size comparison.

Table 2.1: Oligonucleotide primers used in this study.

Primer	**Target**	**Oligonucleotide sequence (5´ - 3´)**	**Annealing temp. (°C)**	**Product size (bp)**	**Reference**
PA-SS F	16S rRNA *P. aeruginosa*	GGGGGATCTTCGGACCTCA	58	956	(Spilker *et al.* 2004)
PA-SS R		TCCTTAGAGTGCCCACCCG			
PAL-1	*oprL* *P. aeruginosa*	ATGGAAATGCTGAAATTCGGC	57	504	(De Vos *et al.* 1997)
PAL-2		CTTCTTCAGCTCGACGCGACG			
LESF9 F	LESF9 (LES)	AACACTTGCTCCATCTGC	56	431	(Fothergill *et al.* 2008)
LESF9 R		CACGATATCCAGCAAGAC			
PS21 F	PS21 (LES)	AAGCAGGCCAGCGTGTCTA	56	364	(Fothergill *et al.* 2008)
PS21 R		AAAACGTAGCAAGCAGTG			
MID1 F	Midlands 1	TTGCGCTCCATCGTTTGA	56	649	(Smart *et al.* 2006)
MID1 R		CTCCAGATGCCTACGAAA			
MA15 F	Manchester epidemic strain	GTCGGCAGATAGCCTTTGTC	56	308	(Lewis *et al.* 2005)
MA15 R		CGACTAATACCCGTCGCTTC			
EXOU F	*exoU*	CCGTTGTGGTGCCGTTGAAG	58	134	(Ajayi *et al.* 2003)
EXOU R		CCAGATGTTCACCGACTCGC			
EXOS F	*exoS*	GCGAGGTCAGCAGAGTATCG	58	118	(Ajayi *et al.* 2003)
EXOS R		TTCGGCGTCACTGTGGATGC			
FpvAI-1F	Pyoverdine receptor type I	CGAAGGCCAGAACTACGAGA	55	326	(de Chial *et al.* 2003)
FpvAI-1R		TGTAGCTGGTGTAGAGGCTCAA			
FpvAII-2F	Pyoverdine receptor type II	TACCTCGACGGCCTGCACAT	55	897	(de Chial *et al.* 2003)
FpvAII-2R		GAAGGTGAATGGCTTGCCGTA			
FpvAIII-3F	Pyoverdine receptor type III	ACTGGGACAAGATCCAAGAGAC	55	506	(de Chial *et al.* 2003)
FpvAIII-3R		CTTCTTCAGCTCGACGCGACG			

2.3 Clondiag/Alere Array Tube genotyping

Bacterial samples isolated as from single colonies were grown overnight at 37°C on Columbia agar (Oxoid) and a sweep of the organism collected using a 5 µl inoculating loop and suspended in 1 ml sterile distilled water. Following centrifugation at 13 000 rpm for 2 min the supernatant was removed and the pellet resuspended in 200 µl sterile distilled water. The suspension was transferred to a 0.5 ml Eppendorf tube and heated at 99°C for 5 min. Following further 2 min centrifugation at 13 000 rpm, 5 µl supernatant was added to a 5 µl Master Mix containing 4.9 µl Labelling buffer and 0.1 µl biotin-dUTP Labelling enzyme. Amplification was then carried out in a linear PCR programme which consisted of initial denaturation at 96°C for 5 min and then 50 cycles of 62°C for 20 s, 72°C for 40 s and 60°C for 1 min. The linear PCR uses only one primer per target, instead of a pair, which produces only single stranded products and thus limits the amount of amplification and controls cross-contamination. The primers are all of a similar length and the sequences and melting temperatures are shown in the appendix, table A2.

Before proceeding with hybridisation, the array tubes were washed twice in hybridisation buffer for 5 min at 550 rpm. Amplified single stranded DNA (ssDNA) was transferred to the AT and hybridisation completed by the addition of 90 µl hybridisation buffer and incubation at 60°C for 1 h. The ssDNA/hybridisation buffer mix was then carefully removed from the AT by pipetting. Care was taken to avoid any contact between the pipette tip and the AT chip as any contact could damage the probes.

The tubes were then washed 3 times with 500 µl AT wash buffer 1 to limit non-specific hybridisation; once without mixing or incubation and twice incubated at 30°C for 5 min at 550 rpm. Following washing, 100 µl horseradish streptavidin-peroxidase (HRP) solution was added to allow conjugation. Tetramethylbenzidine (TMB) was then added to allow precipitation staining and complete the process. AT images were acquired using an ATR 03 Colorimetric reader and AT-Iconclust software (Alere Technologies, Jena). Figure 2.2 shows a simplified step-by-step protocol for AT genotyping; a more detailed protocol can be obtained from the manufacturer (Alere

Technologies, Jena). Six control spots are present on the AT chip that bind directly to HRP which allow indication of a correct test performance and aid in orientating the image for analysis (figure 2.3).

Table 2.2: Table of buffers and solutions used in gel electrophoresis, extraction of genomic DNA, and AT genotyping.

Buffer/Solution/Media	Ingredients	Instructions
Columbia agar (Oxoid)	Special peptone (23 g) Starch (1 g) NaCl (5 g) Agar (10 g)	All components were added to 1 l distilled water and boiled to dissolve completely. Media was then sterilised by autoclaving at 121°C for 15 mins. Media was allowed to cool to 50°C before being poured into plates, allowed to set, and then stored at 4°C until use.
Luria broth (LB)	Tryptone (10 g) Yeast extract (5 g) NaCl (10 g)	All components dissolved in 1 l distilled water and sterilised by autoclaving at 121°C for 15 mins. Media was then cooled and stored at room temperature.
Glycerol LB (5% V/V)	LB (475 ml) Glycerol (25 ml)	All components combined and mixed well. Media sterilised by autoclaving at 121°C for 15 mins. Media was then cooled and stored at room temperature.
TBE buffer (0.5 x W/V)	Tris (162 g) EDTA (11.16 g) Boric acid (83.5 g)	All components were added to 2.5 l distilled water and then mixed on a stirring plate until completely dissolved. Volume was then made up to 3 l with distilled water
Nuclei lysis solution (Promega)	2-Amino-2-(hydroxymethyl)-1,3-propanediol Sodium dodecyl sulphate (60% W/V)	
1 kb+ DNA ladder (Invitrogen)	1 Kb Plus DNA Ladder™ (1 µg μl^{-1}) Tris-HCl (10 mM, pH 7.5) NaCl (50 mM) EDTA (1 mM)	All components combined to give a final concentration of 1 µg μl^{-1} 1 Kb Plus DNA Ladder ™. For loading into gel the solution was diluted 1:6 with 6X DNA loading dye (Thermo Scientific).
AT wash buffer 1 (2 x SSC/0.01% Triton X100)	NaCl (175.3 g) Sodium citrate (88.2 g)	NaCl and sodium citrate were dissolved in 800 ml sterile distilled water. The pH was adjusted to 7.0 and the volume made up to 1 l with sterile distilled water. The solution was then diluted 1:10 in H_2O and Triton X100 was added to a final concentration of 0.01% V/V.
AT wash buffer 2 (0.2 x SSC)	AT wash buffer 1 diluted 1:100	
Hybridisation buffer	Formamide 60 - 100% (125 ml)	
HRP conjugation solution	Luminol (3-aminophthalhydrazide) (125 ml)	

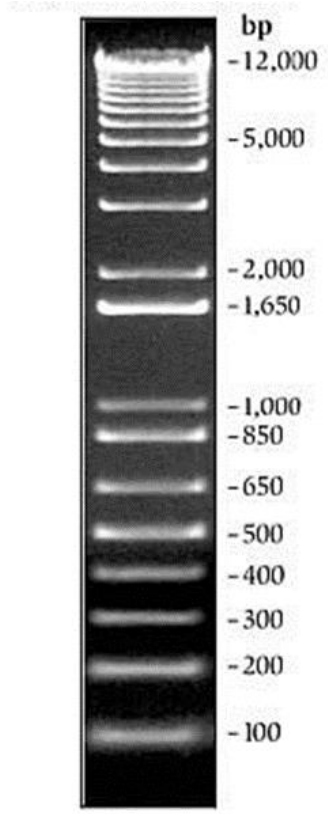


Figure 2.1: 1 kb+ DNA ladder (Invitrogen) (table 2.2) The 1kb plus DNA ladder is composed of 20 double-stranded DNA bands ranging from 100 bp to 12,000 bp, with a quick orientation band at 1,650 bp that forms a distinct doublet with the 2 kb band, and seven bands of round sizes below 1 kb.

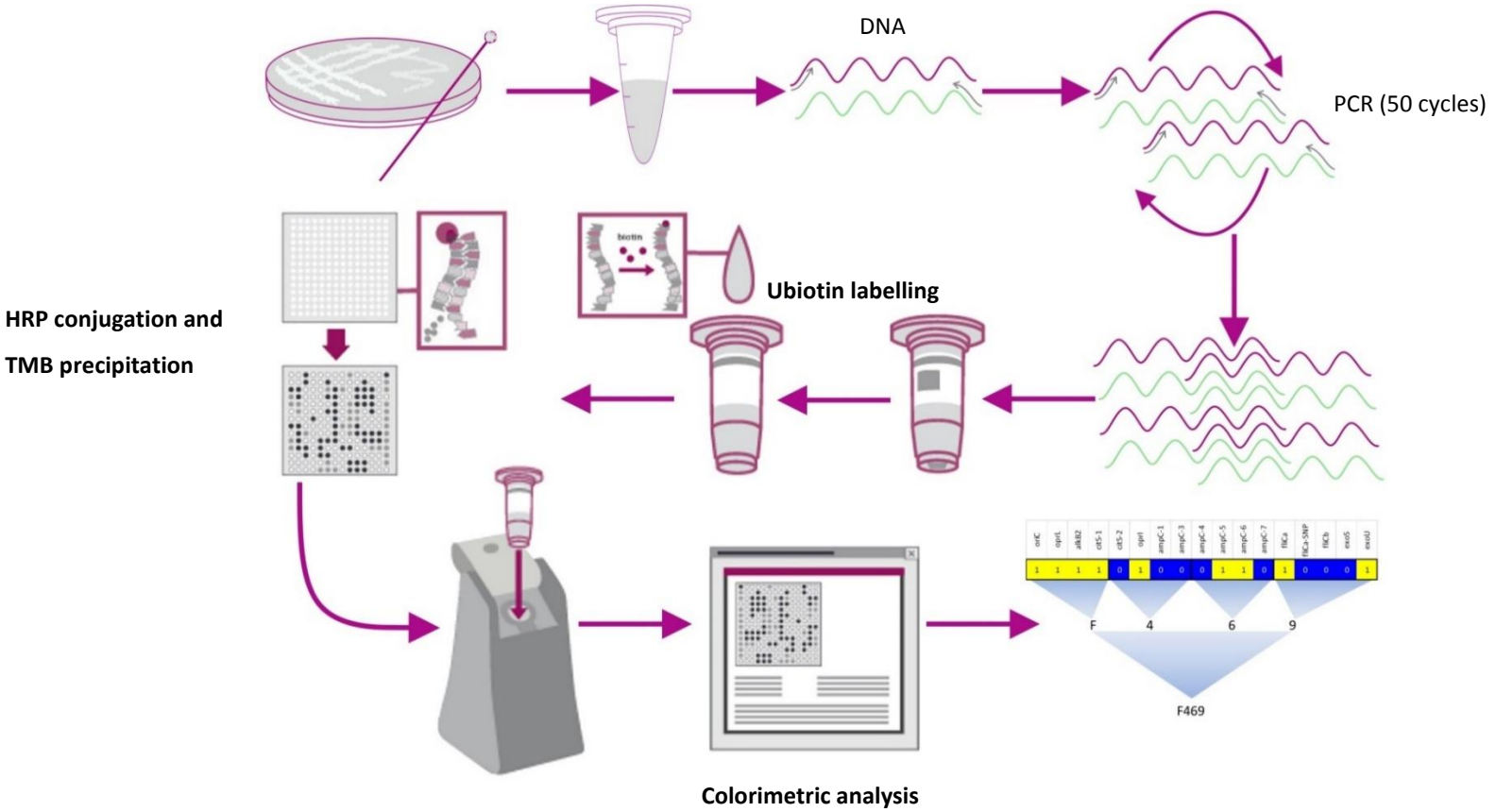


Figure 2.2: Stages of the Clondiag Array Tube system adapted from Alere Technologies: hybridisation, streptavidin-horseradish peroxidase (HRP) conjugation, tetramethylbenzidine (TMB) precipitation and colorimetric analysis (http://alere-technologies.com/en/products/lab-solutions/platforms/arraytube-at.html).

2.3.1 Layout of the AT chip

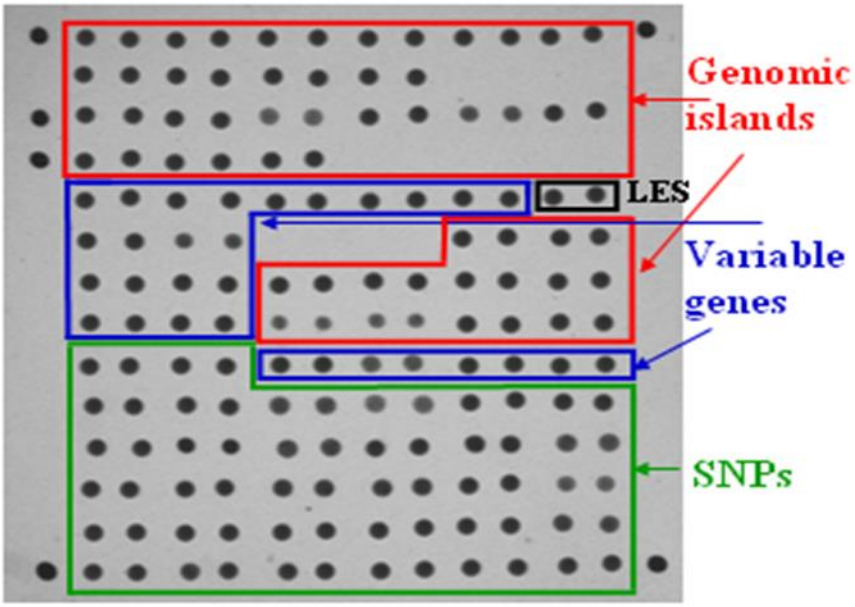
The AT system was developed in 2007 and consists of 77 oligonucleotides immobilised and embedded in a microchip in the base of a tube (Wiehlmann *et al.* 2007). The layout is divided in to 3 sections (figure 2.3): genomic islands, variable genes, and SNPs. The chip contains 29 markers for a range of genomic islands and islets, 15 markers for variable genes, a LES PS21 marker, and 16 markers relation to SNP loci. The markers for the genomic islands, variable genes, and the LES PS21 marker are represented by two spots on the chip, and the SNPs by four spots.

2.3.2 Interpretation of the AT chip

Analysis of the 13 SNP patterns at 7 conserved loci (*ampC, citS, alkB2, fliCa, oprI, oprL,* and *oriC*) and the presence or absences of 3 variable genes encoding the type III secretion virulence proteins ExoU and ExoS and the flagellin protein FliC provides a genetic profile, initially read as a 16 digit binary code, and then translated into a 4 digit hex code. The genomic islands and variable genes are represented by two oligonucleotides and the presence of the spots on the chip following hybridisation indicates whether the target is present. If the gene is present the isolate is assigned a “1” for that locus; if the gene is not present it is assigned a “0”. Each SNP is represented by four oligonucleotides: two on the left which have sequences matching with PAO1 and two on the right with sequences that do not match with PAO1 (these are considered a “mutant strain”). If the hybridisation spots are stronger on the left (PAO1 sequence) the isolate is assigned a “0” for that locus. If the hybridisation spots are stronger on the right (“mutant” sequence) the isolate is assigned a “1” for that locus. Figure 2.4 shows examples of binding for both wild type (PAO1) and non-PAO1 (mutant) variants of the hybridisation spots to act as a guide when reading the AT image (figure 2.4). Interpretation of the AT chip is carried out manually and so the interpreted results can be somewhat subjective.

2.3.3 Hexadecimal code conversion

The resulting hexadecimal binary code can then be converted to a 4 digit code as shown in figure 2.5 and a genetic fingerprint is produced and assigned to the strain. This code can then be compared to isolate information from several large, previously described databases (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2014; De Soyza *et al.* 2014). A combined dataset was created including information from all 761 isolates from the previous databases. The comparison allows for further information to be acquired about the strain in question, in particular if it has previously been assigned a clone type and environments from which isolates of the same strain type have been previously collected.



C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1
PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6		
pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1-Pilich	PAPI-1-LuBiPr.
pKLC-unknown	pKLC-adhesion	pKLC-metabol			
Pyov. Rec. I	Pyov. Rec. IIa	Pyov. Rec. IIb	Pyov. Rec. III	Pyov. Rec. B	LES
PA0636	PA0722			PAGI-1	PA0980
PA0728	PA2185	Fla-island-1	Fla-2-orfA	47D7-1	PAPI-2-Actr
PA2221	PA3835	Fla-2orfI	Fla-2orfj	47D7-2	PAPI-2-xF1753
ampC-7		fliCa	fliCb	exoS	exoU
ampC-4		ampC-5		ampC-6	
oprI		ampC-1		ampC-3	
citS-1		citS-2		oprI	
fliCa		fliCa		alkB2	
oriC		oprL a		oprL b	

Figure 2.3: Positions of SNPs, genomic islands, and variable genes on the AT chip (Wiehlmann *et al.* 2007).
C45, C46, C47 – gene island in Clone C , **PAGI** – Pathogenicity Island, **pKLC** – pKLC102 plasmid in Clone C, **pyov.rec 1,2a,2b,3** – pyoverdine type receptors I,II and III fpvA, **pyov.rec B** – pyoverdine type I receptor fpvB, **LES PS21** marker, **PA0636, PA0722, PA0728, PA2185, PA2221, PA3835** – PAO1 sequences, **TB –C47-1, TB- C47-2** - TB, pKLC102 related gene island integrated in tRNA(Lys), **fla-islands 1,2 orfA, orfI** and **orfJ** – flagellin glycosylation islands. , **fliCa, fliCb** – Flagellin proteins, **ExoU/ExoS** – Type III secreted virulence factors, **ampC** – βlactamase, **oprL** – outer membrane lipoprotein, **citS** – citrate synthase, **oprI** – outer membrane lipoprotein, **alkB2** – alkaline hydroxylase alkB2

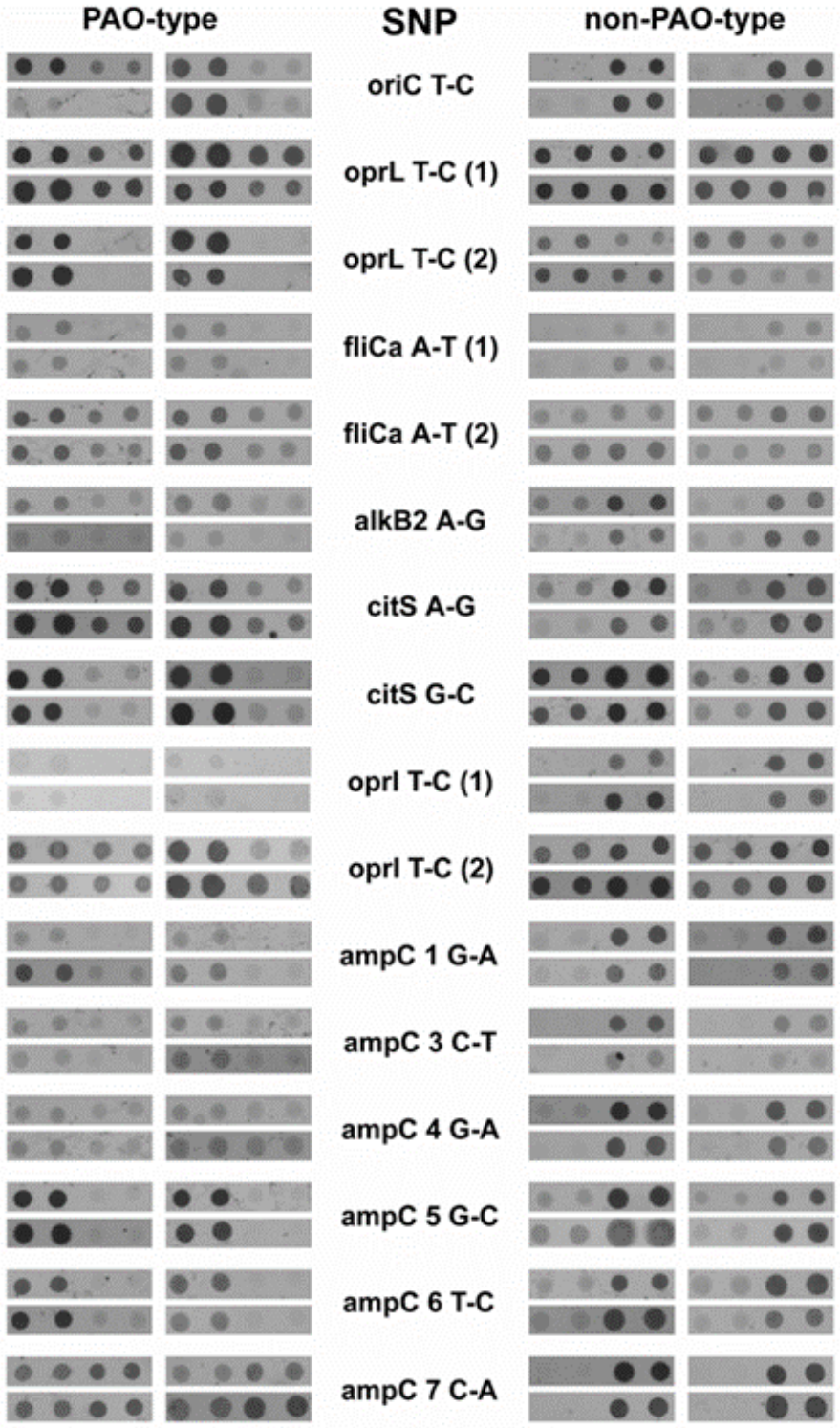


Figure 2.4: Predetermined table for comparison of hybridisation patterns for the seven conserved loci used for AT chip interpretation (Wiehlmann *et al*. 2007)

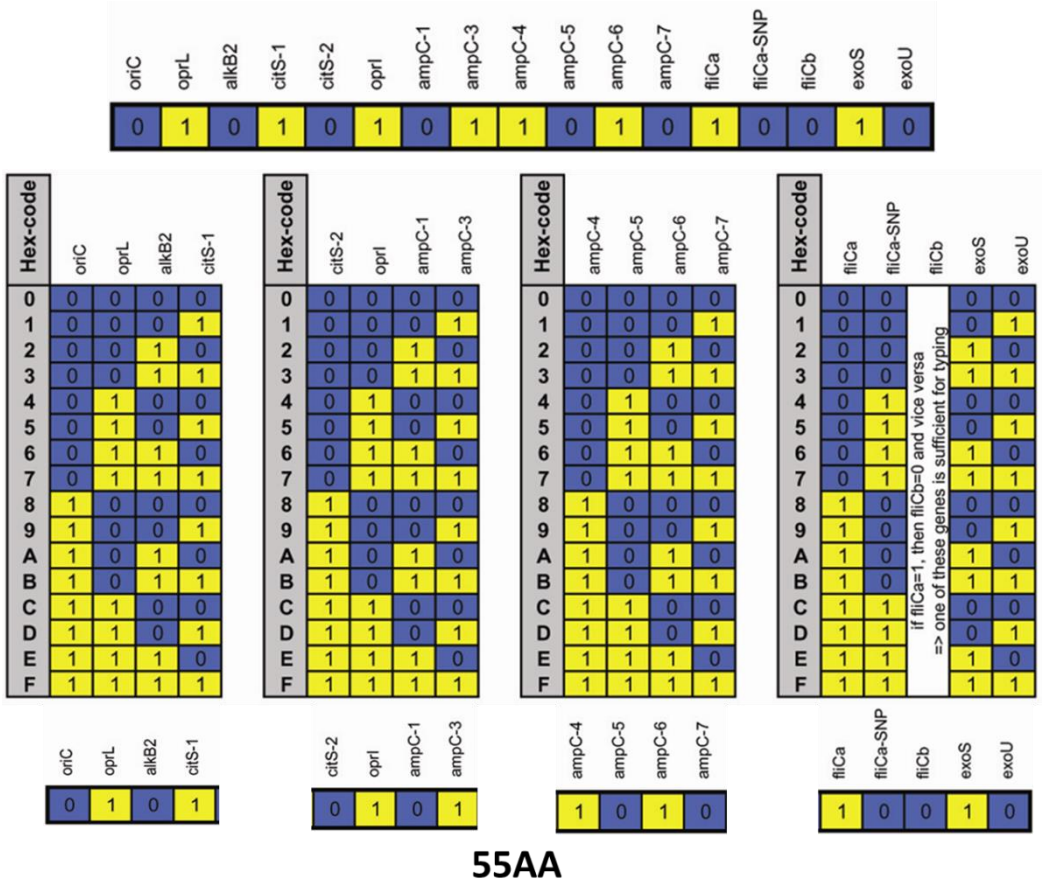


Figure 2.5: Conversion of the hexadecimal binary code, adapted from Wiehlmann *et al.* (2007).

2.4 Genomic DNA extraction for Illumina sequencing

A total of 191 isolates, isolated from single bacterial colonies, were selected to undergo whole genome sequencing. Genomic DNA (gDNA) was extracted from these isolates using a Promega Wizard Genomic DNA Purification Kit. The basic protocol was followed, with some minor adjustments to improve yield and purity. Asterisks indicate where the method used deviates from that provided by the manufacturer.

A single colony was picked and grown overnight in 5 ml LB broth in a shaking incubator at 37°C and 180 rpm. To pellet the cells, 1 ml overnight culture was centrifuged for 2 min at 14 000 rpm and the supernatant discarded. Cells were lysed by adding 600 µl Nuclei Lysis Solution and mixing gently. The tubes were then incubated at 80°C for 5 min and then cooled on ice for a further 5 min*. Once cool 3 µl RNase A Solution (Promega) was added and gently mixed. The tubes were then incubated at 37°C for 1 h and then cooled on ice for 5 min*. Protein was precipitated by adding 400 µl Protein Precipitation Solution* and vortexing immediately. The tubes were kept on ice for 5 min and then centrifuged at 14 000 rpm for 5 min*. The supernatant was then transferred to a tube containing 600 µl room temperature isopropanol and mixed by inverting the tube several times. Following centrifugation at 14 000 rpm for 15 min* the supernatant was removed. Once dry, 600 µl room temperature 70% ethanol (V/V) was added to each tube and the samples were centrifuged at 14 000 rpm for 15 min*. The supernatant was then removed and the tubes left to air dry completely. The DNA pellet was then rehydrated by adding 100 µl DEPC-treated water and storing overnight at 4°C.

2.4.1 Quantification of genomic DNA by Qubit fluorometer

Quantification of gDNA in samples was carried out using a Qubit 3.0 fluorometer and Qubit dsDNA broad range assay kit (Life Technologies). A working solution was made using concentrated assay reagent and dilution buffer in a 1:200 dilution. 190 µl working solution was added to 10 µl of each of the two pre-diluted standards for a total reaction volume of 200 µl. 2 µl sample was used per reaction,

and made up to a final volume of 200 µl with working solution. The tubes were vortexed for 2 – 3 seconds and then incubated at room temperature for 2 min before being inserted into the Qubit fluorometer to measure fluorescence. Samples were required to contain >20 ng μl^{-1} gDNA in order to be sent for sequencing.

2.4.2 Quantification and purity testing of genomic DNA by NanoDrop spectrophotometer

Further quantification and purity testing of gDNA in samples was carried out using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Extracted DNA had previously been eluted in DEPC-treated water and so the same was used as a blank for NanoDrop measurements. 2 µl DEPC-treated water was carefully pipetted on to the lower measurement pedestal of the spectrophotometer and the sampling arm then closed to form a liquid column to allow the machine to make a blank measurement. 2 µl sample was pipetted in the same manner on to the lower measurement pedestal of the machine and measurements made. Both the upper and lower measurement pedestals were wiped clean with a soft clean tissue between sample measurements.

NanoDrop measurements provide a wide range of information on the absorbance of the sample but the measurements most highly relating to the purity of the sample are the ratios of sample absorbance at 260/280 nm and 260/230 nm. 260/280 ratio of absorbance assesses purity of DNA present in the sample. A ratio of ~1.8 is generally accepted as “pure” for DNA samples. If the ratio is much lower than 1.8 it may indicate the presence of protein or other contaminants that absorb strongly at 280 nm. 260/230 ratio of absorbance is a secondary measure of DNA purity and 260/230 ratios for “pure” samples are often higher than the respective 260/280 ratios, often in the range of 1.8 – 2.2. A much lower ratio may indicate the presence of co-purified contaminants.

2.5 Whole genome sequencing

2.5.1 Data acquisition

Following gDNA extraction and quantification, samples were sequenced and analysed at the Centre for Genomic Research at the University of Liverpool. Shotgun libraries were prepared from the normalised samples using TruSeq Nano library preparation kit. Following library preparation, paired-end sequencing (2 x 100 bp) was performed by multiplexing into one lane of the Illumina HiSeq platform and sequenced with SBS V4 chemistry.

Following processing, the raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 (Martin 2011). The option –O 3 was used to that the 3' end of any reads which match the adapter sequence for 3 bp or more were trimmed. The reads were further trimmed using Sickle (https://github.com/najoshi/sickle) version 1.200 with a minimum window quality score of 20. Reads shorter than 10 bp after trimming were removed. If only one read of a pair passed this filter, it was included in the R0 file, with files R1 and R2 containing corresponding paired-end sequences.

2.5.2 Genome assembly

Genome assembly, core genome extraction, phylogenetic analysis, and variant calling were performed by Matthew Moore (University of Liverpool). Quality filtered and adapter trimmed short reads were *de novo* assembled and scaffolded using SPAdes-3.5.0 (Bankevich *et al.* 2012). Genome assembly quality metrics such as N50, largest contig, and overall number of contigs were produced using QUAST (Gurevich *et al.* 2013). Pairwise comparisons between assembled genomes were performed using progressiveMauve (Darling *et al.* 2004).

2.5.3 Core genome extraction

The core genome was extracted using Panseq (Laing *et al.* 2010) and was defined as 500 bp fragments of all genomes in this study which matched with at least 85% similarity.

2.5.4 Phylogeny

A phylogenetic tree was approximated from core genome polymorphic sites, not including gaps or ambiguous bases by maximum likelihood with inner node bootstrap (n = 100) and 10 discrete gamma categories. All phylogenetic analyses were performed using MEGA6 (Tamura *et al.* 2013) and visualised using the iTOL software (Letunic & Bork 2007; Letunic & Bork 2011). Long branches were reduced for clarity.

2.5.5 Variant calling

Short reads were mapped to a reference genome PAO1 (Stover *et al.* 2000) using bwa-0.7.5 (Li & Durbin 2009) (-mem). Resulting sequence alignment map (*.sam*) files were ordered, converted to binary alignment map (*.bam*) format, and duplicates marked. The *.bam* files were de-duplicated using picardtools-1.8.5 (http://sourceforge.net/p/picard/wiki/Main_Page/) and variants called using GATK-3.3 (McKenna *et al.* 2010) HaplotypeCaller module. All variants were filtered using vcffilter (https://github.com/ekg/vcflib) and annotated using snpEff (http://snpeff.sourceforge.net/SnpEff_manual.html).

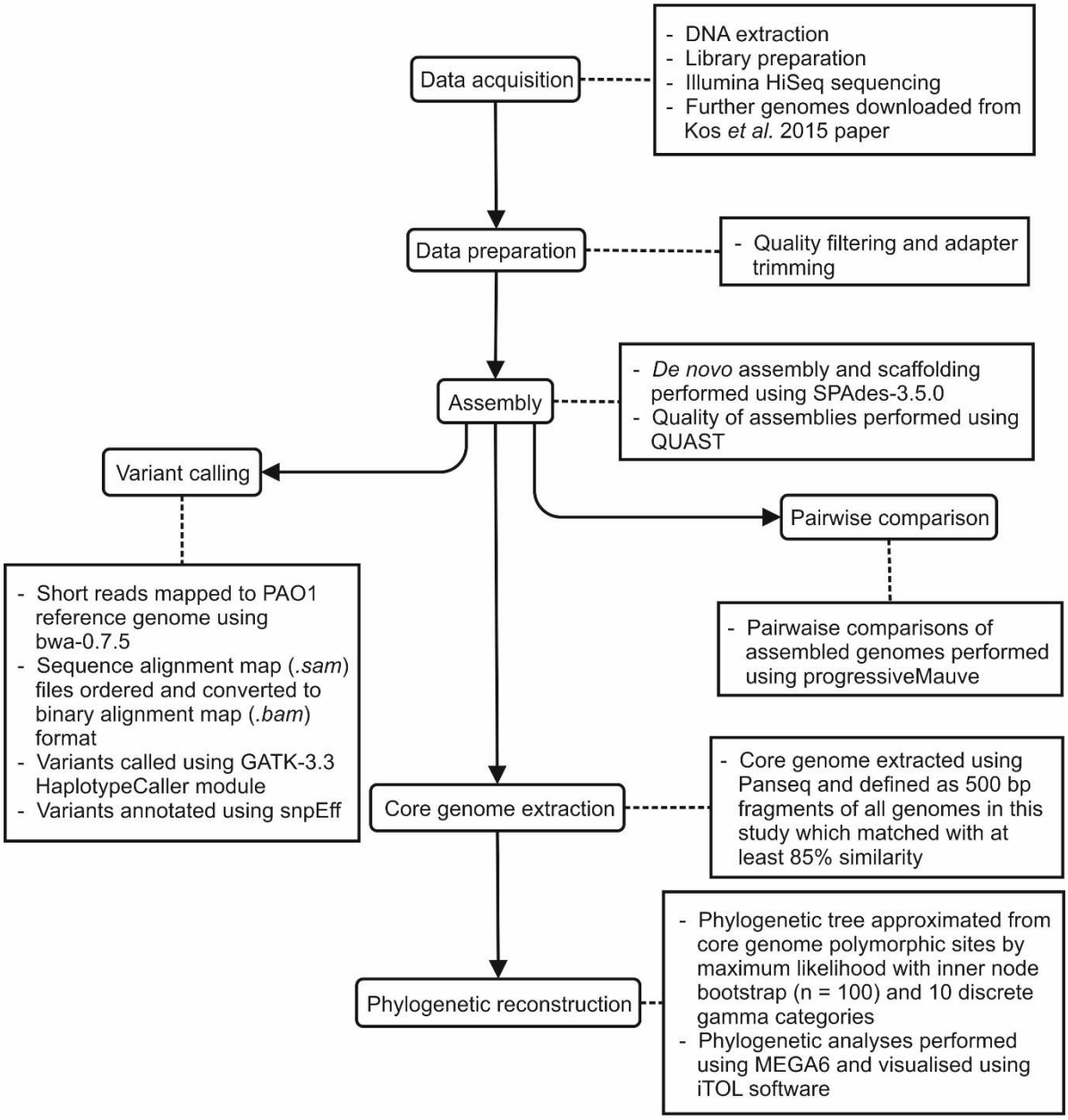


Figure 2.6: Flow diagram illustrating the processes undertaken in the computational analysis of genomic data from this study. All processes described in more detail in sections 2.5.1 – 2.5.5.

2.6 eBURST algorithm

The eBURST algorithm (http://eburst.mlst.net) was originally devised and developed by Ed Feil from the University of Bath (Feil *et al.* 2004). An enhanced version was later developed and integrated into the MLST websites (Spratt *et al.* 2004) and the latest version (eBURSTv3) has been developed with funding from the Wellcome Trust, and is hosted by Imperial College London. The algorithm displays the relationships between closely-related isolates within a bacterial population using a simple model of bacterial evolution based on a founding or ancestral genotype that diversifies to produce a cluster of related genotypes. The algorithm predicts descent of genotypes from the ancestor and displays the output as a radial diagram. The eBURST algorithm has mainly been applied to MLST data though in this case it has been used to display the relationships between genotypes identified through AT genotyping. For use with the AT genotypes, the profiles were converted to a tab delineated text file and uploaded to the eBURST site. For analysis, the programme was set to estimate the relatedness based on 16 loci, with a minimum of one identical loci for group definition, a single locus variant (SLV) count of 0 for subgroup definition, and number of re-samplings for bootstrap support set to 1000.

The data is divided into groups of sequence types (STs) that have a level of similarity in their SNP profiles. Within a single group all STs must be an SLV of at least one other ST in the group. The primary founder of any group is defined as the ST that differs from the largest number of other STs at only a single locus. In the case of two STs having the same number of SLVs, the one with the greater number of double locus variants (DLVs) is selected as the founding member. More than one group can be displayed, appearing as a cluster, in a single eBURST diagram along with any unlinked STs. The eBURST diagram shows the patterns of descent within a group in a radial fashion with lines connecting the founder to each of its SLVs, and lines connecting these STs to other STs varying from them at only one locus. The size of the node representing an ST indicates the relative abundance of that ST within the population.

Chapter Three

Longitudinal study of chronic *Pseudomonas aeruginosa* infections in non-cystic fibrosis bronchiectasis patients

3.1 Introduction

3.1.1 Maintenance of *Pseudomonas aeruginosa* strains in cystic fibrosis patients

Pulmonary infections are the biggest cause of morbidity and mortality in cystic fibrosis (CF) patients. Throughout childhood patients may be infected with a wide range of microbial pathogens, although colonisation by the most prevalent pathogens tends to be age-related (figure 3.1): *Staphylococcus aureus* is acquired during infancy*, Haemophilus influenzae* in the early years of development, and *P. aeruginosa* in adolescence (Govan & Deretic 1996). Unless detected early and rapidly treated with an aggressive course of antibiotics, *P. aeruginosa* infections persist within CF patient lungs often for the rest of their life. Longitudinal studies of both adult and paediatric CF patients have been previously carried out by many authors (Şener *et al.* 2001; Leone *et al.* 2008; Burns *et al.* 2001; Jelsbak *et al.* 2007; Mahenthiralingam *et al.* 1996) and have provided insights into transmission and maintenance of *P. aeruginosa* strains between and within patients.

A study carried out by Jelsbak *et al.* (2007) using array tube (AT) genotyping in a Danish CF clinic investigated diversity of 45 *P. aeruginosa* isolates taken from 7 patients diagnosed as chronically infected (defined as being infected for a period of >12 years) over a period of 1 year. Amongst these 45 isolates only 5 different genotypes were identified, with considerable clonal overlap between patients. The group also carried out a long-term longitudinal study on 6 of the 7 patients which

revealed chronic infection as a dynamic process in which genotypes carried by patients are periodically replaced by different clones. Two genotypes were found to be particularly dominant, with one genotype found to have infected all six patients at some point during the period of the study. The results of this study demonstrated clearly that the two dominant clones among these patients were capable of inter-patient transmission, and so it is likely that the clones have a strong selective advantage in colonising the CF airway.

Leone *et al.* (2008) used pulsed-field gel electrophoresis (PFGE) and O serotyping to analyse the genotypic characteristics of isolates from 55 adult CF patients over a 5 year period. The discriminatory powers of PFGE and O serotyping were determined to be too low to define the relationships between phenotypic and genotypic characteristics of the isolates or to elucidate any possible transmission events between patients in the centre; however the results of the study did suggest long-term persistence of strains of *P. aeruginosa* in some patients. Hypermutable strains, defined by an increase in frequency of spontaneous mutations (Oliver 2000), were also revealed to be present in some instances, with variations in PFGE profiles and antibiotic susceptibility.

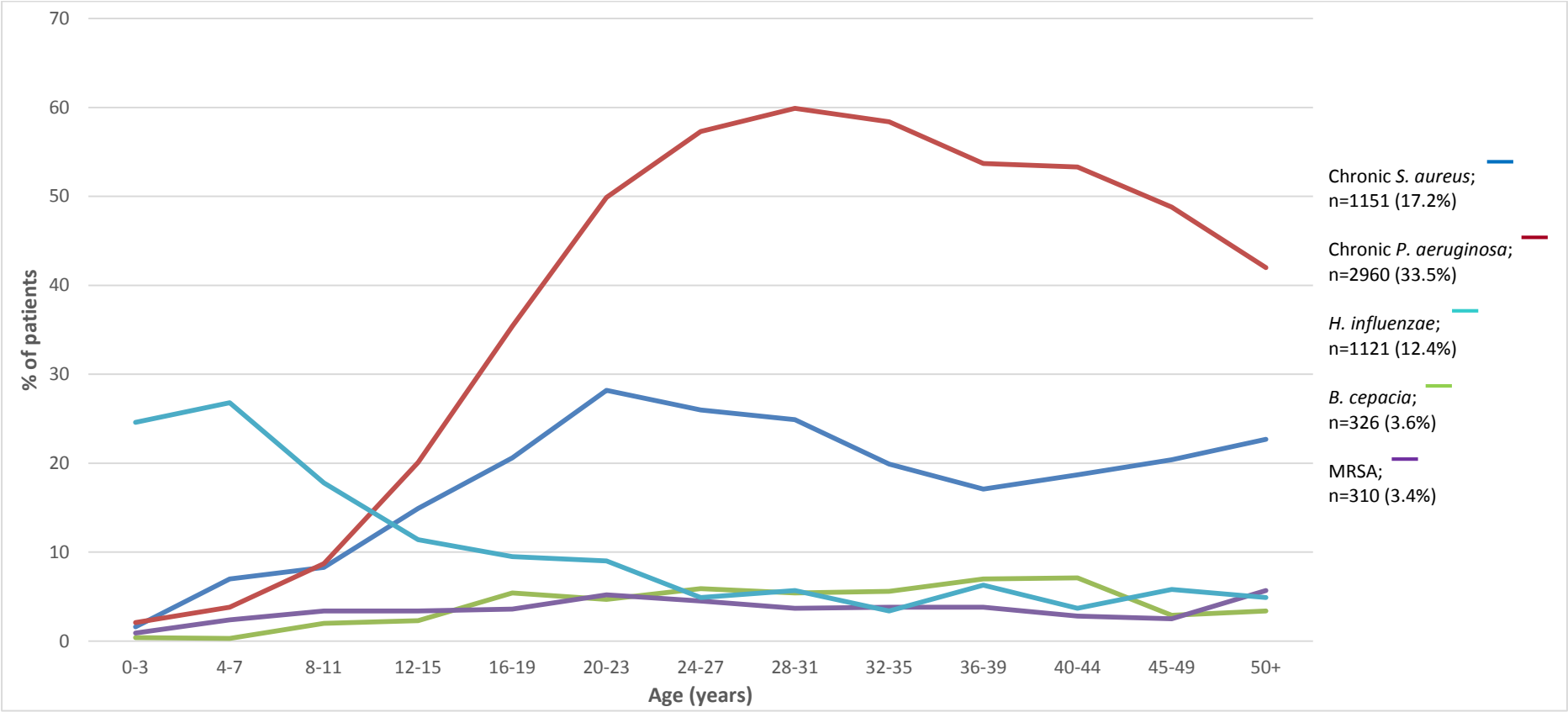


Figure 3.1: Data from CF Registry Annual data report (Cystic Fibrosis Trust 2014) indicating the percentage of patients affected by bacterial infection by common pathogens at different ages. Chronic infection with *S. aureus* or *P. aeruginosa* were identified at annual review. Data on *Burkholderia cepacia*, *H. influenzae*, and methicillin-resistant *S. aureus* (MRSA) were collected from culture results at annual review.

Analysis of *P. aeruginosa* isolates from 20 CF patients over a period of 10 years by Şener *et al.* (2001) used random amplified polymorphic DNA fingerprinting-PCR (RAPD-PCR) in combination with phenotypic tests to further understand the dynamics of *P. aeruginosa* populations in CF lung infections. The study revealed that many patients were colonised with strains of varying phenotypes but sharing a consistent genotype, although other patients were colonised with strains of varying genotypes. From the 20 patients, 24 unique RAPD types were identified from RAPD-PCR. Seven patients shared the same genotype, which Şener *et al.* (2001) speculated may have originated in one of the patients, with the earliest strain being isolated in May 1992, and spread to the others through close contact in CF clinic sessions. Of these seven, six patients consistently harboured this same genotype during repeated sampling over a 12 month period and one patient was also found to harbour a second genotype in one sample. Of the 20 patients, 13 harboured a single persistent colonising genotype; defined as the same genotype isolated from all specimens (at least 5) from a patient collected over the study period. Nine patients also showed transient colonisation with genotypes other than the persistent coloniser genotype. Genotyping of sequential isolates from these patients revealed that each of the patients was colonised with strains of a predominant RAPD type which remained stable over a period of up to 6 years.

3.1.2 Transmissible strains of *Pseudomonas aeruginosa* in CF patients

Generally, it has been accepted that CF patients with chronic lung infections acquire a strain of *P. aeruginosa* from the environment that, over time, becomes adapted to living in the niche environments within the CF lung and that these strains are usually unique to the patient in question (Fothergill *et al.* 2012). Phenotypic typing systems have previously suggested that CF siblings in close contact with one another often harbour the same *P. aeruginosa* strain, and it has been repeatedly confirmed that a single strain cross-infects in these cases (Speert & Campbell 1987; Grothues *et al.* 1988; Renders *et al.* 1997), as opposed to independent acquisition of a clonal strain. Independent acquisition of strains can occur when certain strains are naturally more abundant within the environment, hence increasing the likelihood

that multiple patients will acquire the same strain. Clone C, for example, is distributed worldwide and clone C strains are highly prone to infecting CF patients in several countries including the UK, Canada, and Germany (Römling *et al.* 2005) although the mechanisms of acquisition of the strain are unclear, and may vary completely from patient to patient.

Since the 1980s studies have suggested that unique environmental acquisition may not exclusively be the method by which chronic *P. aeruginosa* infections are acquired by CF patients. A strain of *P. aeruginosa* caused an epidemic among patients attending a Danish CF centre early in 1983 and was described by Pedersen *et al.* (1986). Later, Cheng *et al.* (1996) reported that a high proportion of children in a single CF centre in Liverpool were colonised with a β-lactam resistant epidemic strain of *P. aeruginosa*. It was determined by use of PFGE and flagellin genotyping that 55 patients were infected with the same strain of *P. aeruginosa*, now known as the Liverpool Epidemic Strain (LES). Several years later a study conducted in an adult CF centre in Liverpool indicated that 63 out of 80 *P. aeruginosa*-positive patients were infected with the LES (Panagea *et al.* 2003).

A nationwide study into the prevalence of transmissible strains of *P. aeruginosa* was undertaken by (Scott & Pitt 2004). Strains with unique genotypes were found to be harboured by at least 72% of all patients, although small clusters of related strains were found to be present in some centres, indicating limited transmission of local strains. The most prevalent strain was one indistinguishable from the previously described LES which accounted for approximately 11% of patient isolates from CF centres in England and Wales (Scott & Pitt 2004). The Midlands 1 strain was found to be harboured by 86 patients in 9 centres, and the Manchester Epidemic Strain (MES) was found in 3 centres. Inter-patient transmission and superinfection by strains of *P. aeruginosa* are now accepted as common methods of infection among CF patients, yet lack of funding has impeded research into similar transmission events in non-CF bronchiectasis (NCFBr) patients.

3.1.2 Maintenance and transmission of *Pseudomonas aeruginosa* strains in non-cystic fibrosis bronchiectasis patients

Lack of research into the maintenance and transmission of *P. aeruginosa* strains among NCFBr patients has meant that many key issues regarding patient care that are addressed in CF guidelines, in particular the issue of segregation, have been ignored in the British Thoracic Society (BTS) guidelines for NCFBr patients (Pasteur *et al.* 2010). Work by De Soyza *et al.* (2014) was undertaken to investigate cross-infection with *P. aeruginosa* among adult NCFBr patients attending a single centre. A total of 56 isolates were selected for analysis; 50 from 40 NCFBr patients, collected between 2008 and 2011, and 6 isolates from CF patients to be used a laboratory controls. This was undertaken at an adult bronchiectasis service in the north-east of England that is separate from the regional CF centre (situated 2 miles away). The service was started in 2007 as a weekly specialist clinic, with no *Pseudomonas*-specific clinic. 36 patients who regularly attended the bronchiectasis service were randomly selected and paired longitudinal isolates were included from 10 patients with a mean of 16 months between isolates (2 – 35 months). Single isolates were also chosen from 4 NCFBr patients who did not attend the clinic and who had not been hospitalised to act as potential patient controls. Two genotyping methods were used in this study: AT genotyping (described in chapter 2) and variable number tandem repeat (VNTR) analysis based on the number of DNA repeats at specified sites across the genome at nine variable loci and comparison to a VNTR database.

De Soyza *et al.* (2014) found that the vast majority of the 36 NCFBr patients harboured their own strains of *P. aeruginosa*. Four patients, 2 patients attending the clinic and 2 from the non-hospitalised control group, had distinct variants of clone C, which is found environmentally worldwide (Römling *et al.* 2005). Three pairs of patients from the remaining 34 attending the adult NCFBr service were found to harbour *P. aeruginosa* isolates which shared very similar profiles. For one pair of patients the similarity was confirmed by PFGE of *Spe*I-digested genomic DNA; the profile was unusual and the fact that the two patients shared a strain most likely reflects inter-patient transmission. Mapping of the NCFBr isolates among a wider population structure of *P. aeruginosa* isolates, based on AT genotype, demonstrated

that the NCFBr isolates were widely distributed and AT analysis found several matches with known clone types in the AT database. Data from the longitudinally paired isolates were compared and showed that in 9 of the 10 patients the first and second isolates were indistinguishable by both AT and VNTR, which confirms persistence of the initial strain. Longitudinal isolates from the final patient were identified as identical by VNTR typing but not by AT type.

The key findings of this study were a lack of dominant clones of *P. aeruginosa* among the NCFBr population, with many patients harbouring "unique" strains, and little evidence of cross-infection among patients. Isolates from the NCFBr population were widely distributed, with little to no distinct clustering. Clone C was found in 6% of the NCFBr patients but is found widely in the environment; differences at several loci in the VNTR profiles of these isolates suggests that the isolates were independently acquired, as opposed to inter-patient transmission. Standard infection control and prevention measures are less stringent in NCFBr clinics than in CF clinics (De Soyza *et al.* 2014) and so the apparent absence of cross-infection by *P. aeruginosa* is unlikely to be due to the standards of infection control practice in NCFBr clinics.

3.2 Aims

The aims of this chapter are to:

1. **Use AT genotyping to investigate the maintenance of *P. aeruginosa* strains in chronic lung infections in NCFBr patients over periods of up to 84 months;**
2. **Compare four digit AT codes to those in databases from previous studies to place NCFBr *P. aeruginosa* isolates in the context of the wider *P. aeruginosa* population.**

3.3 Results

3.3.1 Panel of isolates

A collection of 48 isolates from 20 patients (table 3.1) regularly attending an adult bronchiectasis centre, separate from the regional CF centre, in the north east of England were selected based on the number of sequential isolates available for each patient, and the period of time between the collection of the early and late isolates. The minimum time period between collection of the early and late isolates was 12 months, although the period between collection of early and intermediate isolates was less than 12 months in some cases. The mean period between early and late isolate was 40 months (range 17 – 84). A total of 40 isolates were subjected to AT genotyping as part of this study, and the remaining 8 isolates (marked with an asterisk in table 3.1) included were previously analysed by (De Soyza *et al.* 2014).

3.3.2 Array Tube hexadecimal codes

The captured AT images were read and converted as described in chapter 2 (appendix, table A3) and it was found that in 15 out of 20 patients, all isolates from each patient had the same hexadecimal code (figure 3.2). Patient 11 had a total of 4 isolates with 3 different AT codes; the early and first intermediate isolates shared the same code but the second intermediate and late isolates both had vastly different AT codes. Patients 14 and 22 had no intermediate isolates and the early and late isolates had very different AT codes in both patients. Patient 30 had an early isolate, two

intermediate isolates and a late isolate; the early and late isolates shared an AT code and both the intermediate isolates shared a different AT code. The two codes varied only in the presence or absence of the *fliCa* genomic island. Patient 32 had only early and late isolates and the AT codes for the two isolates were very different.

3.3.3 Array Tube accessory genome markers

As well as the 17 core genome single nucleotide polymorphisms (SNPs) that determine the genotype of isolates, the AT has markers for 41 genomic islands and variable genes, including the PS21 marker for LES, which represent the accessory genome. The hybridisation at markers for genomic islands and variable genes shows presence or absence of the element in a particular isolate and the results were determined as described in chapter 2 (appendix, table A4). However, isolates sharing a core genotype may have variation in their accessory genomes. Figures 3.3a and 3.3b show AT images for isolates B21 and B22, both taken from patient 11. Both isolates share genotype C40A (clone C) but also share the same hybridisation pattern across the variable genes and genomic islands present on the AT chip. Conversely, figures 3.4a and 3.4b show AT images for isolates B79 and B113, both taken from patient 18, which also share a genotype (B420) but have variations in hybridisation at some of the genomic islands and variable genes present on the AT chip. Faint hybridisation is present at the markers for three genomic islands (C47, fla-island-1, and 47D7-1) and one variable gene (PA2185) in isolate B79 but no hybridisation is seen for these loci in isolate B113.

Table 3.1: Summary of isolates used in the longitudinal study. The table shows isolates used, their collection dates, and the time between the early isolate and subsequent isolates. Asterisk indicates that the isolate was included in data from De Soyza *et al.* (2014).

Patient	Isolate	Date collected	AT code	Months since early isolate	
8	B1	Jul-08	0C1A		
8	B3	Nov-08	0C1A	4	*
8	B7	Oct-14	0C1A	75	
9	B10	Oct-11	D421		
9	B16	Nov-13	D421	25	
10	B17	Jul-11	741E		
10	B20	May-13	741E	22	
11	B21	Jul-08	C40A		
11	B22	Jan-09	C40A	6	*
11	B24	Jul-11	AC2E	36	*
11	B35	Oct-14	3C2A	75	
12	B36	Jul-11	F42A		
12	B38	May-13	F42A	22	
13	B40	Oct-07	3C52		
13	B45	Nov-11	3C52	49	
14	B46	Oct-12	3C28		
14	B49	Oct-14	D421	24	
15	B50	Mar-11	AC2A		
15	B62	Sep-14	AC2A	42	
16	B63	Jul-11	F469		
16	B71	Jul-14	F469	36	
17	B72	Jun-08	239A		
17	B74	Jul-11	239A	42	*
17	B77	Sep-13	239A	63	
18	B79	Oct-07	B420		
18	B84	Oct-08	B420	12	*
18	B90	Jul-11	B420	45	*
18	B113	Oct-14	B420	84	
19	B114	Apr-12	0C4A		
19	B126	Feb-14	0C4A	22	
20	B127	Oct-13	6852		
20	B139	Oct-14	6852	12	
21	B141	Jul-11	D421		
21	B150	Apr-14	D421	33	
22	B151	Nov-10	1BAE		
22	B152	Apr-12	2C12	17	
23	B156	Mar-09	059A		
23	B162	Sep-14	059A	66	
25	B164	Mar-12	CC60		
25	B169	Oct-14	CC60	31	
29	B185	Sep-11	4C8A		
29	B186	May-13	4C8A	20	
30	B187	Dec-07	682A		
30	B190	Jul-10	682E	31	*
30	B192	Jul-11	682E	42	*
30	B194	Sep-13	682A	69	
32	B199	Oct-11	F429		
32	B202	Aug-13	0C2E	22	

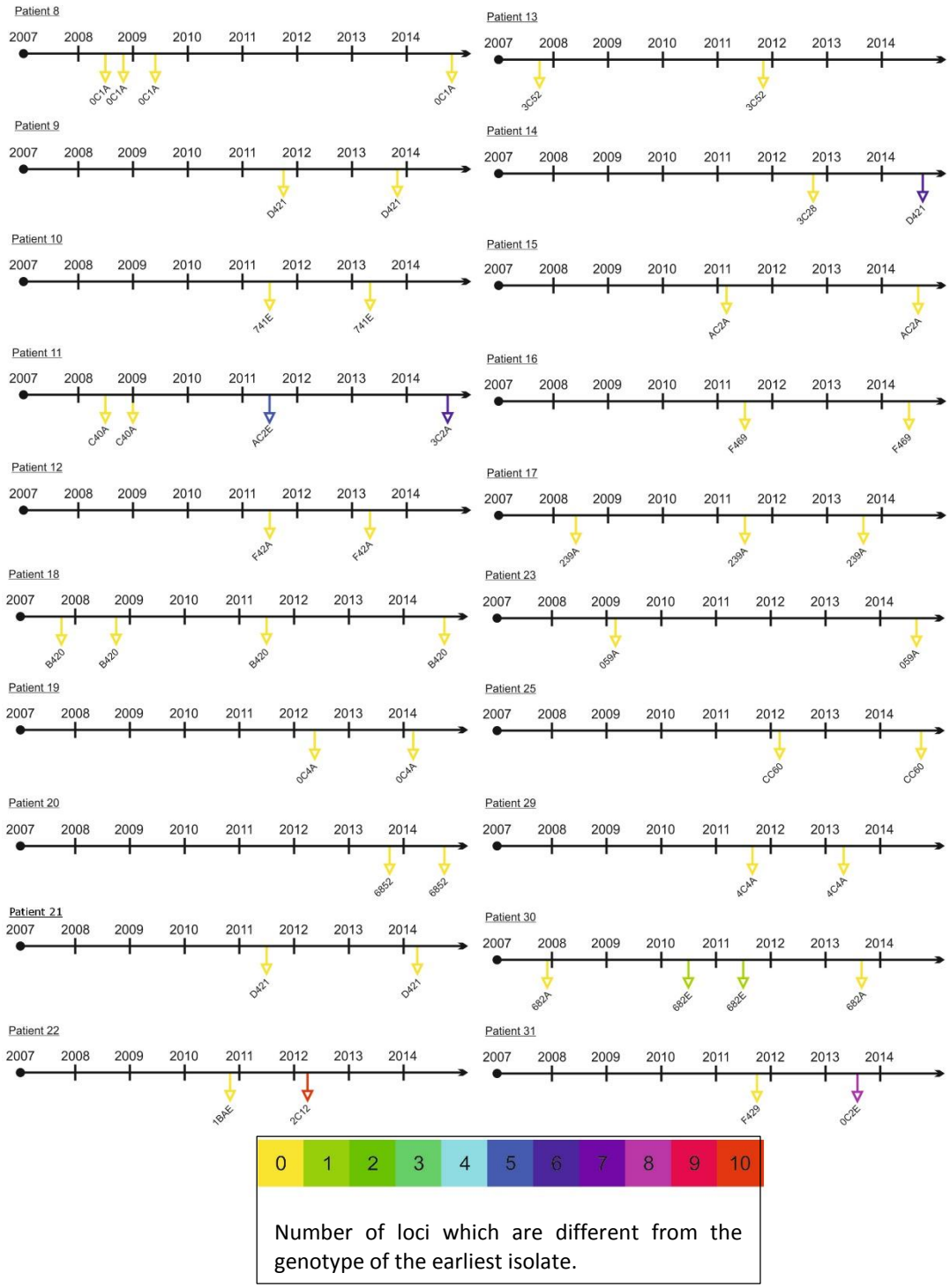


Figure 3.2: Timelines of infection for all 20 patients included in the longitudinal study. The timelines show all isolates included in the longitudinal study, approximately when they were collected and the AT code assigned to them following genotyping. The colour of the arrow indicates number of loci in the 16 digit binary code that vary from the earliest isolate collected from that patient.

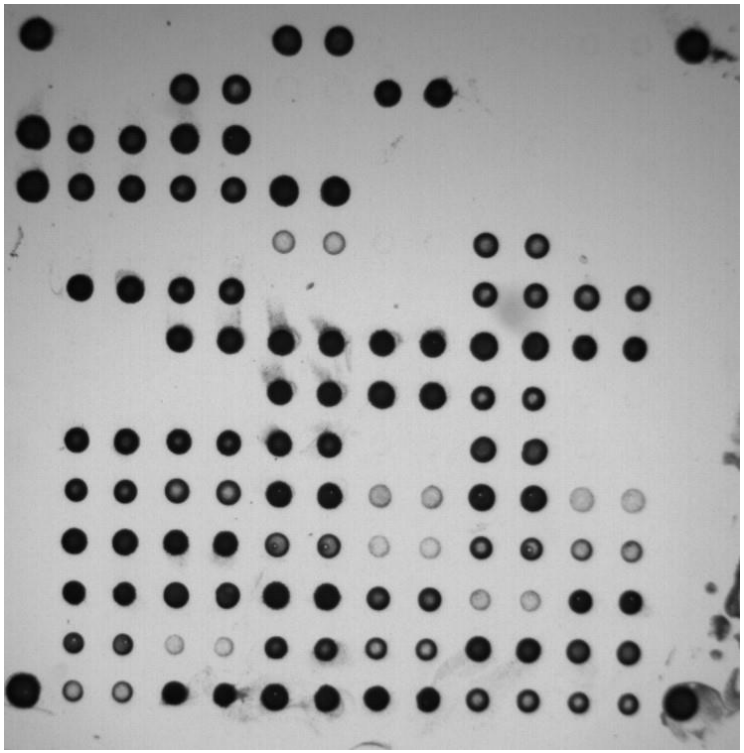


Figure 3.3a: AT image for isolate B21.

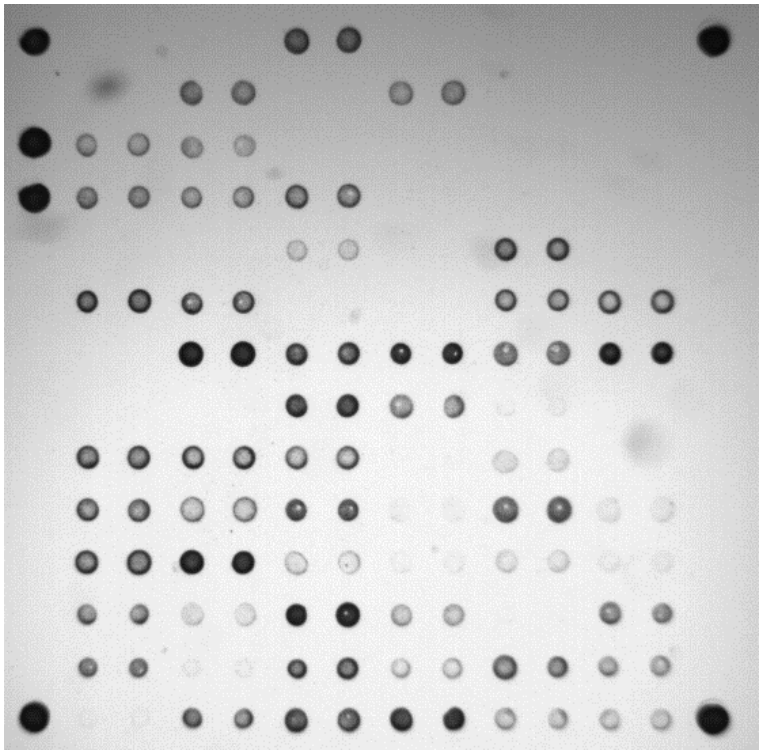


Figure 3.3b: AT image for isolate B22.

Figures 3.3a & 3.3b: AT images for isolates B21 and B22 from patient 11. Both isolates are genotype C40A (clone C) and also share the same hybridisation pattern at the markers which represent the accessory genome.

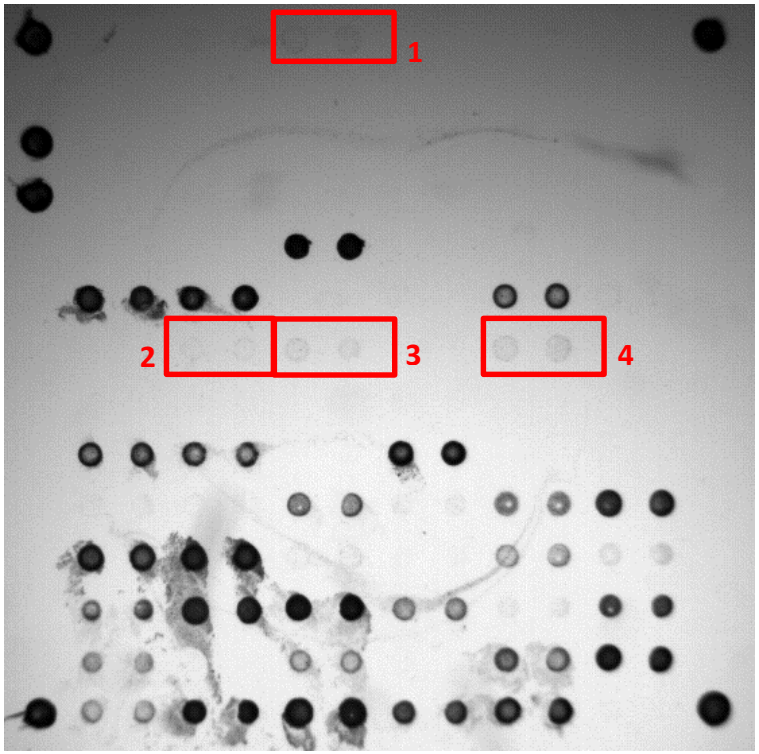


Figure 3.4a: AT image for isolate B79.

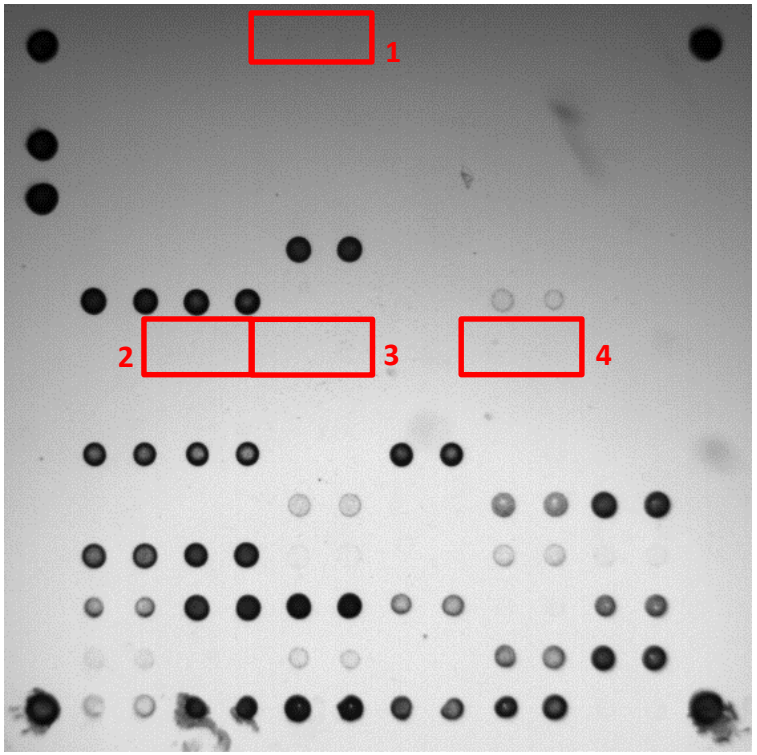


Figure 3.4b: AT image for isolate B113.

Figures 3.4a & 3.4b: AT images for isolates B79 and B113 from patient 18. Both isolates are genotype B420 but they do not share identical hybridisation patterns at the markers representing the accessory genome. Isolate B79 shows hybridisation for the markers for C47[1], fla-island-1[3], and 47D7-1[4] genomic islands and PA2185[2] variable gene.

3.3.4 Presence of highly prevalent *Pseudomonas aeruginosa* clones

Analysis of the four digit AT codes found that several strains had matches with known clones in the AT database (Wiehlmann *et al.* 2007). Patients 9, 14, and 21 harboured clone A which is represented by strain PA14 and is highly abundant throughout Europe. Patient 11 initially harboured clone C, although the two later isolates from this patient were of differing strain types: one unidentified clone and clone U.

Figure 3.5 shows a comparison between the prevalence of 40 common clones of *P. aeruginosa*, as identified by Wiehlmann *et al.* (2007), in a compiled database of AT data from 1175 isolates from previous studies (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2013; Hall *et al.* 2014) and AT data from 61 NCFBr isolates from this study (n = 24) and work by De Soyza *et al.* (2014) (n = 37). The isolates in the compiled dataset span a period of more than 70 years, with the earliest isolate having been collected in 1943, and have been collected from countries around the world; including Germany, Japan, the UK, and the USA. The isolates in the compiled dataset come from a wide range of sources: animal, environmental, and clinical isolates from several sources including; chronic CF lung infections, chronic non-CF lung infections (*i.e.* COPD), acute lung infections, keratitis, and bacteraemia. The NCFBr dataset was compiled from AT data collected in this study and data from De Soyza *et al.* (2014). In order to reduce sampling bias, isolates from a patient which shared the same genotype were only included once per patient. For example, all four isolates collected from patient 18 (table 3.1) share the same genotype, B420, and so this AT code was only included as one entry for this patient in the dataset.

In the compiled dataset 45.36% (533) of the isolates had AT codes that did not correspond to any of the 40 common clones (figure 3.5) previously identified by Wiehlmann *et al.* (2007); in the NCFBr dataset 57.38% (35) isolates did not have AT codes corresponding to any of the common clone types. In both datasets the most prevalent of the identified clones were clone A (D421) and clone C (C40A). The prevalence of clone C (7.06%) was significantly higher than that of clone A (4.60%) in

the compiled dataset (P = 0.0134) but these two clones were present at the same prevalence in the NCFBr dataset (both 6.56%). Despite the apparent differences in percentage prevalence of each clone between the two datasets, a Fisher's exact test showed that the differences in prevalence were not statistically significant (P = >0.05). Further statistical analysis revealed that the differences in prevalence between the two datasets for any of the clone types were not statistically significant (P = >0.05), possibly due to the small size of the NCFBr dataset in comparison to the compiled dataset from multiple geographical, clinical and environmental sources.

There were also some clone types that were common in the compiled dataset (>1% prevalence) but were not found to be present in any of the NCFBr data. Clones B (2.81%, n = 33), F (1.62%, n = 19), H (1.28%, n = 15), P (1.28%, n = 15), and V (2.55%, n = 30) are among the most common clone types found in the general population of *P. aeruginosa* (figure 3.5) but they were not identified in data from this study or from De Soyza *et al.* (2014). Clones B, F, H, and V were identified from a wide range of sources (e.g. acute infections, environmental, CF, chronic obstructive pulmonary disease (COPD), and keratitis) and were found to be widespread across northern Europe and the USA. Clone P was isolated almost exclusively from CF patients in Germany, with three UK CF isolates and one environmental isolate from Belgium (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2013; Hall *et al.* 2014).

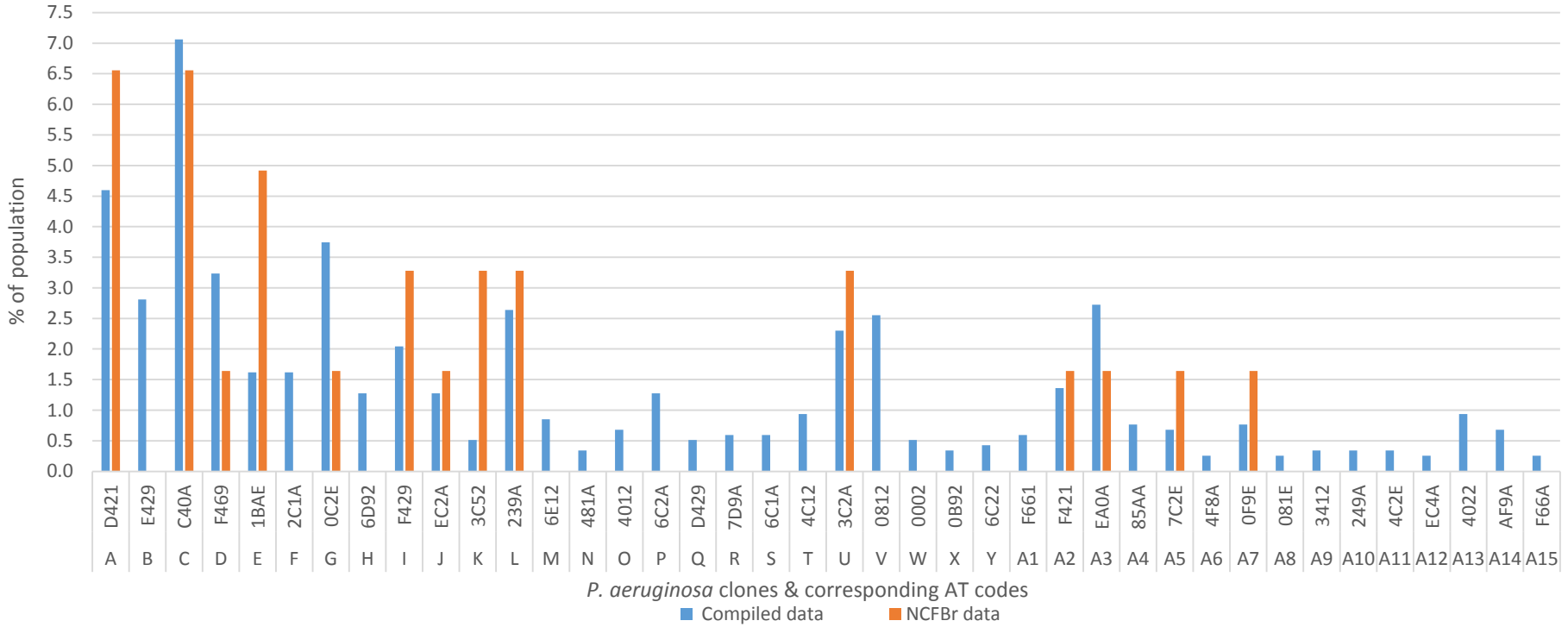


Figure 3.5: A comparison of the prevalence of common clonal strains of P. aeruginosa, as identified by Wiehlmann et al. (2007), in a compiled dataset (blue) from Wiehlmann et al. (2007); Cramer et al. (2012); Shankar et al. (2012); Hall et al. (2013); Hall et al. (2014), and data from this study combined with further NCFBr data from De Soyza et al. (2014) (orange). The compiled database contains AT data for 1175 isolates from environmental, CF, and non-CF sources. The combined data from this study (n = 24) and from De Soyza et al. (2014) (n = 37) is made up of AT data from 61 isolates, with isolates being excluded when the same genotype was present more than once in an individual patient to reduce bias. Although there are several genotypes for which the proportional representation within the given population seems to differ, the P values determined by Fisher's exact test showed that the differences are not statistically significant (P = >0.05).

3.3.5 Population structure of non-cystic fibrosis bronchiectasis *Pseudomonas aeruginosa* isolates

The population of NCFBr isolates, from this study and De Soyza *et al.* (2014), was analysed along with the wider population of *P. aeruginosa* isolates from the compiled dataset (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2013; Hall *et al.* 2014) using the eBURST algorithm (http://eburst.mlst.net) to determine the distribution of isolates from NCFBr patients amongst the wider *P. aeruginosa* population. The eBURST algorithm identifies mutually exclusive groups of related genotypes within a population. Details of how the algorithm was used to map the genotypes of these isolates can be found in chapter 2. Figure 3.6a shows the distribution of AT genotypes of NCFBr isolates from this longitudinal study alone among the wider population of isolates taken from environmental and other clinical sources. Figure 3.6b shows the distribution of AT genotypes combining NCFBr isolate data obtained in this study and from the earlier De Soyza *et al.* (2014) to provide a fuller representation of the population structure of *P. aeruginosa* isolates from NCFBr patients. Each node represents a defined strain type (ST) and lines between nodes represent a single locus difference between two nodes. The size of each node indicates the relative abundance of that particular genotype within the population. The eBURST diagram consists of one large main cluster of nodes, with the remaining smaller clusters (consisting 2 – 5 nodes) and single nodes distributed radially. All but 8 of the 40 common clones described by Wiehlmann *et al.* (2007) are found in the main cluster of isolates (figure 3.7). As well as being well distributed among the main cluster of nodes, several genotypes from NCFBr isolates are located individually or as part of smaller clusters away from the main group (figures 3.6a & 3.6b). Hence, NCFBr isolates are widely distributed amongst the general population of *P. aeruginosa*.

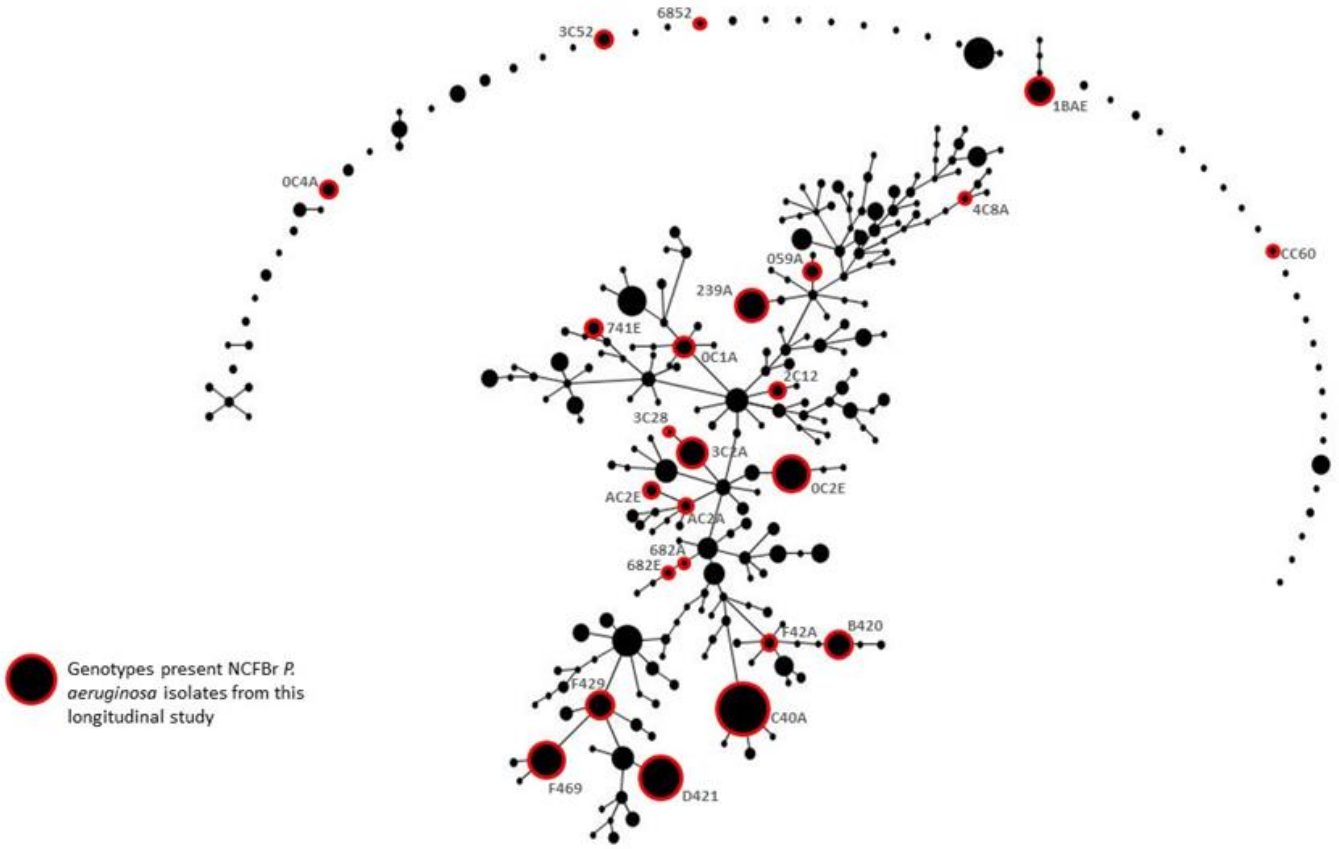


Figure 3.6a: eBURST diagram showing the distribution of *P. aeruginosa* genotypes identified in this study among the general population. eBURSTv3 (http://eburst.mlst.net), developed and hosted by Imperial College London (Feil *et al.* 2004; Spratt *et al.* 2004), was used.

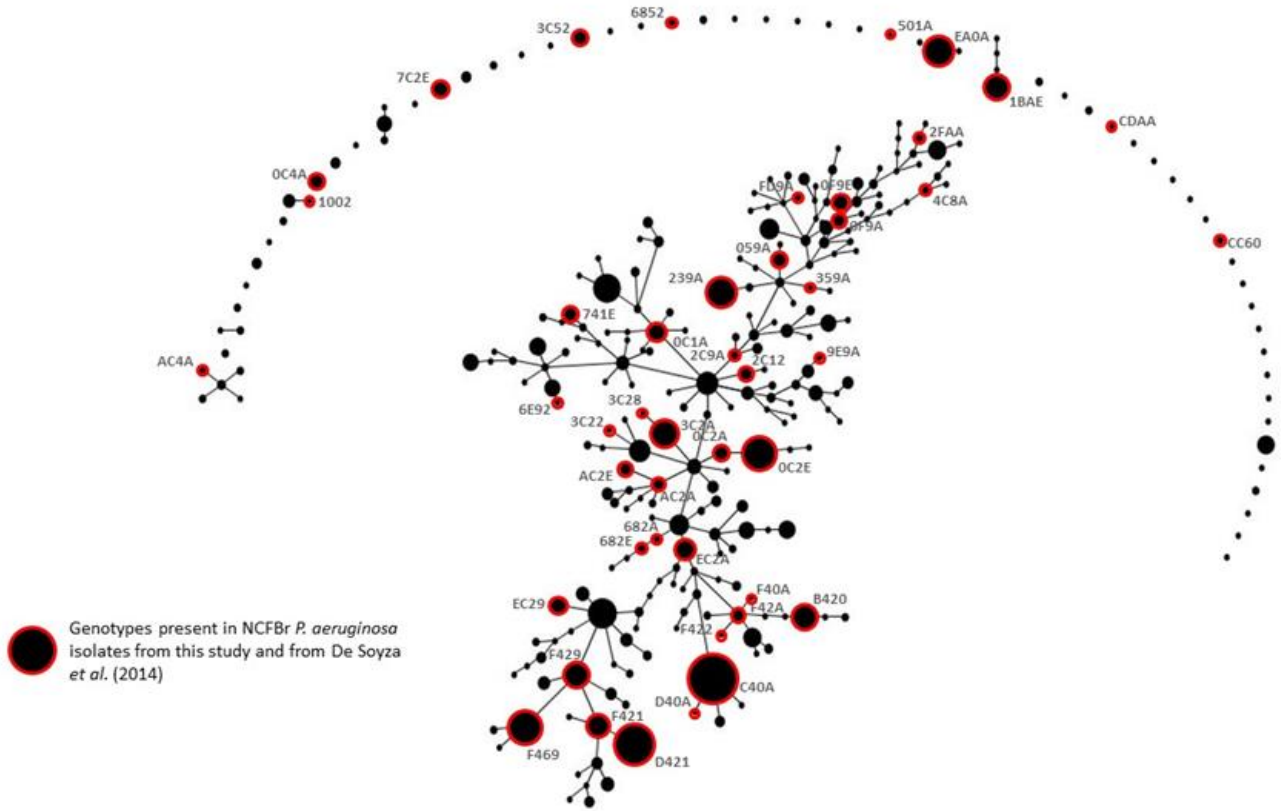


Figure 3.6b: eBURST diagram showing the distribution of *P. aeruginosa* genotypes identified in this study and by De Soyza *et al.* (2014) among the general population. eBURSTv3 (http://eburst.mlst.net), developed and hosted by Imperial College London (Feil *et al.* 2004; Spratt *et al*. 2004), was used.

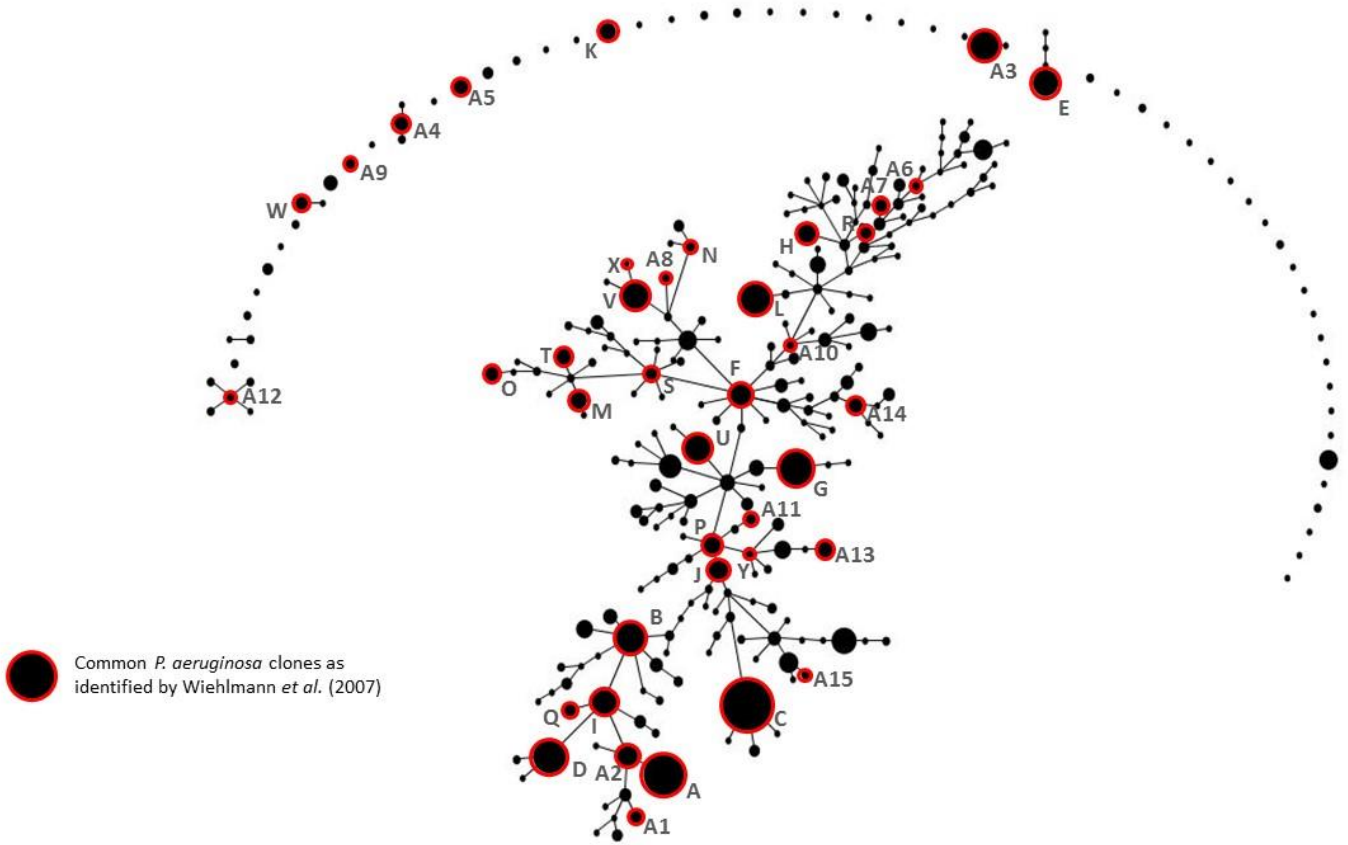


Figure 3.7: eBURST diagram showing the distribution of the 40 common *P. aeruginosa* clones as described by Wiehlmann *et al.* (2007). eBURSTv3 (http://eburst.mlst/net), developed and hosted by Imperial College London (Feil *et al.* 2004; Spratt *et al.* 2004), was used.

3.3.6 Poor hybridisation at *exoS* and *exoU* variable gene markers

It had been noted previously that one of the more serious limitations of the AT system is poor hybridisation at some markers on the chip (Hall 2015, personal communication), leaving interpretation of the results open to ambiguity. In particular, hybridisation was frequently poor at the markers for *exoS* and *exoU* variable genes. Figures 3.8a and 3.8b are AT images from isolates B164 and B169 (table 3.1) respectively. When the AT images were read initially, B164 was assigned the code CC60 and B169 was assigned the code CC62; the difference in codes was due to the variance in *exoS* hybridisation. In figure 3.8b there appears to be faint hybridisation at the *exoS* marker although there is no hybridisation at either *exoS* or *exoU* in figure 3.8a. A PCR assay was carried out using specific primer sets (table 2.1) to determine the presence or absence of both variable genes. Gel electrophoresis was performed on the PCR products and the results are shown in figure 3.9. Both B164 and B169 were confirmed negative for both *exoS* and *exoU*, despite the original decision that B169 was positive for *exoS*, which resulted in the two isolates appearing to be of differing strain types. Similarly, isolates B1 and B7 were originally assigned different AT codes (0C1C and 0C1A respectively). Figure 3.10a and 3.10b are AT images for isolates B1 and B7 (table 3.1), in which there appears to be no hybridisation at either *exoS* or *exoU* markers in figure 3.10a and faint hybridisation at *exoS* marker in figure 3.10b. A PCR assay was also carried out on these isolates and showed that both B1 and B7 were positive for *exoS* (figure 3.11). There were 8 other isolates for which PCR assays were needed to determine the presence or absence of the *exoS* and *exoU* variable genes (figures 3.12a & 3.12b).

3.3.7 Transmissible strains of *Pseudomonas aeruginosa* in non-cystic fibrosis bronchiectasis patients

As part of the initial PCR screening of the panel of isolates (chapter 2.2), four isolates taken from patient 10 tested positive for MID1, indicating that they were the Midlands 1 strain of *P. aeruginosa* (figure 3.13). Two of these isolates were subsequently selected as a pair of longitudinal isolates to undergo AT genotyping (isolates B17 and B20). However, both isolates were assigned AT code 741E, which is

not consistent with the previously defined AT code for the Midlands 1 strain, 2C1A (Wiehlmann *et al.* 2007). Hence, these were in fact false PCR-positives.

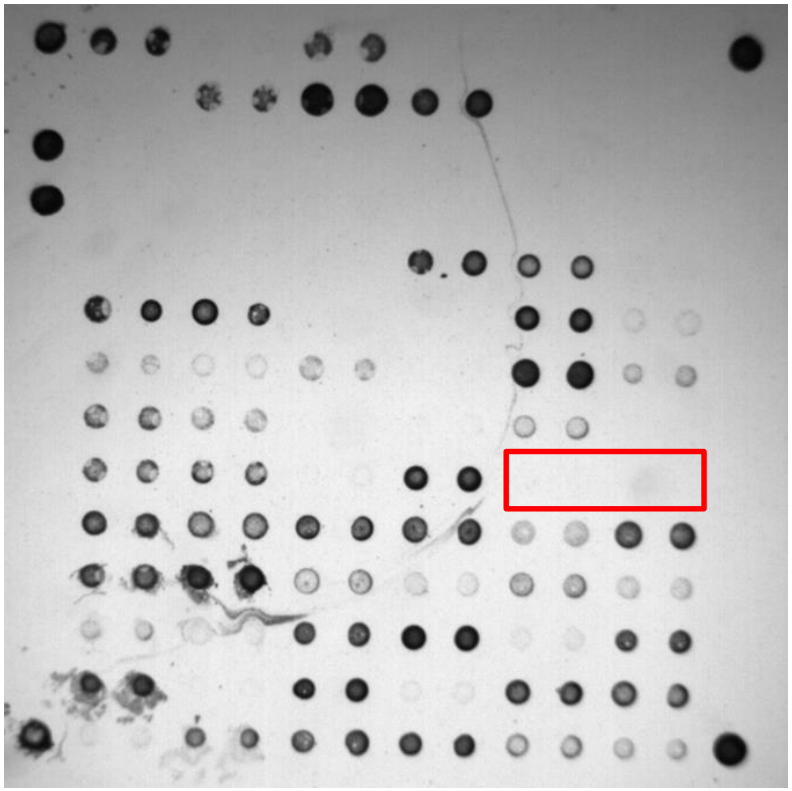


Figure 3.8a: AT image for isolate B164

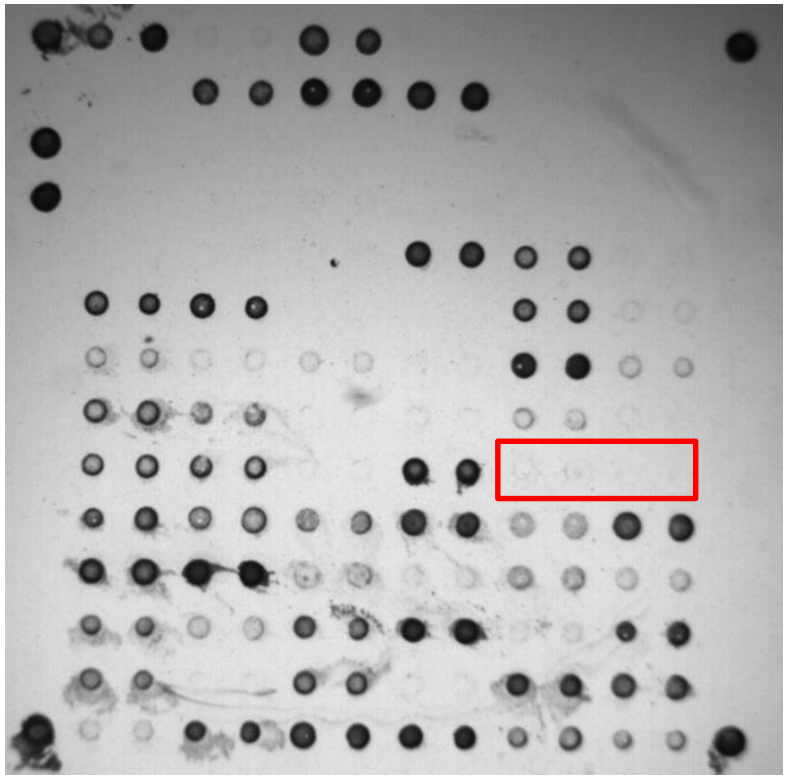


Figure 3.8b: AT image for isolate B169

Figures 3.8a & 3.8b: AT images for isolates B164 and B169 from patient 25. Highlighted are hybridisation markers for the *exoS* and *exoU* variable genes. In figure 3.8a there is no clear hybridisation at either variable gene. However, in figure 3.8b there appears to be faint hybridisation at *exoS*.

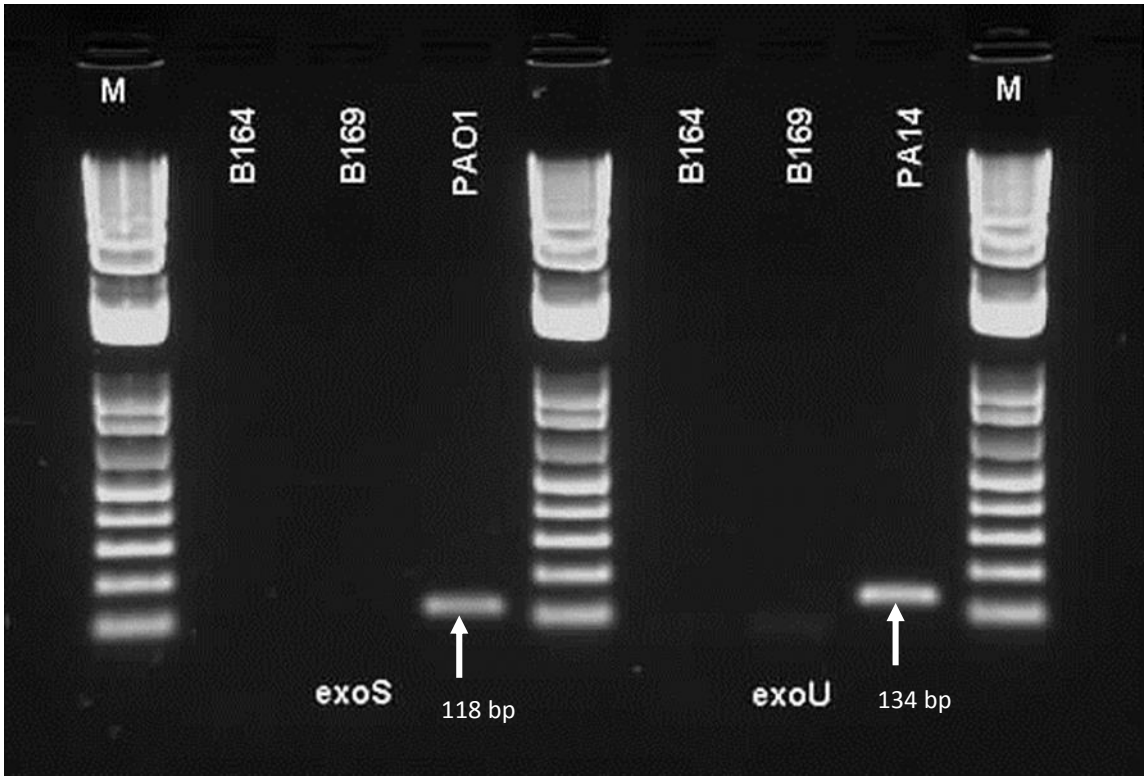


Figure 3.9: PCR assay using primers to detect *exoS* and *exoU* variable genes performed on patient 25 isolates to confirm hexadecimal AT code with PAO1 and PA14 serving as positive controls respectively. Both B164 and B169 showed no amplification of either region. Gel electrophoresis performed on 1.5% agarose gel at 50 V for approximately 2 h. Marker: 1 KB+ DNA ladder (Invitrogen).

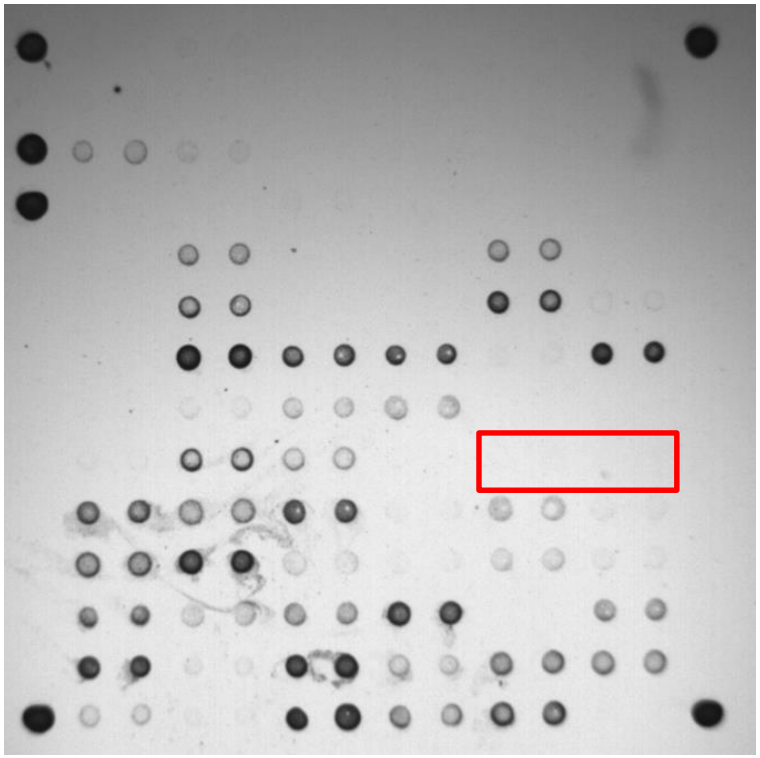


Figure 3.10a: AT image for isolate B1

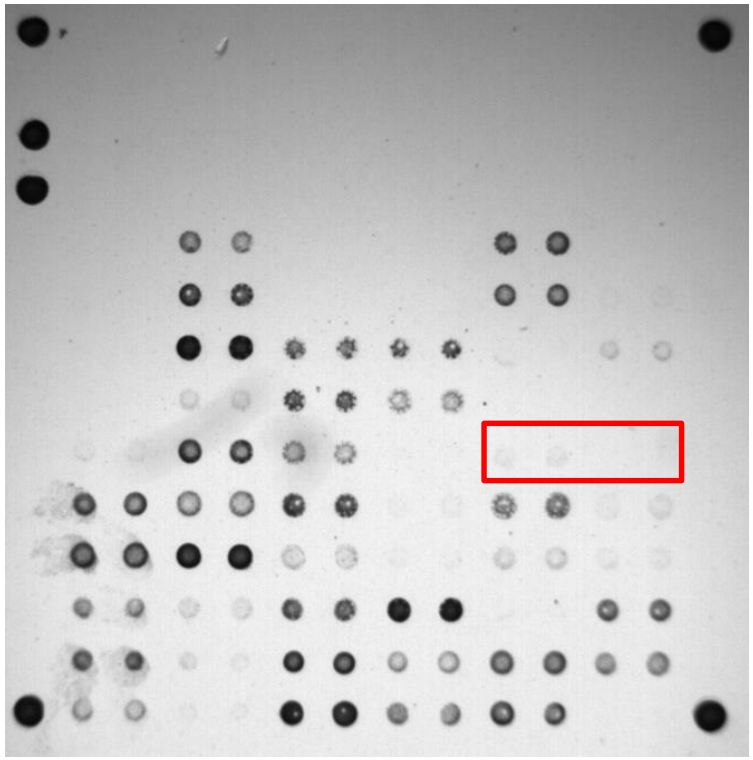


Figure 3.10b: AT image for isolate B7

Figures 3.10a & 3.10b: AT images for isolates B1 and B7 from patient 8. Highlighted are the hybridisation markers for the *exoS* and *exoU* variable genes. There is no visible hybridisation at either *exoS* or *exoU* markers in figure 3.10a. There is faint hybridisation at the *exoS* marker in figure 3.10b.

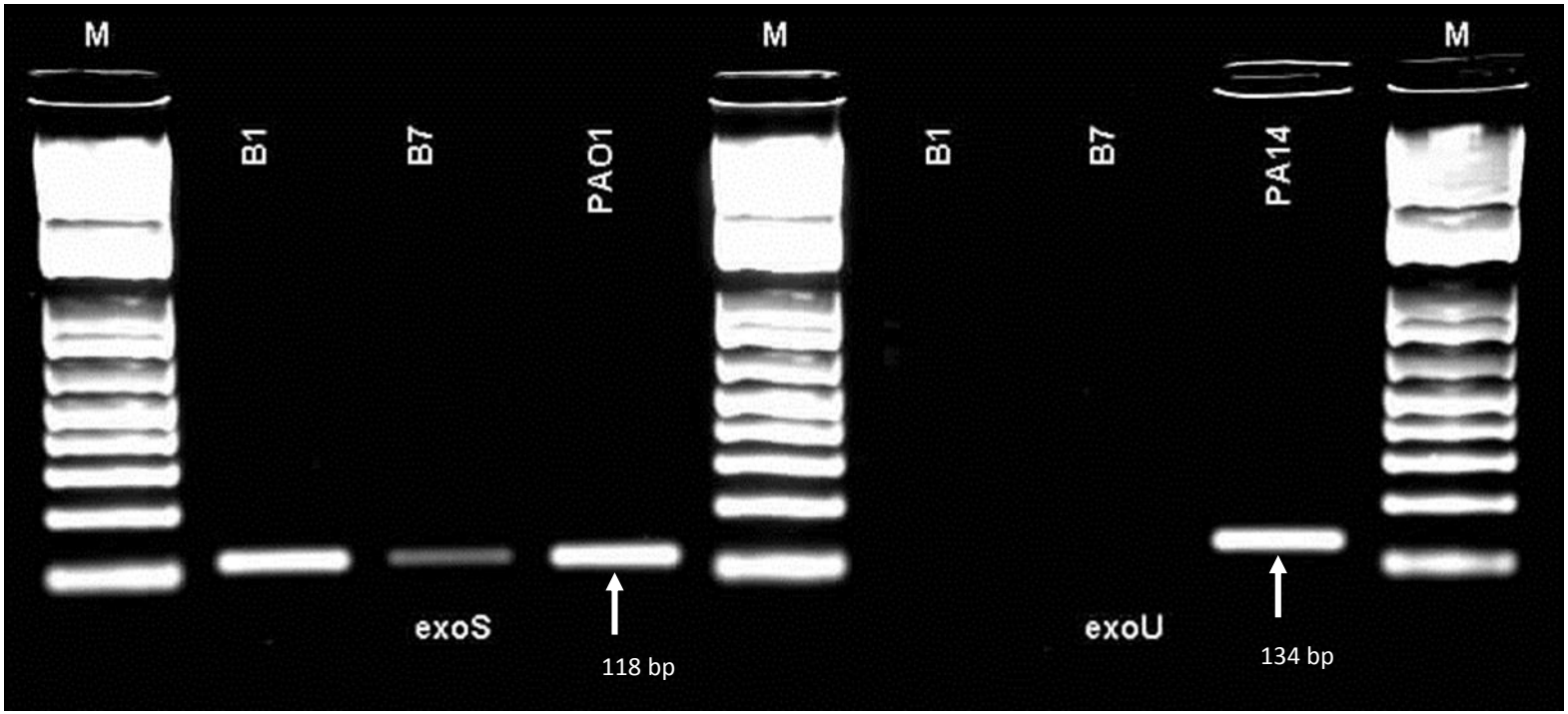


Figure 3.11: PCR assay using primers to detect *exoS* and *exoU* variable genes performed on isolates from patient 8 to confirm hexadecimal AT code with PAO1 and PA14 serving as positive controls respectively. Both B1 and B7 appear show strong amplification of exoS regions and no amplification of *exoU* region. Gel electrophoresis performed on 1.5% agarose gel at 50 V for approximately 2 h. Marker: 1 KB+ DNA ladder (Invitrogen).

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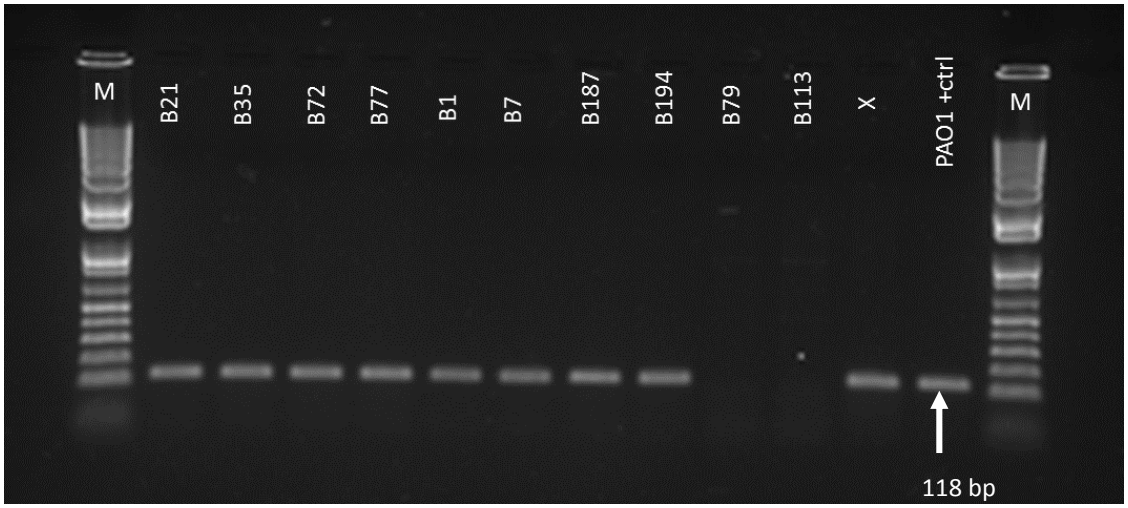

Figure 3.12a: PCR assay to determine presence of *exoS* variable gene in *P. aeruginosa* isolates. PAO1 was used as a positive control. Isolate X was assayed in the same run as other isolates but is not part of this study. Marker: 1 KB+ DNA ladder (Invitrogen).

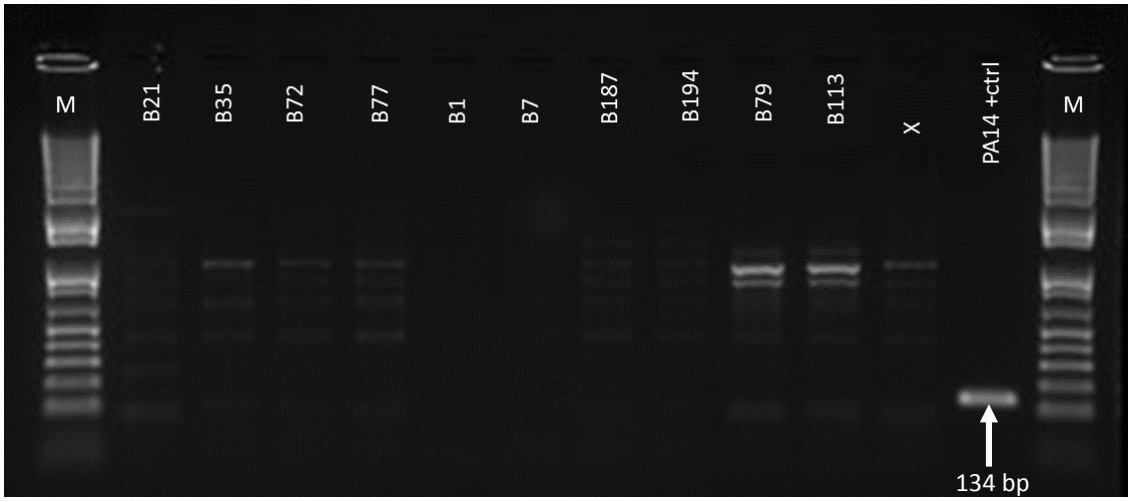

Figure 3.12b: PCR assay to determine presence of *exoU* variable gene in *P. aeruginosa* isolates. PA14 was used as a positive control. Isolate X was assayed in the same run as other isolates but is not part of this study. Marker: 1 KB+ DNA ladder (Invitrogen).

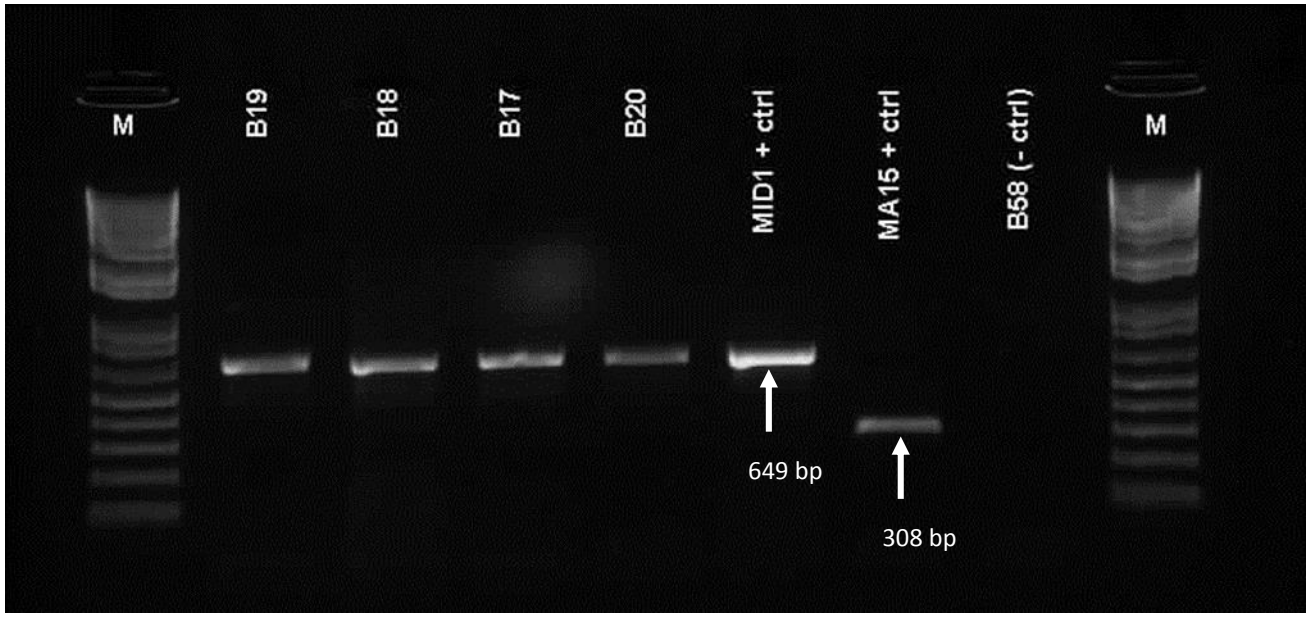

Figure 3.13: Multiplex PCR assay using primers to determine presence of MES and Midlands 1 epidemic strain of *P. aeruginosa*. All 4 isolates tested come from patient 10 and appear to be positive for MID1. Gel electrophoresis performed on 1% agarose gel at 90 V for approximately 1 h. Marker: 1 KB+ DNA ladder (Invitrogen).

3.4 Discussion

3.4.1 Maintenance of *Pseudomonas aeruginosa* strains in non-cystic fibrosis bronchiectasis patients

There have previously been very few studies investigating populations of *P. aeruginosa* among NCFBr patients. In fact, there have been very few long-term studies investigating the progression of NCFBr as a disease. Studies that have been performed (Wilson *et al.* 1997; Loebinger *et al.* 2009), though often not published in recent years, implicate *P. aeruginosa* chronic infection in an increase in morbidity and mortality. In early studies *P. aeruginosa* infection was shown to lead to increased disease progression, with a more rapid decline in lung function (Evans *et al.* 1996; Wilson *et al.* 1998), although the role of *P. aeruginosa* as a source or marker of disease progression was unclear. The study by Loebinger *et al.* (2009) examined a large number of variables in disease progression, and those that were determined not to be independently predictive were discarded to provide a multivariate modelling strategy to draw mechanistic conclusions from, as opposed to a predictive tool for individual patients. By this method Loebinger *et al.* (2009) suggested that *P. aeruginosa* infection in NCFBr patients was more likely to impact on survival than to act as a marker of severity of disease. It is, therefore, important to understand the population of *P. aeruginosa* within NCFBr patients in order to better understand disease progression, given that there are many known mutations associated with chronic infection in CF patients (Smith *et al.* 2006; Amiel *et al.* 2010; Bjarnsholt *et al.* 2010; Manos *et al.* 2013).

A small longitudinal study of isolates from NCFBr patients was undertaken by De Soyza *et al.* (2014) as a part of a study investigating the occurrence of cross-infection in NCFBr patients from a single adult clinic. Aside from this, there has been no further study into the maintenance of strains of *P. aeruginosa* in chronically infected NCFBr patients. This work builds upon the data from De Soyza *et al.* (2014) to provide evidence of maintenance of clonal strains of *P. aeruginosa* in NCFBr patients.

In 15 of the 20 patients from whom isolates were collected for this study, the same strain of *P. aeruginosa* was maintained over a mean (range) period of 40 months (12 – 84 months), indicating that for the majority of patients infection with the same strain can be maintained for considerable periods of time. Two of these patients, 9 and 21 (table 3.1), were chronically infected with *P. aeruginosa* clone A (D421). Isolates from patients 9 (B10 and B16) were not only categorised as the same clonal type of *P. aeruginosa* by analysis of the SNPs and variable genes on the AT chip, but they also shared an identical hybridisation pattern for the markers used to represent the accessory genome (genomic islands and further variable genes). Isolates B141 and B150 from patient 21 shared AT codes but varied in hybridisation at the variable gene and genomic island markers. The early and late isolates for patient 21 (B141 and B150 respectively) were collected 33 months apart. Although the strain type appears to have been maintained, the data suggest that the accessory genome of the strain has undergone some changes. The late isolate has lost a genomic island and a variable gene that was present in the early isolate but also appears to have gained a genomic island not present in the early isolate. These changes in the accessory genome are most likely evidence of the occurrence of horizontal gene transfer between *P. aeruingosa* strains within the lungs of the patient. The early isolate (B141) was positive for both pyoverdine receptor types 3 and B variable genes (figure 2.2) whereas the late isolate (B150) is only positive for pyoverdine receptor type B (Stover *et al.* 2000; de Chial *et al.* 2003). The late isolate also exhibits no hybridisation at the fla-2 *orfA* and fla-2 *orfJ* genomic island markers, which indicates a loss of the genomic island essential for glycosylation of flagellin (Arora *et al.* 2004) and shows hybridisation at the C47 marker, which indicates the presence of a strain-specific gene island into a $tRNA^{Gly}$ gene (Larbig *et al.* 2002).

These apparent changes in the accessory genome could be explained in a number of different ways. It is possible that the infecting strain population has changed due to environmental factors driving adaptation and evolution of the *P. aeruginosa* populations, as has been described in a number of CF studies (Thomas *et al.* 2000; Ciofu *et al.* 2005; Smith *et al.* 2006; Fothergill *et al.* 2010a). This may be due to new regimens of treatment, changes in patient lifestyle, other members of the

microbial community, host responses or other factors influencing the conditions present in the lung environment in which the bacteria thrive. However, it has been reported in CF that during chronic infections, *P. aeruginosa* populations diversify both phenotypically and genomically (Mowat *et al*. 2011; Workentine *et al.* 2013; Williams *et al.* 2015). It has been shown that genomic instability, in particular the transfer of genetic material between strains, can lead to misleading results when various genotyping methods are used (Fothergill *et al.* 2010a; Worby *et al.* 2014). These observations also reflect the intra-population diversity that occurs in CF, with accessory genome variations very common. It has been demonstrated in CF patients that multiple divergent lineages of *P. aeruginosa* can co-exist within a single chronically infected patient. Work by Williams *et al.* (2015) sought to investigate the diversity of *P. aeruginosa* isolates within CF patients using whole genome sequencing of 40 isolates from 9 patients. Initially, one patient was found to have two divergent lineages (A and B) sharing a common ancestor. Lineage A was characterised by 55 shared SNPs, whilst lineage B shared 24 SNPs. A difference of 79 SNPs separated the co-existing lineages, however only 42 SNPs separated the most recent common ancestor (MRCA) from the earliest known LES (LESB58), used as a reference genome. In addition to this patient, two divergent lineages were found to be present in a further 6 of the 8 remaining patients, indicating that the plasticity of the *P. aeruginosa* genome may regularly allow for in-patient diversity of chronic strains (Williams *et al.* 2015). Hence, the observations in this study could reflect the fact that, as in CF, *P. aeruginosa* populations in the NCFBr lung can diversify and exhibit genomic instability. Due to the analysis of only a single isolate per patient, per time point in this study it is not possible to confirm this explanation of accessory genome variation from the data obtained.

One other patient (14) was also infected with *P. aeruginosa* clone A (D421), although in this case clone A was only present in the late isolate collected 24 months after the early isolate, and varied from the early isolate by 6 SNPs in the AT binary code. The hybridisation of variable genes and genomic islands in this isolate (B49) were notably different to those of any of the other four isolates with the D421 AT code, indicating that these isolates were likely not shared among the patients and

were acquired environmentally, which is unsurprising given the prevalence of clone A in the environment among the general population of *P. aeruginosa* (figure 3.14).

3.4.2 *Pseudomonas aeruginosa* strains from non-cystic fibrosis bronchiectasis patients in the context of the wider CF population

There have been many attempts to elucidate the population structure of *P. aeruginosa* in an attempt to better understand the acquisition of bacterial infection and the pathogenicity of strains found in clinical isolates although there is a general consensus that clinical isolates of *P. aeruginosa* are generally indistinguishable genotypically, chemotaxically, and functionally from environmental isolates (Pirnay *et al.* 2002). It was noted by Römling *et al.* (1994) that the major clone (C) identified in CF patients (28%) was also present at a high frequency in aquatic environments (21%). By use of outer membrane lipoprotein gene sequences (*oprI, oprL,* and *oprD*), DNA fingerprinting, serotyping, and pyoverdine type Pirnay *et al.* (2002) concluded that *P. aeruginosa* has an epidemic population structure, similar to that of *Neisseria meningitidis* (Feil *et al.* 2001), comprised of a number of widespread clones originating from a larger number of unrelated genotypes. These clones are abundant and widespread throughout the natural environment, with no discernible correlation between habitat or geographical location and clone type, and are therefore expected to be prevalent in clinical isolates. Investigation into the population structure of *P. aeruginosa* was later revisited by this group in 2009, using a wide range of traits to analyse a network of relationships between 328 unrelated *P. aeruginosa* isolates. This work confirmed the nonclonal epidemic population structure; providing a view of a superficially clonal structure in which frequent recombination occurs and successful epidemic clones occasionally arise (Pirnay *et al.* 2009).

Further work carried out by Cramer *et al.* (2012) also confirmed this population structure among a large database of *P. aeruginosa* isolates from CF centres, aquatic environments, patients with ventilator associated pneumonia (VAP), keratitis, or patients with COPD. There was no evidence of a widespread transmissible clone among CF patients, and the five most common clones found in CF patients were found to be among the ten most common clones found in the

environment and in VAP, COPD, and keratitis (Cramer *et al.* 2012). Conversely it has been demonstrated that certain genetic characteristics are implicated in the adaptation of *P. aeruginosa* to cause corneal infections (Stewart *et al.* 2011; Shankar *et al.* 2012). A set of 63 isolates underwent AT genotyping and were analysed by Stewart *et al.* (2011). Using eBURSTv3 it was shown that the majority of keratitis isolates were represented in a closely related sub-group of *P. aeruginosa*. Twitching motility, due to type IV pili, in *P. aeruginosa* is associated with keratitis infection and 46% of isolates with the most common serotype found in keratitis (O11) were found to carry a distinctive *pilA* which encodes the pilin of type IV pili. This indicates that keratitis isolates are associated with specific characteristics and, therefore, that a subpopulation of *P. aeruginosa* is adapted to cause corneal infection (Stewart *et al.* 2011). Work by Shankar *et al.* (2012) also investigated clustering of *P. aeruginosa* isolates from keratitis infections. A total of 123 isolates were collected over two different time periods (2003 – 2004 and 2009 – 2010) and analysed using the AT genotyping system. When compared to a database of isolates from a non-ocular origin it was discovered that 71% of the UK keratitis isolates clustered together, with no major evidence for variations in the distribution of the clone types between the two collections. The "core keratitis cluster" appeared to be related to the *P. aeruginosa* eccB clonal complex which is associated with an adaptation to survive in water, suggesting that adaptation to an aquatic environmental habitat is a key factor in the ability of *P. aeruginosa* isolates to cause ocular infections (Shankar *et al.* 2012).

There appeared to be no such clustering or associations within this collection of NCFBr isolates. The eBURST diagrams in figures 3.6b and 3.7 show the distribution of genotypes found in NCFBr isolates and 40 common clone types (Wiehlmann *et al.* 2007) respectively among the general population of *P. aeruginosa* isolates from a compiled dataset (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2013; Hall *et al.* 2014; De Soyza *et al.* 2014). Comparison of the two diagrams shows that the distribution of NCFBr isolates is similar to that of the abundant and widely distributed clones described by Wiehlmann *et al.* (2007), indicating that there are no widespread epidemic clones present in the NCFBr population, much like the population of CF isolates. Figure 3.14 shows the sources

from which genotypes found in this study have previously been isolated. Many genotypes have been isolated from multiple sources, both clinical and environmental, although genotypes 059A, 0C4A, and AC2E have only previously been isolated in patients with CF (figure 3.14). The 16 most common of the 40 clones described by Wiehlmann *et al.* (2007) (those with a frequency of ≥1% in the compiled dataset) were generally isolated from a wide range of sources, with a broad geographical spread.

Some isolates with novel genotypes (meaning they had not previously been described in the compiled dataset) were also present in this NCFBr data. Six genotypes (3C28, 6852, CC60, 4C8A, 682A, and 682E) from isolates in five patients had no matches with isolates previously identified by the AT genotyping method. Genotypes 6852 and CC60 are represented in figure 3.6b as single nodes, distributed radially away from the main cluster of genotypes whereas the remaining novel genotypes are present in the main cluster. Genotype 3C28 is only present in the early isolate of patient 14. The late isolate from this patient was collected 24 months later and was identified as clone A (D421). It is possible that patient 14 acquired a unique strain of *P. aeruginosa* (*i.e.* ST 3C28) independently from the environment which was subsequently replaced by the common clone A. It is also possible that this patient was continuously co-infected with both strains, but by sampling only one isolate per patient per time point we are unable to determine the exactitudes of this patient's infection.

Clone P (6C2A) was almost exclusively found in CF patients from Germany and the UK, with one isolate being collected from an environmental water source in Belgium (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al.* 2012; Hall *et al.* 2013; Hall *et al.* 2014) but was not found to be present in the NCFBr data. Isolates of this clone type were collected from CF patients at clinics in Hannover, Jena, and Frankfurt between 1996 and 2001 (Wiehlmann *et al.* 2007), and from CF patients in Liverpool, UK (Hall *et al.* 2013). Although there is no evidence of clustering of *P. aeruginosa* isolates from CF patients, the limited sources from which this clone has been previously isolated is interesting. It is possible that it does not fall within the "core keratitis cluster" as described by Shankar *et al.* (2012) and so is not adapted to

cause ocular infection, and therefore is not recovered from keratitis patients. There may also be sampling bias in the compiled dataset, in that many of the isolates were collected in order to study *P. aeruginosa* populations within CF patients or clinics and so the clone appears to be recovered more frequently from CF patients than the environment. In the case of this study, isolates were collected from patients attending only one bronchiectasis service which is geographically separate from the regional CF centre. It is possible that in a study including a wider range of bronchiectasis services, including some which share facilities with a CF service, that this clone would also be recovered from NCFBr patients.

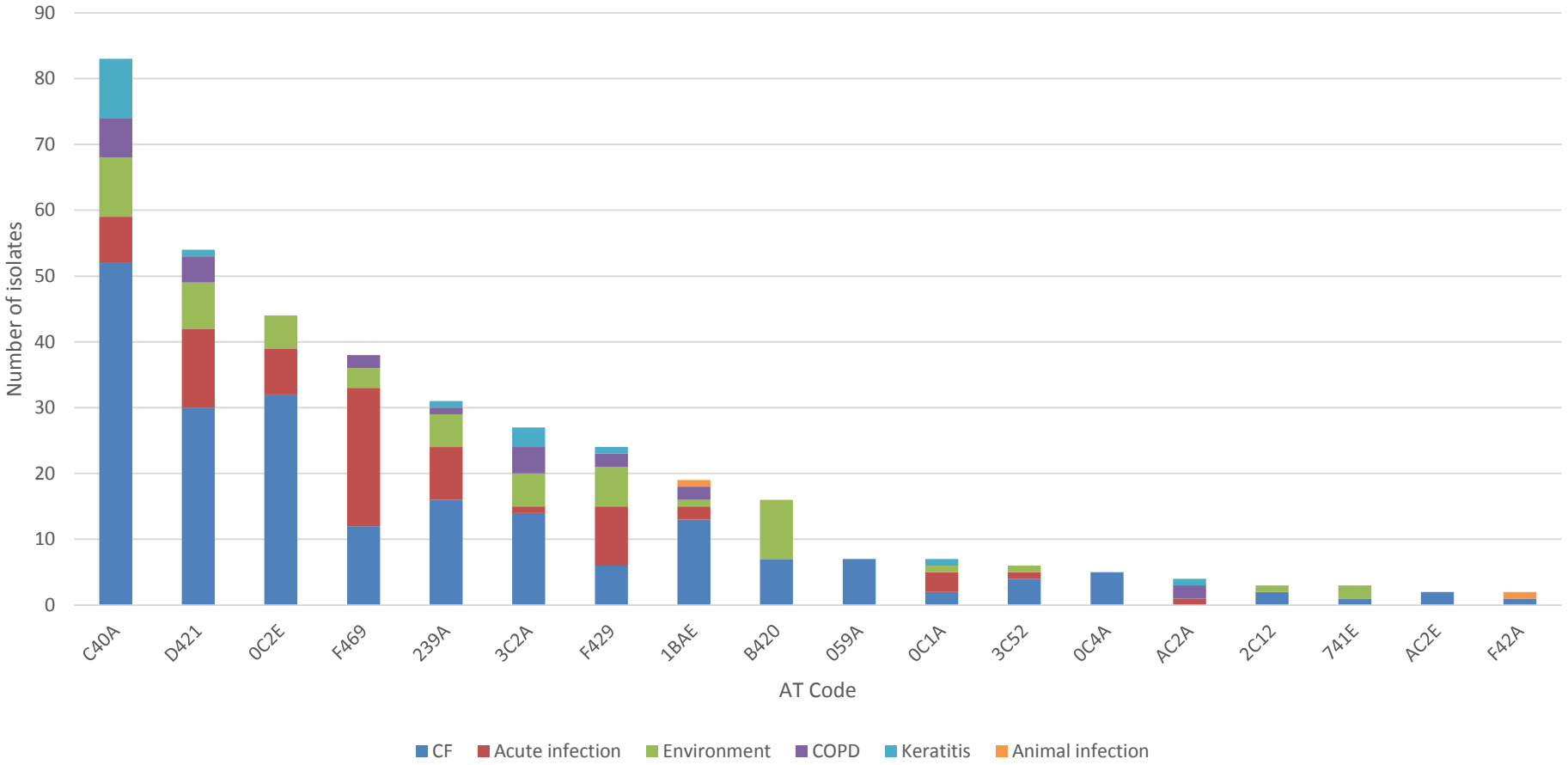

Figure 3.14: Sources of genotypes identified in this study that have previously been identified in the compiled dataset (Wiehlmann *et al.* 2007; Cramer *et al.* 2012; Shankar *et al*. 2012; Hall *et al*. 2014). Genotypes which showed no match with the compiled dataset were not included.

3.4.3 Epidemic strains of *P. aeruginosa* in NCFBr patients

Transmissible (epidemic) strains of *P. aeruginosa* have been a major concern in CF units, and they can have severe consequences in terms of morbidity and mortality. After the initial description of the LES by Cheng *et al.* (1996) further work was carried out to investigate the possibility of superinfection by transmissible strains of *P. aeruginosa*. Four adult patients who had previously been colonised with unique strains of *P. aeruginosa* were studied after transferring from either a local paediatric CF centre or a distant hospital CF clinic to the regional adult centre (McCallum *et al.* 2001). Longitudinal monitoring of sputum samples from these patients revealed that after inpatient stays they had become superinfected with the strain referred to as AH (LES). In two of the four patients, after a number of months only strain AH was cultured from sputum samples. In the case of the remaining two patients other strains that had previously been isolated from sputum samples were also present alongside strain AH (McCallum *et al.* 2001). Once it had been established that superinfection by LES was a threat among mixed populations of *P. aeruginosa*-positive individuals it became important to discover the effects of infection with an epidemic strain on patient health and survival. It was shown by Al-Aloul *et al*. (2004) that a group of CF patients infected with the LES experienced a greater deterioration in pulmonary function and nutritional state than paired LES-negative patients. Chronic infection with LES also led to an increased treatment burden on the affected patients. Given that LES tends to be multiresistant to antipseudomonal antibiotics this increased burden is important as it has been shown that the repeated use of antibiotics to which the strain is susceptible are nephrotoxic and cause renal damage in CF patients (Al-Aloul *et al.* 2002). Antibiotic resistance tends to increase in both LES and other strains of *P. aeruginosa* over time in chronically infected patients, although the LES has been found to exhibit significantly higher levels of resistance in collected isolates (Ashish *et al.* 2012).

Very little is known about cross infection in relation to NCFBr patients infected with *P. aeruginosa*. In this cohort of patients, no known UK CF transmissible strains were found. As part of initial screening, all isolates were assayed by PCR for LES, Midlands 1 (Scott & Pitt 2004), and Manchester epidemic strain (Jones *et al.*

2001) (table 2.1) and all 4 isolates collected from patient 10 tested positive for MID1, the marker for the Midlands 1 transmissible strain (figure 3.13). However, the Midlands 1 strain isolates assayed as part of work by Smart (2007) correspond to AT code 0C1A whereas isolates B17 and B20 were assigned code 741E. The MID1 primers were designed using suppression subtractive hybridisation (SSH) by Smart *et al.* (2006) as a diagnostic test for use in CF centres to rapidly identify patients with epidemic strains of *P. aeruginosa*. Through the use of SSH, sequences from the accessory genome of various *P. aeruginosa* strains were compared to identify sequences unique to the Midlands 1 strain. The MID1 primers (table 2.1) target a bacteriophage-related site-specific recombinase (Smart, Walshaw, et al. 2006). The observations presented here suggest that the selection process used was not 100% effective at ensuring that the target sequence was specific for the Midlands 1 strain. This is the first reported example of a false PCR-positive for the Midlands 1 strain and it suggests that there are other strains of *P. aeruginosa* that share this accessory genome region. Work by Fothergill *et al.* (2010) investigated anomalous results of PCR assays to detect LES isolates from CF patients. The diagnostic PCR for LES uses two primer sets (table 2.1): PS21 and LESF9. Originally the PS21 marker was tested alone as a marker for LES-positive isolates, but false positives were recognised (Lewis et al. 2005; Smart, Walshaw, et al. 2006) and so the test was expanded to include the LESF9 marker (Smart *et al.* 2006a; Fothergill *et al.* 2008). Fothergill *et al.* (2010) identified four false positives and 11 false negatives for the PS21 marker from a panel of 24 isolates. Analysis also revealed 10 false positives and one false negative for the LESF9 marker (Fothergill, White, et al. 2010). There were also three false positives for both PS21 and LESF9 markers. The large size of the *P. aeruginosa* genome and, in particular, the plasticity of its accessory genome means that it is likely that bacteriophage-related sequences are shared among more than one strain of the bacteria, leading to false positives in the diagnostic PCR. The data presented here further confirms the need for using more than one typing method in order to unequivocally identify strains.

3.5 Conclusions

The main conclusions that can be drawn from this study are:

1. **In the majority of NCFBr patients with chronic *P. aeruginosa* infections, infecting strains of *P. aeruginosa* are maintained;**
2. **Strains of *P. aeruginosa* isolated from NCFBr patients are widely distributed in much the same way as the general population.**

Chapter Four

Use of genome sequencing to study the diversity of *Pseudomonas aeruginosa* isolates from non-cystic fibrosis bronchiectasis patients

4.1 Introduction

4.1.1 Diversity of *Pseudomonas aeruginosa* isolates in cystic fibrosis patients

There have been many studies undertaken into the genetic diversity of *P. aeruginosa* isolates in cystic fibrosis (CF) patients, using many different methods (Hall *et al.* 2013), in order to establish the prevalence of certain strains of *P. aeruginosa* (Cramer *et al.* 2012), investigate the methods by which the bacterial population adapts from an environmental habitat to the lungs (Marvig *et al.* 2014), and the effects of selective pressures, such as the administration of intravenous antibiotics, on *P. aeruginosa* populations (Fothergill *et al.* 2010a). The diversity of *P. aeruginosa* populations can be studied at different levels, such as within-patient, inter-patient, and both nationwide and globally (Freschi *et al.* 2015), and using techniques of varying degrees of depth.

During chronic infection *P. aeruginosa* exhibits both adaptation and diversification, and the mechanisms underlying many of the chronic-stage adaptations, such as the switch to a mucoid phenotype and a loss of motility (Govan & Deretic 1996; Goodman *et al.* 2004), have been well established. The acquisition of mutations in important regulatory genes can happen rapidly following initial infection in the CF lung environment (Wilder *et al.* 2009; Rau *et al.* 2010). The conditions experienced by bacterial populations living within the CF lung environment are the drivers of these mutations, which can confer a selective advantage in niche environments. Mutation

commonly occurs in the gene encoding the quorum sensing regulator LasR. In comparison with wild type strains *lasR* mutants exhibit a significant metabolic shift with decreased oxygen consumption and increased nitrate utilisation, both of which are predicted to offer increased fitness in the nutrient conditions provided in CF lungs (Hoffman *et al.* 2010). This shift by the *lasR* mutants also conferred increased resistance to tobramycin and ciprofloxacin, both of which are commonly used in CF care. Selection for resistance in these mutants *in vitro* did not require previous antibiotic treatment. Increases in antibiotic resistance among strains of *P. aeruginosa* have been previously reported, particularly among the Liverpool Epidemic Strain (LES) (Salunkhe *et al*. 2005; Ashish *et al.* 2012) and among hypermutator strains that are commonly found in CF patients (López-Causapé *et al.* 2013). To further investigate the genotypic changes experienced by *P. aeruginosa* isolates inhabiting the CF lung the genomes of 12 isolates, including 4 environmental isolates, underwent whole genome sequencing and comparative genomic analysis (Stewart *et al*. 2014). Paired nonmucoid and mucoid isolates from three separate patients were compared. Analysis revealed that two of these three pairs were derived from the same strain and that the third pair was not, suggesting that this patient may be suffering either a multi-strain infection or undergoing a strain succession event (Stewart *et al.* 2014). One of the related pairs of isolates was found to be near-isogenic but it was noted that the mucoid strain had a smaller genome than that of its nonmucoid progenitor. Significant evidence for genome shrinkage during bacterial adaptation to a host has previously been observed (Toft & Andersson 2010) and it is possible that shrinkage occurred in the case of this pair of isolates. Notably the mucoid isolate is lacking the filamentous phage PF1 and the *exoY* gene, due to a 65 kbp deletion relative to its putative parental strain (Stewart *et al.* 2014). The core genomes of 55 isolates, including the 12 sequenced isolates, were also analysed using a core genome threshold of 55 and sequence identity of 90%. Only one isolate of these 55 was found to be closely related to taxonomic outlier PA7 (Roy *et al*. 2010) and the remainder fell into one of two major subgroups of *P. aeruginosa* known as group 1 and group 2 (Stewart *et al.* 2014). Group 1 was the larger of these two groups and contains notable

strains DK2, PAO1, LESB58, and PAK. Group 2 is significantly less populous than group 1 and contains PA14. Isolates from all sources, including CF sputum, were found to be well represented in both groups but at present it is unclear whether group 1 isolates are naturally more abundant than group 2 isolates or if a bias has been introduced due to selection of strains for sequencing projects (Stewart *et al.* 2014).

Much of the previous work undertaken to investigate changes in *P. aeruginosa* populations in CF patients has been designed to reveal the genetic changes over the course of a longitudinal study, meaning that often only single isolates are taken per patient and analysed at the cost of sampling depth. Evidence from phenotypic studies has suggested that there is widespread heterogeneity among isolates from individual patients (Mowat *et al.* 2011; Workentine *et al.* 2013) and so extensive sampling for whole genome sequencing was undertaken to further understanding of the diversity of *P. aeruginosa* isolates from CF patients. A total of 360 isolates were collected from 9 patients attending an adult CF centre in Liverpool, UK to undergo whole genome sequencing in order to ascertain the diversity among 40 isolates per patient sputum sample. The patients were all known to have been LES positive for a number of years prior to the study (Williams *et al.* 2015). Isolates from all nine patients were analysed, revealing that 7 of the 9 patients harboured coexisting, divergent lineages of the LES. These divergent lineages were typically found to be more closely related to lineages found in other patients than to each other. Other studies have also highlighted within-population variation for *P. aeruginosa* during CF infections (Darch *et al.* 2015; Diaz Caballero *et al.* 2015; Jorth *et al.* 2015). There are obvious clinically-relevant consequences to this. It has been shown that conventional antimicrobial susceptibility tests, which rely on a single isolate to represent infection, are not accurate predictors of response to therapy (Smith *et al.* 2003) and this may be in part due to the apparent diversity of isolates within chronically infected patients (Williams *et al.* 2015).

A study carried out in a Danish CF centre (Marvig *et al.* 2014) sought to further understanding of how *P. aeruginosa* evolves during long-term infection in human hosts by sequencing whole genomes of 474 isolates collected from the airways of 34 children

and young adults with CF (aged between 1.4 and 26.3 years). The collection of isolates was intended to give insight into the longitudinal progression of the early stages of *P. aeruginosa* infection and so the initial and several subsequent isolates from each patient were selected for sequencing. From these 474 isolates 53 genetically distinct clonal groups were identified. Genomes from isolates with the same clone type differed on average by 122 single nucleotide polymorphisms (SNPs) (median: 9 SNPs, range: 0 – 1 333 SNPs). Genomes from isolates in different clonal complexes differed by >10 000 SNPs. 10 clone types were identified that were present in multiple patients. In 19 of these patients the genomes of the isolates were found to be separated by at least 50 SNPs and, taking into account the average mutation rate of *P. aeruginosa* within patients (2.6 SNPs/year) (Marvig *et al.* 2013), it was concluded that the presence of the same clone type in these patients was unlikely to be due to recent or direct transmission between the patients. Alternatively, the presence of these genetically distant clonal isolates may be due to transmission from a patient not included in this study or as a result of independent acquisition of a prevalent clone from the environment (Marvig *et al.* 2014). In a few cases (5 patients) the difference between clonal isolates from different patients were only a few SNPs (range = 0 – 29) which suggested the occurrence of recent interpatient transmission events. Information on patient visits was retrieved and confirmed that in all cases of suspected patient-to-patient transmission there were overlaps in the patients' time spent in the hospital. Heterogeneity of *P. aeruginosa* within patients can mislead attempts to elucidate the direction and source of transmission (Worby *et al.* 2014), although it is suspected in this case that the clone types were transmitted from the older patients, in whom the clones were identified first, to the younger patients.

The diversity of *P. aeruginosa* isolates among CF patients varies worldwide. Many different studies, covering centres across various countries, have drawn conclusions about the precautions necessary for infection control within their respective CF centres. In one study in the UK the majority of patients were found to harbour their own unique strains of *P. aeruginosa* although around one fifth of those sampled carried one of two

epidemic strains (LES and Midlands 1) (Scott & Pitt 2004). A third common genotype previously identified in Germany (Dinesh *et al*. 2003), clone C, was also widespread in UK CF centres. The LES accounted for 11% of isolates in this study and was found to be present in 48% of centres surveyed. The Midlands 1 strain was also highly prevalent and accounted for 10% isolates, being found in 29% of centres. The findings were unexpected but provided strong evidence of cross-infection both within and between UK CF centres (Martin *et al*. 2013). More recently whole genome sequencing has been used to further understanding of the LES. The genomes of seven LES (UK) and LESlike (Canada) isolates were sequenced in order to undertake comparative genomic analysis (Jeukens *et al.* 2014).In six of the seven isolates at least one large deletion (40 – 50 kbp) was revealed in comparison to the LESB58 reference genome. These deletions were shown to correspond to prophages which have been previously shown to increase the competitiveness of LESB58 in chronic lung infections (Jeukens *et al.* 2014). A further 308 non-synonymous polymorphisms were identified, of which 28 were found to be associated with virulence determinants and 52 with regulatory proteins. The majority of the regulatory mutations were shown to be isolate-specific and 29% were predicted to have a high functional impact implicating polymorphism in regulatory genes in variations in phenotype between LES isolates (Jeukens *et al.* 2014).

Two separate studies were carried out in different provinces in Canada (Ontario and British Columbia) and arrived at differing conclusions regarding the risk of interpatient transmission. In Ontario 22% of 446 patients were found to be infected with one of two transmissible strains. One strain (LES) was found to be present in 15% of patients and the second strain, not previously identified as epidemic, was found in 7% of patients; 3 patients (0.6%) were found to harbour both of these strains (Aaron *et al.* 2010). Conversely, in British Columbia there were 157 genotypes identified from isolates collected and 123 of these were unique to individual patients. Only 34 types were shared by two or more patients and these were proved to be epidemiologically linked only in the case of 10 pairs of siblings and one unrelated pair of patients. It was concluded that the risk of transmission of *P. aeruginosa* between CF patients in British Columbia was

low (Speert *et al.* 2002). However, Canada is a vast country and both provinces are large (Ontario: >1 M km^2; British Columbia: >900 K km^2) and sparsely populated, and separated by a distance of over 1000 km meaning that differences in CF patient experience are not completely unexpected.

Patients in Australia underwent a large-scale study to investigate *P. aeruginosa* populations across the country. More than 60% of patients sampled were found to harbour a strain of *P. aeruginosa* indistinguishable from those found in at least one other patient. Small areas of clustering were found among populations in small centres but AUST-01 and AUST-02 strains were found to be highly prevalent across all centres; all 18 centres surveyed were found to have either one or both of these strains present (Kidd *et al.* 2013). Greater than 40% of patients were found to be affected by either AUST-01 or AUST-02. Those harbouring AUST-01 attended healthcare facilities more frequently than those patients infected with unique strains of *P. aeruginosa* which indicates that certain genotypes are causing widespread cross-infection in centres across Australia (Kidd *et al.* 2013). In the Netherlands a large study of two centres, serving 45% of the Dutch CF population, revealed the prevalence of two *P. aeruginosa* sequence types (ST406 and ST497) which were found in 15% and 5% respectively of the 265 patients from whom *P. aeruginosa* was cultured (van Mansfeld *et al.* 2009). Neither of these sequence types were genetically linked to previously described epidemic strains. Of the patients surveyed, 60% were found to harbour a strain of *P. aeruginosa* also found in at least two other patients (van Mansfeld *et al.* 2009).

In Belgium 163 *P. aeruginosa* genotypes were found from 213 patients across all 7 Belgian CF centres; 75% of these patients harboured only one genotype and 80% of patients maintained the same genotype over a 1 year period. Limited clustering of isolates was discovered and therefore it is unlikely that cross-infection is a common occurrence among Belgian CF patients (Van daele *et al.* 2006). There was also no strong evidence of nationwide cross-infection in New Zealand. DNA fingerprinting of 496 *P. aeruginosa* isolates from 102 patients revealed only one cluster of related isolates that was significantly more prevalent than expected in one centre. This cluster involved

isolates from 9 patients, 7 of whom attended the same centre. These 7 patients from the same centre were also revealed to have had more interpatient contact with one another than other patients, unconnected with the cluster, in the same centre. Analysis showed that this clustering was likely due to healthcare-related transmission (Schmid *et al.* 2008). Some previously-identified transmissible strains from the UK and Australia were identified among patients who had previously lived in or visited these countries, although there was no evidence of transmission from these patients to others (Schmid *et al.* 2008).

Patient care standards for CF centres vary around the world and so, therefore, does the population structure of *P. aeruginosa* among CF patients. In areas where transmission within and between centres is a common occurrence (UK, Australia, Ontario) it is necessary to implement segregation of patients based not only on their *Pseudomonas* status, but also based on the strain identified from sputum samples. However these measures can be costly and time-consuming to implement so areas where inter-patient transmission is not a major health risk to CF patients it may be more prudent to continue with current segregation measures.

The relatedness of CF *P. aeruginosa* isolates from within patients, within centres, and between centres has been well studied; although further depth of study can always be implemented. Inter-relatedness of *P. aeruginosa* isolates in non-CF bronchiectasis (NCFBr), along with many aspects of the disease, remains underfunded and understudied.

4.1.2 Diversity of *Pseudomonas aeruginosa* isolates in non-cystic fibrosis bronchiectasis patients

Much of the research into *P. aeruginosa* populations has been carried out with regards to CF patients, with little being done to resolve the population biology of infection within other chronic lung diseases. Some work has been previously carried out to investigate *P. aeruginosa* infections in chronic obstructive pulmonary disease (COPD). *P. aeruginosa* infection is more likely to be associated with advanced COPD and is

associated with the symptoms of exacerbation, although infections in COPD follow one of two possible patterns: (1) short-term colonisation followed by clearance or (2) long-term persistence. Persistence of a strain of *P. aeruginosa* in COPD is associated with the development of serum antibody response but this response, in turn, is not associated with clearance of the infection (Rakhimova *et al.* 2009). In patients where the infection persists *P. aeruginosa* is found to diversify, leading to co-existing isolates with varying morphotypes and antibiotic susceptibilities(Martínez-Solano *et al.* 2008). Work by Martínez-Solano *et al.*(2008) showed that chronic isolates from COPD patients had similar phenotypic profiles to those from chronic CF infections, *i.e.* an increased mutation rate, increased antibiotic resistance, reduced cytotoxicity, reduced motility, and greater biofilm production. On the basis of these results it was suggested that *P. aeruginosa* may cause chronic infections in COPD in much the same manner as in CF. However, the data collected over a 10 year period by Rakhimova *et al.*(2009) showed that a much lower percentage (17.9%) of the COPD population became chronically infected with *P. aeruginosa*, versus 60% of CF patients (Cramer *et al.* 2012) and that the majority of patients experienced short-term or sporadic infection with strains which were sequentially replaced. Although the mechanisms of chronic infection in both COPD and CF may be similar, due to the discrepancies in the rates of chronic infection it is unlikely that the general population of *P. aeruginosa* isolates from COPD reflects the population structure of CF isolates.

Epidemiological studies have been carried out into *P. aeruginosa* populations in NCFBr, though often on a smaller scale than those undertaken in CF research. PCR fingerprinting was been used to investigate the epidemiology of 64 *P. aeruginosa* isolates collected from 17 patients over the course of a longitudinal study. Nine patients were found to have maintained the initially acquired strain of *P. aeruginosa* over a period of between 2 and 38 months. Two patients carried a single major strain which, over a period of between 4 and 18 months, acquired minor variations in genotype. Five patients lost their initially acquired strain within the first year of the study and underwent strain

replacement over a period of 2 – 22 months. One patient was found to be harbouring two distinct strains of *P. aeruginosa* at once (Pujana *et al*. 1999).

The main conclusions of the previous chapter, in which the maintenance of *P. aeruginosa* strains in NCFBr patients was investigated through the use of the array tube (AT) genotyping system, were that the majority of NCFBr patients with chronic *P. aeruginosa* infections maintained the same strain type over an extended period of time. It was also shown using the AT genotyping data that the strains of *P. aeruginosa* isolated from NCFBr patients are widely distributed in a similar population structure to that of the general population of *P. aeruginosa* strains. The AT genotyping system rapidly provides information on both the core and accessory genomes of *P. aeruginosa* isolates; however this information is still limited. Many of the adaptations of *P. aeruginosa* to the lung environment are provided by mutations in genes which cannot be detected by the AT system and so the use of whole genome sequencing in this chapter allows investigation of the diversity of *P. aeruginosa* isolates in far greater depth.

4.2 Aims

The aims of this chapter are to:

1. **Use whole genome sequencing to investigate the diversity and population structure of *P. aeruginosa* isolates from NCFBr patients attending bronchiectasis services throughout England and Wales;**
2. **Use preliminary genomic analysis of whole genome sequences to investigate the heterogeneity of *P. aeruginosa* populations within chronically infected individuals with NCFBr.**

4.3 Results

4.3.1 Panel of isolates

A collection of 191 isolates from 94 patients (appendix, table A5) attending 16 adult bronchiectasis centres throughout England and Wales were selected based on a

number of criteria to undergo whole genome sequencing using the Illumina HiSeq platform (figure 4.1). A total of 86 isolates were chosen from individual patients across all 16 centres to investigate diversity of *P. aeruginosa* isolates from centres around the country. Additionally, two isolates were included that were positive for both LESF9 and PS21 (table 2.1) by PCR assay and so were of particular interest. The remaining 102 isolates were selected from patients from whom multiple isolates had been collected on a single visit. Eighteen of the 88 isolates from individual patients were from patients who had multiple isolates collected on a single visit and so these were also included in the dataset of patient with multiple isolates collected in a single visit. These 18 isolates were combined with 58 others to give a total of 76 isolates collected from 20 patients (mean isolates per patient: 3.8; median: 3) at 10 of the 16 adult bronchiectasis centres were compared to investigate within-patient diversity of *P. aeruginosa* isolates in NCFBr patients. Three patients from Liverpool were chosen to undergo closer investigation of within-patient diversity of *P. aeruginosa* and so 15 isolates per patient were picked from direct patient sample plates to be sequenced and compared. One isolate (A138, patient 149) did not produce long enough reads to be of a high enough quality and so was excluded. The reads for a second isolate (B191, patient 30) could not be assembled, although the cause of this is yet to be confirmed, and so it was also excluded leaving a total of 189 assembled genomes for comparison.

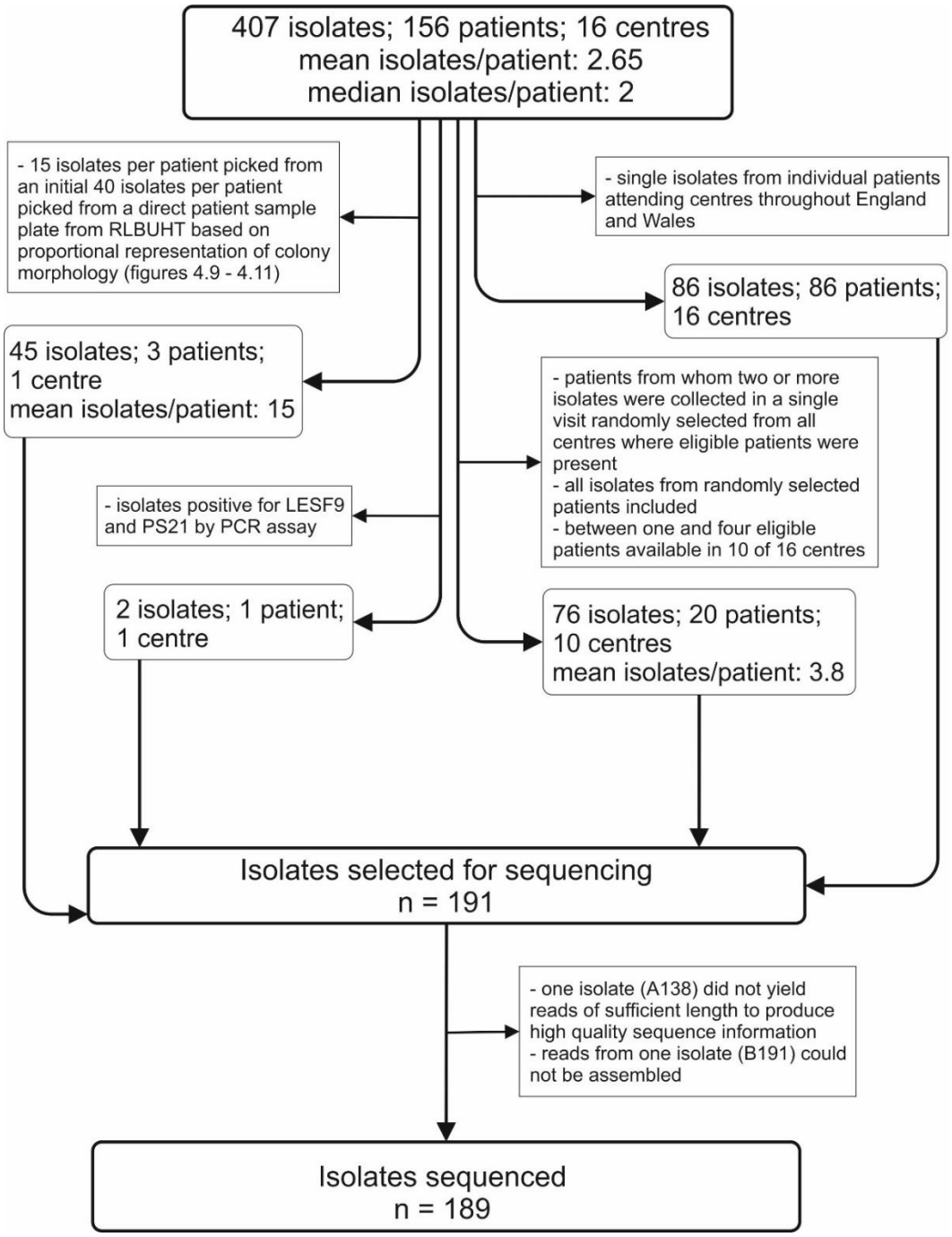

Figure 4.1: Flow diagram illustrating the selection process for *P. aeruginosa* isolates used in this study. The total number of isolates with complete genome sequences used in analysis was 189. A total of 191 isolates were selected for sequencing but two were excluded due to poor quality reads and issues with genome assembly. There is some overlap between the 86 isolates selected from individual patients and the 76 isolates selected as multiple isolates from 20 patients and this is explained in more detail in section 4.3.1.

4.3.1.1 Multiple isolates from three patients to investigate within patient diversity of *Pseudomonas aeruginosa*

In order to investigate the possible heterogeneity of multiple isolates taken from NCFBr patients further, isolates were picked from direct patient sample plates of three patients attending the Liverpool adult bronchiectasis service. Patient 147 is known to have been *Pseudomonas*-positive since at least 2004 and patient 148 since at least 2010. The direct sample plate from patient 149 from which isolates were collected is the first example of this patient culturing *P. aeruginosa* (2015). A summary of the isolates from these patients is shown in table 4.1 along with the observed phenotype of the isolate when grown on Columbia agar (Oxoid) and the ST assigned to each isolate. Isolates collected from patient 147 were observed to have one of two phenotypes, broadly described as “white” and “mucoid-1”, with white isolates growing in small, flat, white colonies and mucoid-1 isolates growing in larger colonies with typical mucoid appearance (figure 4.2). Isolates from patient 148 were also observed to have one of two phenotypes, broadly described here as “mucoid-2” and “brown”. The mucoid-2 isolates exhibited blue-green pigmentation and had a typical mucoid appearance. Isolates exhibiting the brown phenotype grew in small, brown colonies (figure 4.3). Only one phenotype was observed for isolates from patient 149, also described as “brown” and matching the description above (figure 4.4). The intention of recording observed phenotypes was to compare variations in phenotype with possible genomic variation between isolates. All 15 isolates from patient 147 were assigned ST17 and cluster closely together on the phylogenetic tree (figure 4.6), along with four isolates from patient 148 and two isolates from patients in other centres. ST17 has been previously identified as clone C and so multiple patients with isolates with this ST are unlikely to have participated in patient-to-patient transmission as it is far more likely that the environmentally abundant strain was acquired independently by these patients.

Table 4.1: Summary of multiple isolates collected from three patients attending the adult bronchiectasis service in Liverpool. A total of 15 isolates were randomly selected per patient but inadequate read lengths for the genome of one isolate from patient 149 meant that the genome was unable to be assembled and so this isolate has been excluded.

Isolate	Patient	Phenotype	ST
A46	147	Mucoid-1	17
A48		Mucoid-1	17
A52		Mucoid-1	17
A53		White	17
A54		Mucoid-1	17
A55		White	17
A56		White	17
A58		White	17
A60		White	17
A70		Mucoid-1	17
A71		Mucoid-1	17
A72		Mucoid-1	17
A73		Mucoid-1	17
A75		Mucoid-1	17
A76		Mucoid-1	17
A77	148	Brown	175
A78		Mucoid-2	17
A80		Brown	175
A81		Mucoid-2	17
A82		Brown	17
A85		Brown	175
A86		Brown	175
A90		Mucoid-2	175
A91		Brown	175
A92		Mucoid-2	175
A95		Brown	175
A97		Brown	175
A100		Mucoid-2	17
A106		Brown	175
A107		Brown	175
A119	149	Brown	667
A122		Brown	667
A123		Brown	667
A126		Brown	667
A130		Brown	667
A134		Brown	667

Isolate	Patient	Phenotype	ST
A137	149	Brown	667
A141		Brown	667
A144		Brown	667
A147		Brown	667
A148		Brown	667
A151		Brown	667
A154		Brown	667
A156		Brown	667

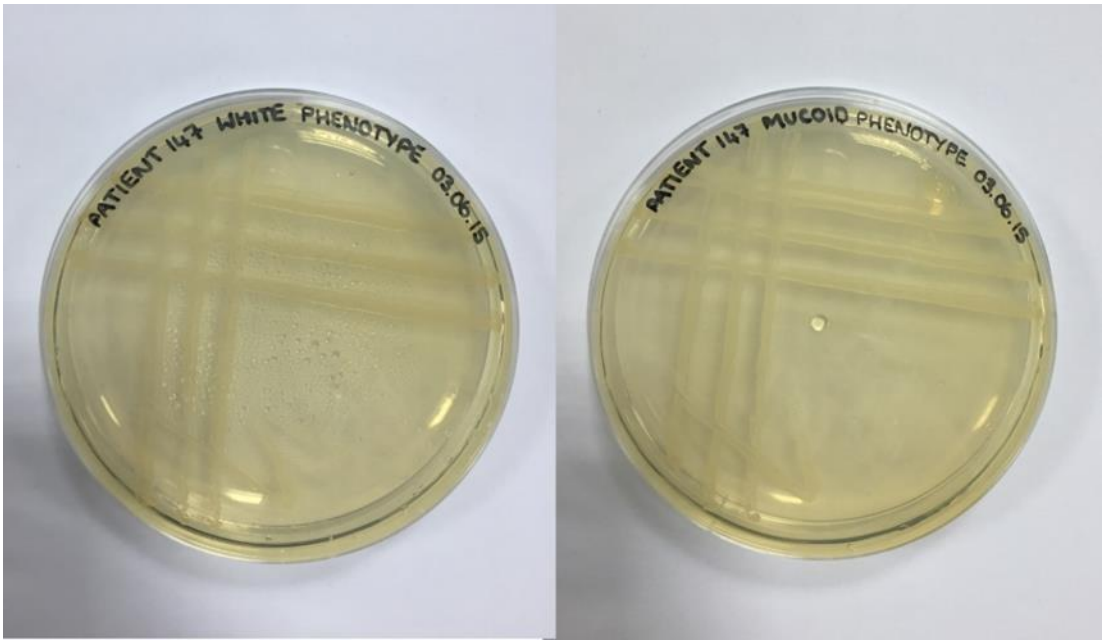

Figure 4.2: Photographs of streak plates of isolates from patient 147 exhibiting differing phenotypes. A single colony exhibiting each of the phenotypes (described as “white” and “mucoid-1”) was picked using an inoculating loop and streaked onto Columbia agar (Oxoid) and grown overnight at 37°C.

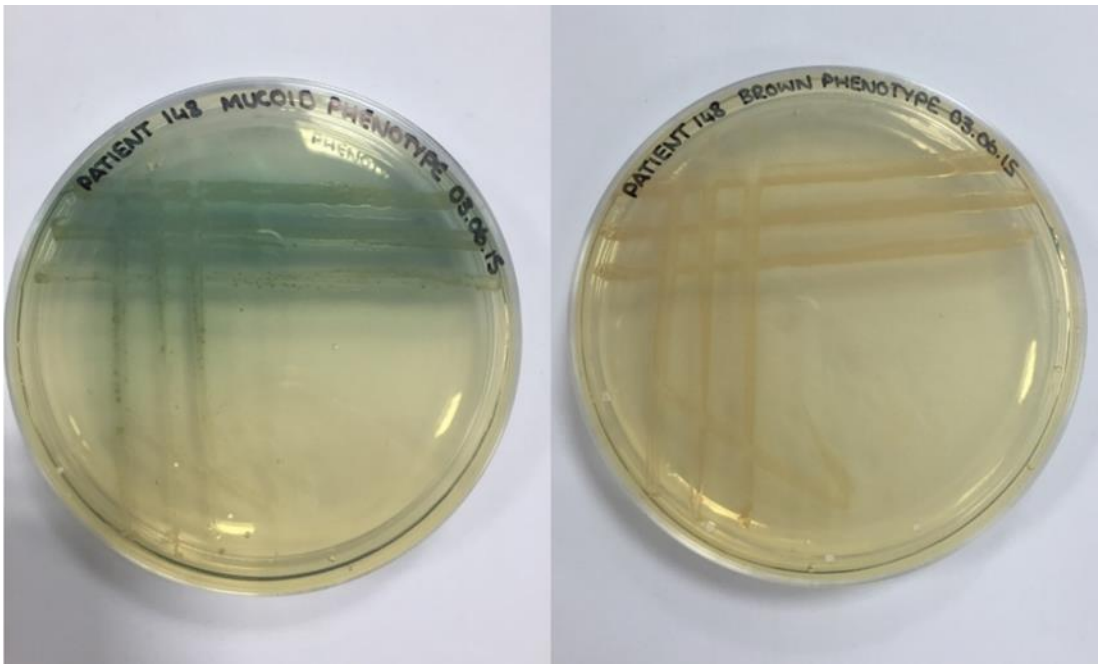

Figure 4.3: Photographs of streak plates of isolates from patient 148 exhibiting differing phenotypes. A single colony exhibiting each of the phenotypes (described as “mucoid-2” and “brown”) was picked using an inoculating loop and streaked onto Columbia agar (Oxoid) and grown overnight at 37°C.

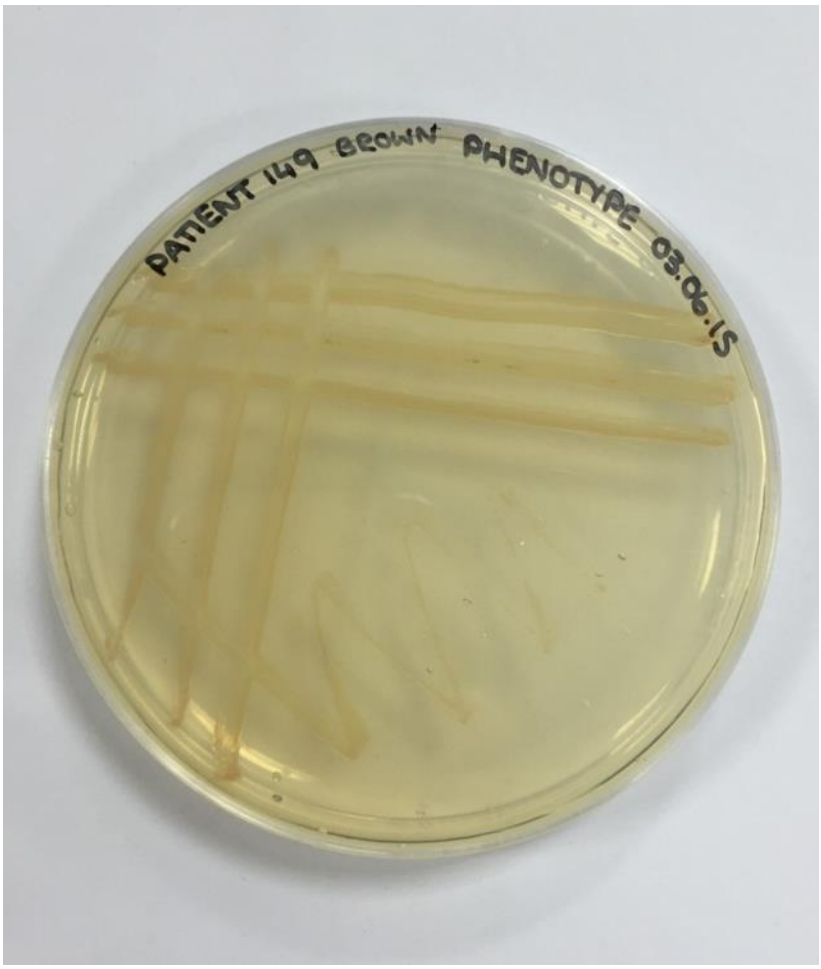

Figure 4.4: Photograph of streak plate of an isolate from patient 149 exhibiting the observed phenotype. A single colony, described as “brown” phenotype, was picked using an inoculating loop and streaked onto Columbia agar (Oxoid) and grown overnight at 37°C.

Figure 4.5: Map of Great Britain showing the locations of the 16 adult bronchiectasis centres from which isolates in this study were collected.

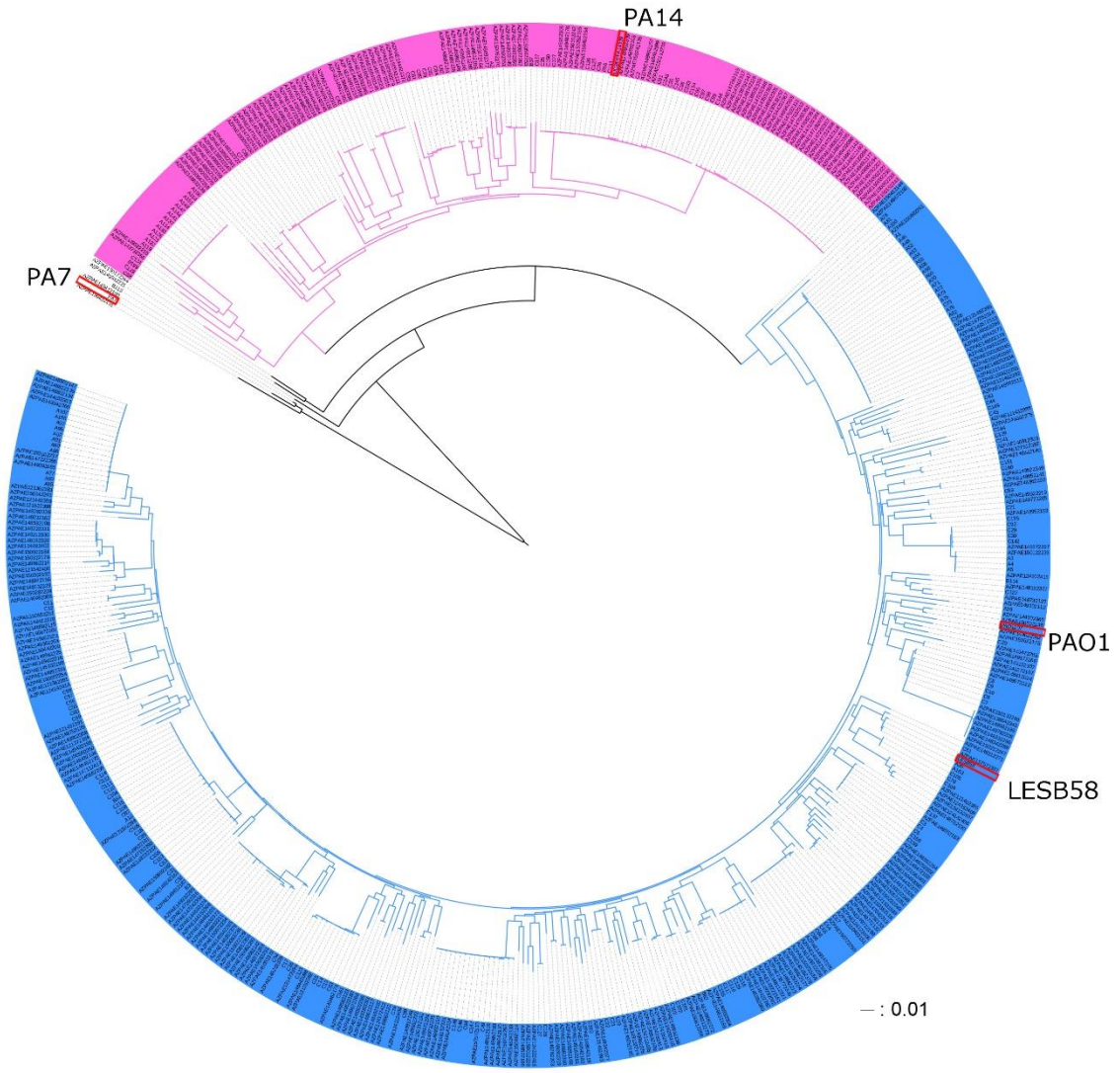

Figure 4.6: Phylogenetic tree showing two main clades of *P. aeruginosa*. Group 1 (labelled blue) includes PAO1, the LES, and clone C isolates. Group 2 (labelled pink) includes lab strain PA14. The locations of PAO1, LESB58, PA14, and PA7 are indicated by red boxes. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

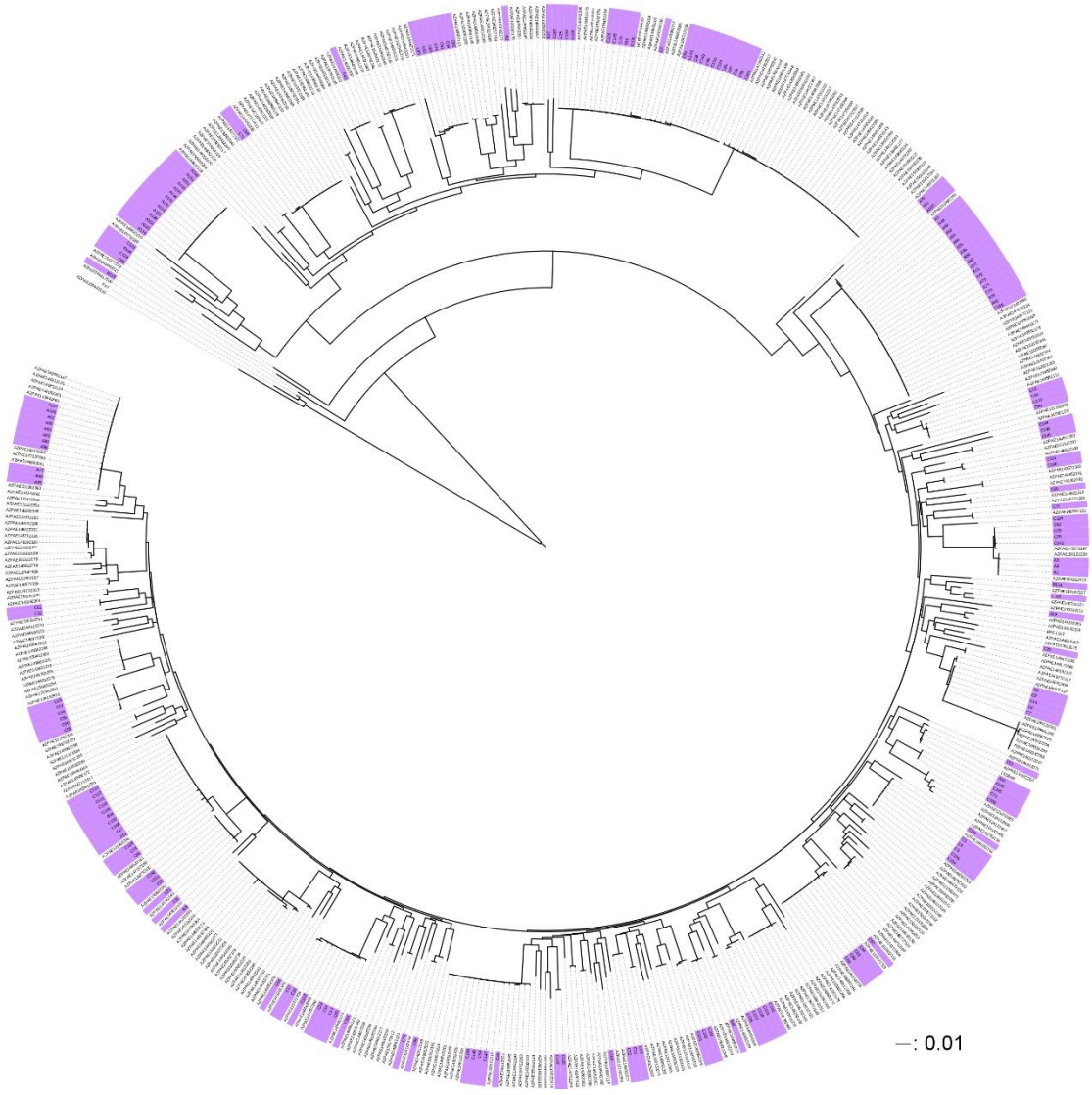

Figure 4.7: Phylogenetic tree of 522 genomes of *P. aeruginosa* isolates. Highlighted in purple are NCFBr isolates from this study. A further 329 isolates from work by Kos *et al.*(2015) are included, alongside the genomes of PAO1 (Stover *et al.* 2000), PA14 (Lee *et al.* 2006), LESB58 (Winstanley *et al.* 2009), and PA7 (Roy *et al.* 2010). Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

4.3.4 Distribution of non-cystic fibrosis bronchiectasis isolates among the general population

Figure 4.7 shows a core genome SNP phylogeny based on all 189 isolates from this study, labelled in purple, distributed on a tree of *P. aeruginosa* isolates which includes an additional 329 genomes from research into the *P. aeruginosa* resistome by Kos *et al.*(2015), the genomes of laboratory strains PAO1 and PA14 (Stover et al. 2000; Lee et al. 2006), LESB58 (Winstanley *et al.* 2009), and the taxonomic outlier PA7 (Roy *et al*. 2010). Small groups of NCFBr isolate genomes appear to cluster together, but generally they are widely distributed across the tree. Figure 4.6 shows two main clades of isolates generally known as group 1, including PAO1, LES, and clone C, and group 2, which includes PA14. *P. aeruginosa* strain PA7 is described as a taxonomic outlier (Roy *et al*. 2010) and is shown as such in figure 4.6 as it falls within neither of the two main clades.

4.3.5 Distribution and population structure of isolates from non-cystic fibrosis bronchiectasis patients in England and Wales

Figure 4.8a shows the distribution of the 88 isolates selected from individual patients at bronchiectasis centres in England and Wales, colour-coded by centre. The isolates from all centres are widely distributed throughout the tree and there is little evidence of extensive clustering of isolates from the same centre. This suggests that transmission between patients in the same centre does not occur regularly, though the sample sizes for each centre are relatively small. In nine of the 16 centres there are examples of pairs of isolates clustering next to one another on the tree, though it is likely that this is due to independent acquisition of strains from the environment, as opposed to transmission events. Isolates from 12 of the 16 centres were present in both the group 1 and group 2 clades (figure 4.6) which indicates a large amount of genetic diversity. Isolates from four centres (Reading, Cardiff, Norfolk & Norwich, and Sunderland) were found to only be present in the group 1 clade, which also includes PAO1, LES, and clone

C. Given that the group 1 clade is much larger than the group 2 clade (figure 4.6) it is unsurprising that some centres have isolates only from this group. There was only one isolate from an individual patient available from the Cardiff centre, so although this isolate can be compared in its relatedness to isolates from other centres it is not possible to investigate the diversity of isolates within this centre.

A small number of isolates cluster together as part of a distinct branch in group 2 (figure 4.8.b). Isolates B199 (Newcastle) and C119 (Hull) appear closest together in this part of the tree, and are apparently more closely related than C119 and C124 despite both isolates being collected from patients in Hull. The remainder of the isolates that are part of this group are from patient 149 (Liverpool). Isolate B113 (Newcastle) does not appear as part of either of the main clades or as part of a cluster (figure 4.8b) and is the closest of the NCFBr isolates to the taxonomic outlier PA7. However, further analysis of B113 in comparison with PA7 with read mapping and genome analysis toolkit (GATK) revealed that the two are not closely related, with a total difference of 262 039 SNPs and 18 305 indels between the two genomes.

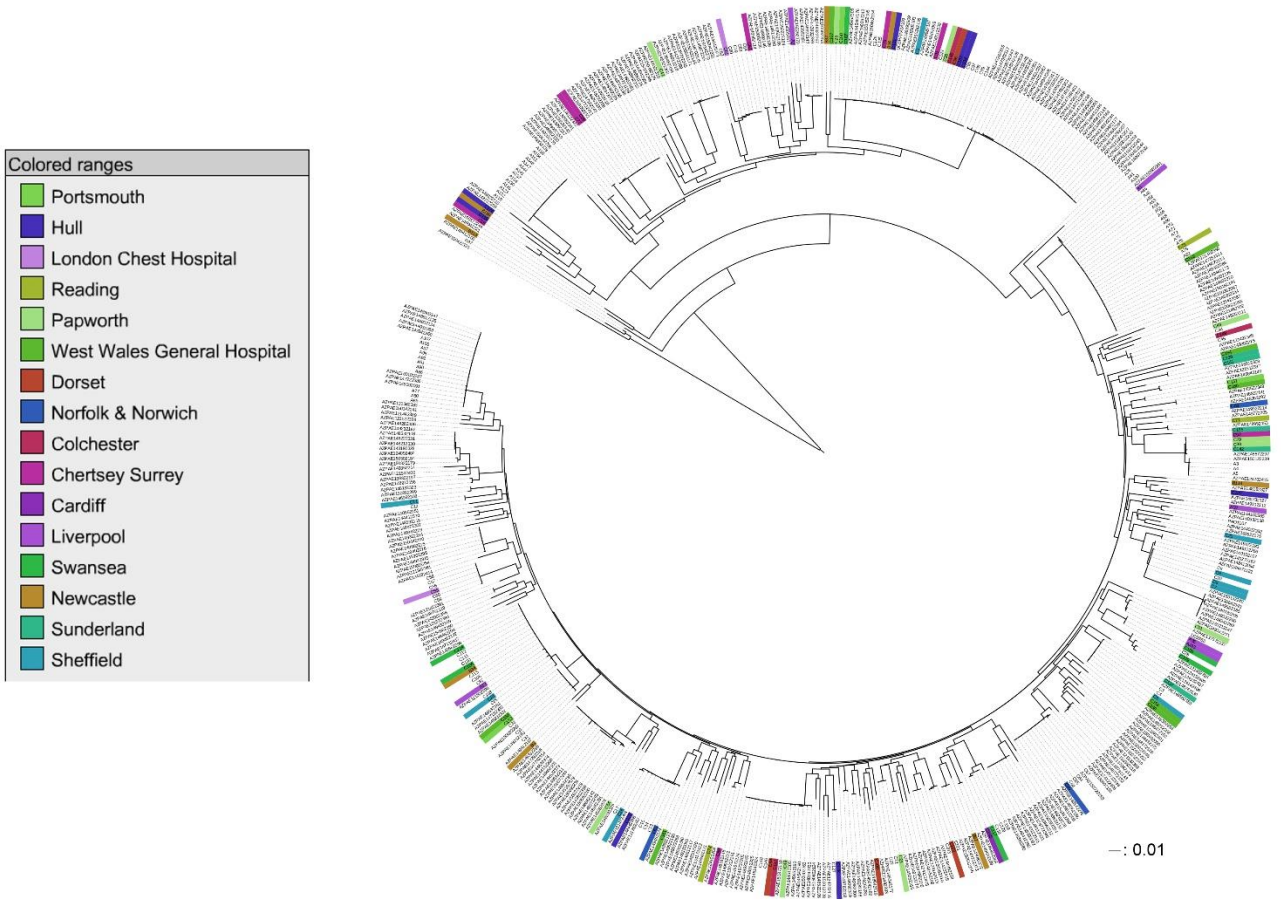

Figure 4.8a: Phylogenetic tree showing the distribution of isolates from individual patients at 16 adult bronchiectasis centres in England and Wales. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

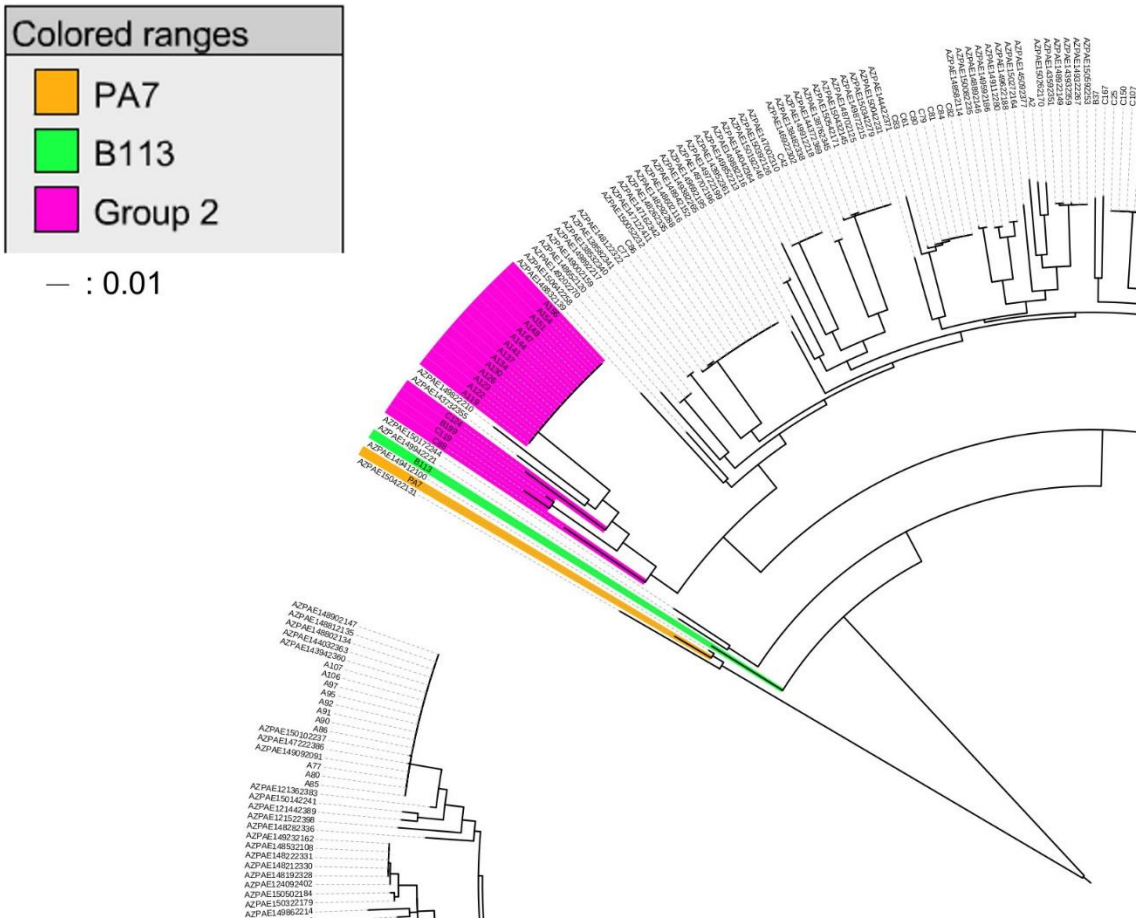

Figure 4.8b: Enhanced view of a section of the phylogenetic tree of NCFBr isolates showing isolate B113 (Newcastle) and taxonomic outlier PA7, which do not fall within either the group 1 or group 2 clades, and isolates which form a distinct group as part of group 2. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

4.3.6 Evidence for multiple *Pseudomonas aeruginosa* strain types within non-cystic fibrosis bronchiectasis patients

As shown in figure 4.9 there is far greater evidence of clustering among isolates taken from the same patient in a single visit than among isolates merely collected from the same centre. The isolates shown in figure 4.9 were collected from 23 patients at 13 bronchiectasis centres in England and Wales during a single visit and should therefore be somewhat representative of the population of *P. aeruginosa* within a given patient at a single point in time. All isolates taken from a patient in a single visit clustered together for the majority of the 23 patients (table 4.2).

Table 4.2: Patients from whom all available isolates cluster together in figure 4.9.

Patient	No. of isolates	Group	Centre	Maximum branch length
79	5	2	Dorset County Hospital	0
147	15	1	Liverpool	0
45	3	1	Papworth	0
37	3	1	Sheffield	0
65	5	1	Norfolk & Norwich	0
102	3	1	Colchester	0
149	14	1	Liverpool	0
86	2	1	Swansea	0
62	6	1	London Chest Hospital	8.96×10^{-4}
40	3	1	Sheffield	8.99×10^{-4}
50	3	1	Papworth	9.03×10^{-4}
3	3	1	Liverpool	9.07×10^{-4}
101	3	1	Colchester	9.09×10^{-4}
55	3	1	Papworth	9.17×10^{-4}
81	3	1	Dorset County Hospital	9.29×10^{-4}
87	3	1	Swansea	1.78×10^{-3}

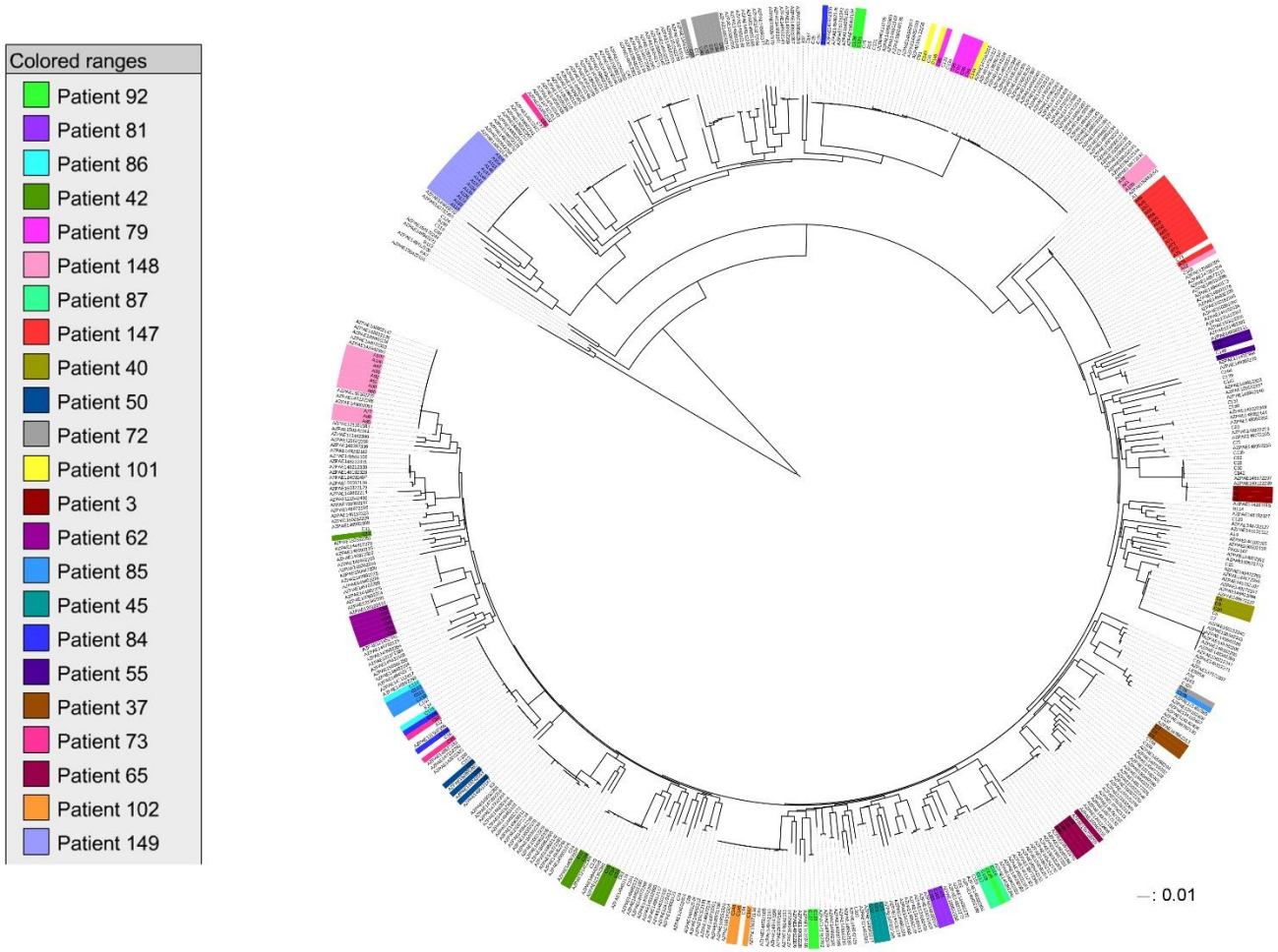

Figure 4.9: Phylogenetic tree showing the distribution of multiple isolates taken from patients in a single visit from 13 centres (not visually represented). Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

Multiple isolates collected from 7 patients did not cluster together (figure 4.9). A total of five isolates were collected from patient 92 (Hull) and these are dispersed across the tree. Three of the isolates are part of the group 1 clade; two of these are closely related (branch length: 0) but the third (C129) is separate and is more closely related to three isolates from a patient from Swansea (patient 87). The remaining two isolates from patient 92 are part of the PA14 clonal complex in group 2. Six isolates were collected from patient 42 (Sheffield) and five of these cluster closely together; three of these have a branch length of 0 and two are slightly further away from these on a branch with a length of 1.75×10^{-3}. The remaining isolate from patient 42 is located much further away on the tree and is in fact more closely related to an isolate from another patient at Sheffield (patient 41). A total of seven isolates were collected from patient 72 (Chertsey, Surrey); six of these cluster relatively closely together in group 2, alongside an isolate from a patient from the London Chest Hospital (patient 63). The final isolate from patient 72 is very distant from these first six isolates and is part of the group 1 clade; isolate C78 clusters with two isolates from two different patients from Swansea. Three isolates were collected from patient 84 (Swansea); two of these isolates are closely related and a part of group 1, though do not appear next to one another on the phylogenetic tree. The remaining isolate is part of the PA14 clonal complex in group 2. Of the three isolates collected from patient 73 (Chertsey, Surrey) two cluster closely together, although not next to one another, in group 1 and the third falls in group 2, most closely related to a single isolate from another patient from the same centre (patient 71). All four isolates collected from patient 85 (Swansea) are found in the group 1 clade; three are closely related and found next to one another, the final isolate is distantly related to these and is most closely related to an isolate from another patient at the same centre and an isolate from a patient from the Chertsey, Surrey centre.

Patient 148 was one of three patients from Liverpool from whom 15 isolates were selected from a direct culture plate. Isolates from patients 147 and 149 cluster by patient; all 15 isolates from patient 147 cluster together as part of group 1; all isolates from patient 149 cluster together as part of group 2. Eleven isolates from patient 148

cluster together in group 1, away from most of the other isolates collected from NCFBr patients and the remaining four isolates cluster near the isolates from patient 147.

4.3.7 Multilocus sequence typing of isolates

Multilocus sequence typing (MLST) data was extracted from the assembled genomes of all 189 isolates and the most commonly identified MLST types are listed in table 4.3. Thirty-one isolates could not be assigned a sequence type (ST) (appendix, table A5). The online MLST database (http://pubmlst.org) was used to identify commonly found STs and clonal complexes.

Table 4.3: Summary of the most abundant STs among the isolates sequenced, and the number of centres each of these STs was identified in. Full list of isolates and STs can be found in the appendix, table A5.

ST	No. of isolates	No. of centres
17	22	3
253	21	9
667	14	1
175	11	1
179	10	4
252	7	4
620	6	2
27	5	3
244	5	1
260	5	3
274	5	1
395	5	3
108	4	2
871	4	2
840	3	2

The LES has previously been identified as ST146 (http://pubmlst.org) and there are two isolates of the 189 that were shown to have this ST. Isolates A36 and A163 (both from a patient in Liverpool) were both found to be positive for LESF9 and PS21 markers by PCR assay (table 2.1) as part of general screening of all isolates. Due to the occurrence of occasional false positives for isolates with either of the LES markers (Fothergill *et al.* 2010a), isolates suspected to be LES positive were subjected to further testing to confirm

their status. This included a PCR assay for pyoverdine receptor (table 2.1). Isolate A36 was confirmed as LES as it was pyoverdine receptor type III positive (the rarest of the three types). Isolate A163 was collected late in the work and so following the initial positive result for both LES markers was submitted for whole genome sequencing as means of confirmation. Both LES isolates (A36 and A163) were compared to LESB58 (high quality, relevant reference genome) using GATK which revealed that both LES isolates were closely related to LESB58. Isolate A36 differed from LESB58 by 109 SNPs and 22 indels, and isolate A163 differed from LESB58 by 113 SNPs and 25 indels. A36 and A163 shared 95 of these SNPs, whilst 14 were unique to A36 and 18 to A163.

Isolates from patients 79 and 101 cluster closely together despite being collected from patients in Dorset and Colchester respectively. These isolates also cluster with a number of isolates from other patients at 7 other centres and PA14 (figure 4.10). All isolates on the highlighted branch of the tree in figure 4.10 have the ST253 assigned by MLST of their genomes.

Clone C is commonly found in the environment and is associated with ST17, as well as with ST142 and ST14 (Curran *et al.* 2004). A total of 22 isolates, from 5 patients at 3 centres, were found to be ST17 (figure 4.11). All 15 isolates collected from patient 147 (Liverpool) were found to be ST17. Four isolates from patient 148, also from Liverpool, were found to be ST17 but due to the prevalence of clone C in the environment it is more probable that these patients acquired strains of *P. aeruginosa* independently rather than this being an example of a transmission event. The remaining 11 isolates from patient 148 are all ST175 which has been identified on numerous occasions from patients in France and Spain (http://pubmlst.org).

All 14 isolates collected from patient 149 (Liverpool) were shown to be ST667 which has not been previously identified in the PubMLST database (http://pubmlst.org). The isolates cluster together as part of group 2 but are only distantly related to PA14 (figure 4.9). Figure 4.12 shows the other isolates from this study which cluster most closely to the isolates from patient 149, along with the STs assigned to them by MLST. Although the leaves appear relatively close to one another on the tree the branch

lengths indicate that the isolates are not closely related and are more likely to cluster together due to genetic differences from the majority of other isolates on the tree. Isolates C88 (Chertsey, Surrey) and C124 (Hull) were assigned STs 1251 and 1753 respectively and occupy their own branches on the tree. Despite not being assigned a ST, isolate C119 (Hull) clusters closely with isolate B199 (Newcastle) which was designated ST1182.

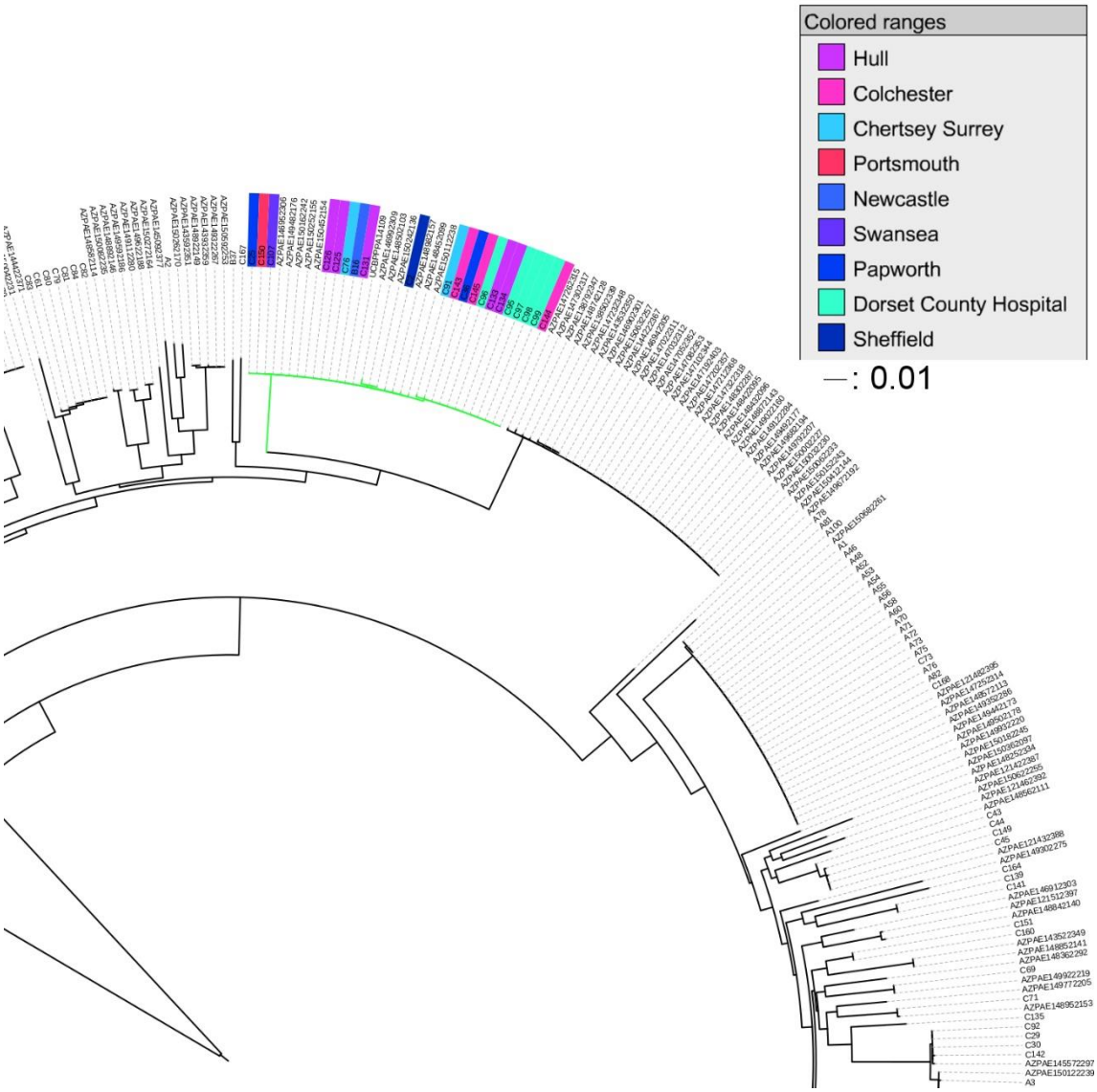

Figure 4.10: Enlarged section of phylogenetic tree showing branch of isolates with ST253. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

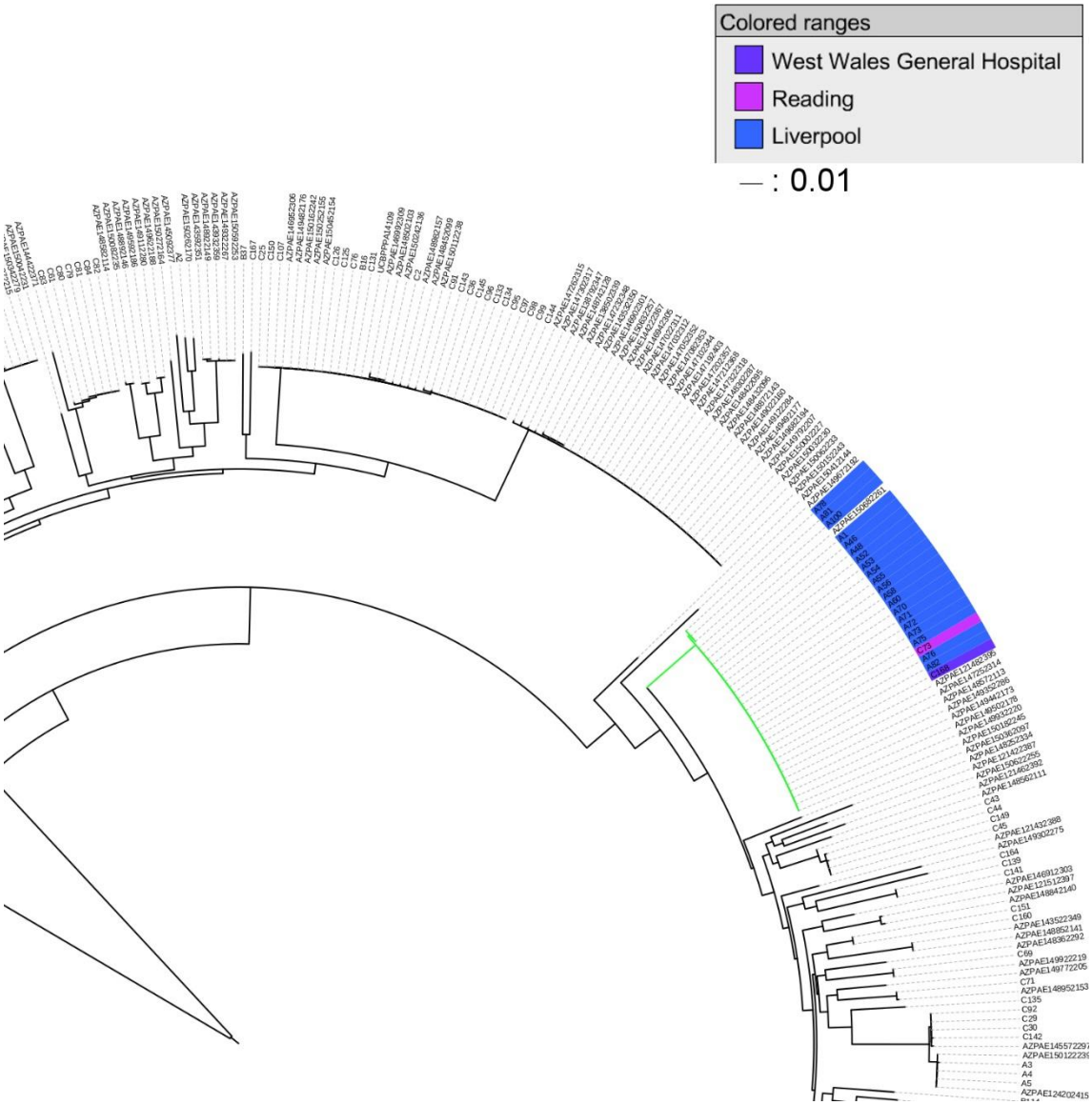

Figure 4.11: Enlarged section of phylogenetic tree showing branch of isolates with ST17. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

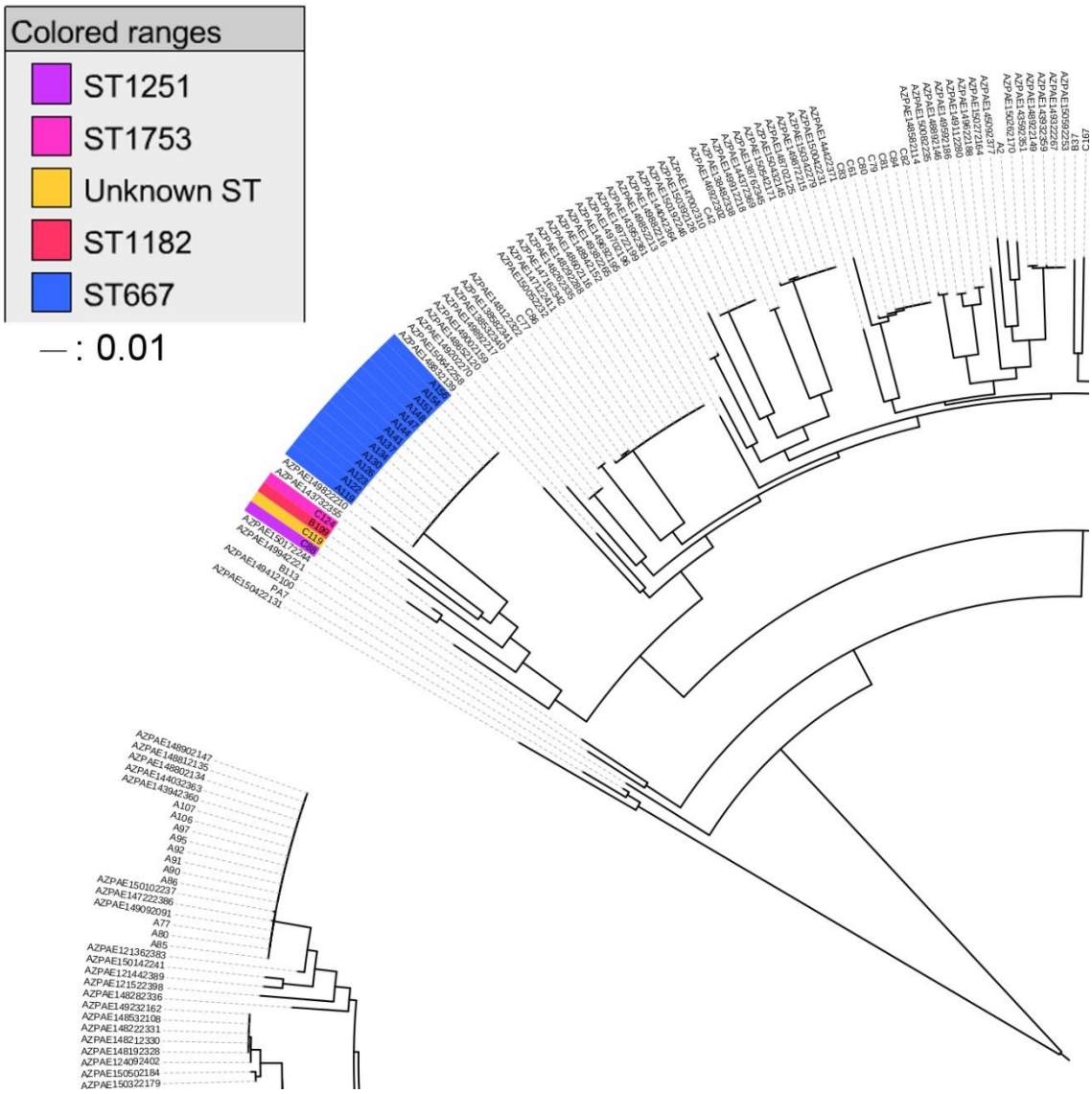

Figure 4.12: Enlarged section of phylogenetic tree showing the isolates with ST667 and its closest relatives. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011)

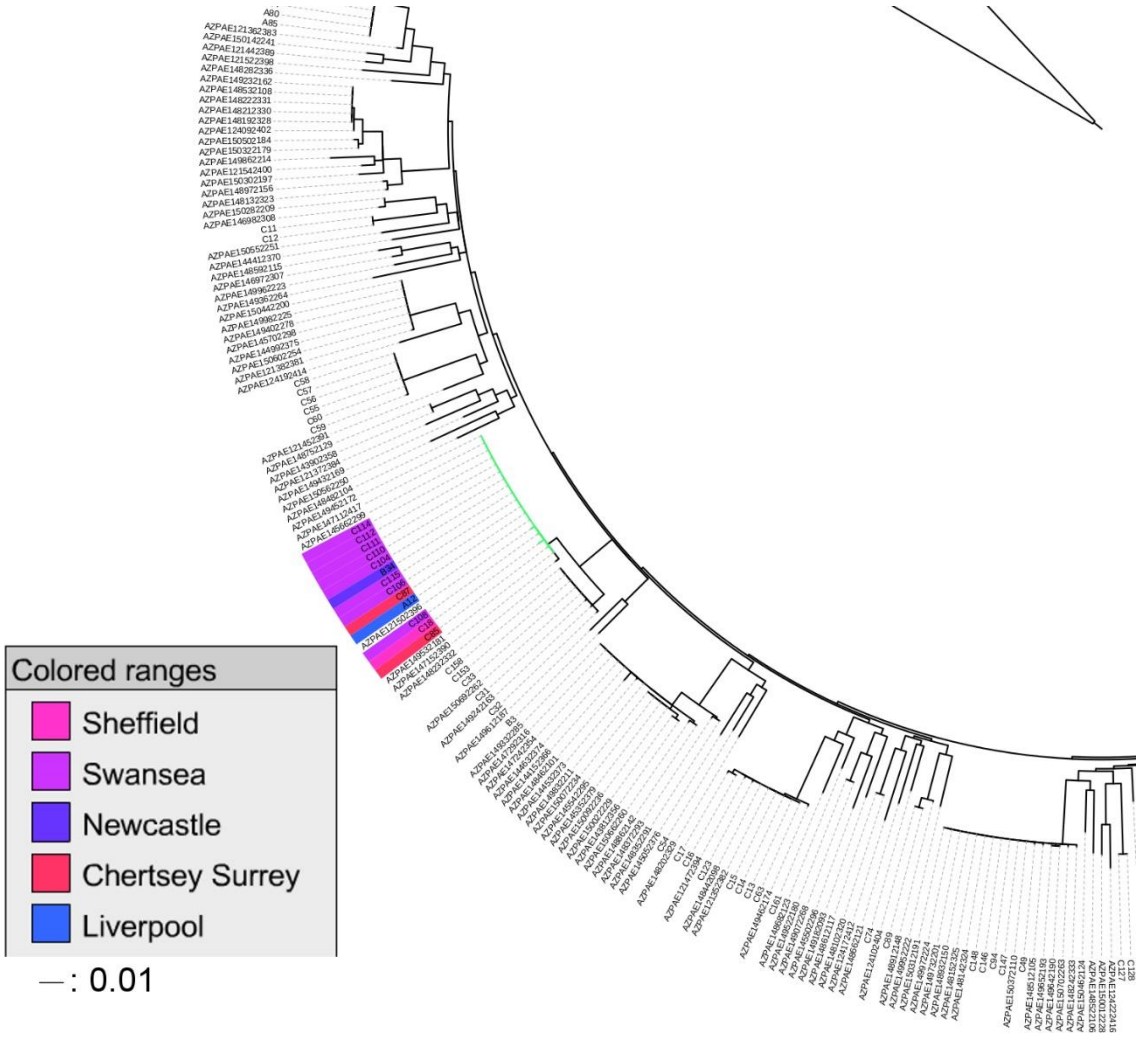

Figure 4.13: Enlarged section of phylogenetic tree showing isolates assigned ST179 and those closely related to these isolates that were unable to be assigned a ST. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

4.4 Discussion

4.4.1 Diversity of *Pseudomonas aeruginosa* isolates from adult bronchiectasis centres in England and Wales

Little is known about the population structure of *P. aeruginosa* isolates from NCFBr patients. Work has been previously undertaken to investigate the wider population of *P. aeruginosa* isolates from patient infections across the UK, including those in CF patients. Combinations of sequenced-based typing techniques have been used to attempt to elucidate the population structure of *P. aeruginosa*. It is generally agreed that *P. aeruginosa* has a non-clonal population structure punctuated by closely related genotypes or clonal complexes (Pirnay *et al.* 2002; Pirnay *et al.* 2009; Curran *et al.* 2004). Large-scale studies of population structure can be subject to bias, in that many databases include over-sampling of isolates from highly studied regions or clinical manifestations of *P. aeruginosa* (*i.e.* CF isolates) and so this is important to take into consideration when making statements about the abundance or relatedness of strains. In broad terms, strains of *P. aeruginosa* can be divided into three genetically distinct groups: group 1, containing PAO1, LESB58, and clone C strains; group 2, containing PA14 clonal complex; and group 3, containing the taxonomic outlier PA7 (Stewart *et al.* 2014; Freschi *et al.* 2015). Work by the International *Pseudomonas aeruginosa* Consortium (IPC) suggests that group 1 strains are naturally more abundant than strains falling in group 2 (Freschi *et al.* 2015) and this is reflected in the results of this work (figure 4.4). Due to the clinical origin of isolates from this study and much of the IPC cohort (Freschi *et al.* 2015) it is possible that sampling bias implies that the abundance of group 1 isolates is naturally higher, especially given that conflicting evidence has been provided using the AT typing method by Wiehlmann *et al.*(2007).

Using the STs assigned to isolates and the *P. aeruginosa* MLST database (http://pubmlst.org) it is possible to investigate the occurrence of commonly reported strains within the NCFBr population in England and Wales. Within each of the two groups

there are several previously reported STs that have often been identified in multiple countries around the world, and from varying sources. In general the NCFBr isolates in group 1 do not form closely related clusters. Two STs with multiple isolates from bronchiectasis centres around the UK were identified. A group of closely related isolates were identified though closer examination of the phylogenetic tree in figure 4.8a. This group consisted of 13 isolates, 10 of which had been assigned ST179; the remaining three isolates could not be assigned a ST. Isolates previously assigned this ST in the *P. aeruginosa* MLST database (http://pubmlst.org) have been collected mainly from Canada and Australia but also from the UK and Spain, and mainly from sputum samples. The isolates in this closely related group (figure 4.13) were collected from five different centres, with the majority (eight) coming from the Swansea centre. These eight isolates were collected from four different patients. The second closely related group of isolates were assigned ST17 which has been previously associated with clone C isolates (Curran *et al.* 2004). A total of 21 isolates were assigned ST17; 19 of these were isolated from patients in Liverpool, and 15 were from one patient. The two remaining isolates were collected from Reading and West Wales General hospital (figure 4.11). There have been several other STs associated with clone C strains of *P. aeruginosa* but none of these were identified among these NCFBr isolates (Curran *et al.* 2004).

Investigation of closely related isolates in group 2 (figure 4.10) reveals two main clusters of closely related isolates which share a common ancestor, although one of these groups is only populated with isolates from work by Kos *et al.*(2015). All NCFBr isolates from the second group of closely related isolates were assigned ST253 which has been previously identified as belonging to the PA14 clonal complex. Isolates assigned ST253 have been found extensively throughout the environment and in human infections in countries around the world, including the UK, France, Spain, Australia, and Brazil (http://pubmlst.org). Another group of isolates was identified on a branch located distantly from the PA14 clonal complex, though still as part of the group 2 clade. All isolates from patient 149 were a part of this group having been assigned ST667. Four other isolates were also located on this branch (figure 4.12) one of which was unable to

be assigned a ST. Although the isolates in this group appear on one branch of the tree they are not closely related to one another. None of the three STs assigned to these isolates were found to be particularly common in the PubMLST database; ST1251 was previously identified in an isolate from a bronchial lavage in Spain, ST1753 had previously been identified in a water isolate from France, and ST1182 was previously found in water isolates from Australia and France, and in isolates from unnamed sources in China and France (http://pubmlst.org).

The most common STs among NCFBr isolates are listed in table 4.2. Five isolates from three patients attending the Sheffield bronchiectasis centre were assigned ST244 which has previously been identified across Europe, Asia, Australia, Africa, and South America from a wide range of clinical and environmental sources (http://pubmlst.org). A clade of isolates assigned ST395 were identified among the isolates collected by Kos *et al*. (2015) and five isolates from three patients, attending three geographically separate bronchiectasis centres were assigned this ST. Previously ST395 has been associated with water sources (Slekovec *et al.* 2012) and it was suggested by Kos *et al.*(2015) that its prevalence among clinical isolates implied that *P. aeruginosa* was easily acquired from the environment in many diseases. There were, however, STs previously acknowledged as being distributed worldwide and that were well represented among the clinical isolates analysed by Kos *et al.*(2015) that were not assigned to any of the 189 NCFBr isolates sequenced as part of this work. ST235 (serotype O:11)and ST111 (serotype O:12) were commonly identified in work undertaken by Maatallah *et al.*(2011) and Woodford *et al.* (2011) and have previously been described worldwide (http://pubmlst.org) but were not identified among the NCFBr isolates. The widespread origins of isolates sharing the most common STs identified among these NCFBr isolates, and their distribution among the other isolates on the phylogenetic tree, implies that the population structure of *P. aeruginosa* among NCFBr patients is nonclonal.

4.4.1.1 Transmissible strains of *Pseudomonas aeruginosa* in non-cystic fibrosis bronchiectasis patients from England and Wales

Further work by Pirnay *et al.* (2009) into the population structure of *P. aeruginosa* led to the conclusion that the nonclonal structure of the population is generally punctuated by outbreaks of epidemic strains that are common in both the environment and in clinical isolates. Various transmissible "epidemic" strains of *P. aeruginosa* have been identified in the CF population in the UK (Scott & Pitt 2004) but the prevalence of these strains among patients with NCFBr has not previously been investigated. Two isolates from a patient attending the Liverpool bronchiectasis service were confirmed to be LES positive. The LES was not found to be present in any other centres in England and Wales and no other known UK epidemic strains (Midlands 1 or Manchester epidemic strain) were identified among any patients. The two LES isolates recovered (A36 and A163) were found to be closely related to one another but the strain is obviously not widespread among NCFBr patients attending the Liverpool centre given its presence in only one of 56 patients (appendix, table A1) surveyed from Liverpool. In total, the LES accounted for fewer than 0.5% of the 408 NCFBr patient isolates sampled from 16 centres which is significantly lower than the prevalence of LES recorded among CF patients in the UK (P = >0.0001) (Scott & Pitt 2004). The low frequency of LES and absence of other known epidemic strains indicates that transmissible strains of *P. aeruginosa* are not as great a cause for concern among NCFBr patients as in patients with CF, although the prognostic implications of LES infections previously studied in CF (Al-Aloul *et al.* 2003) should not be ignored in NCFBr patients.

4.4.2 Diversity of *Pseudomonas aeruginosa* isolates from individual non-cystic fibrosis bronchiectasis patients

In the field of diagnostic microbiology it has been commonplace to use diagnostic results from single bacterial isolates from patient samples to inform the appropriate method of treatment and prognosis. However, more recently it has become apparent than in the case of chronic lung infections, predominantly in CF, that a single isolate is

unlikely to be representative of the true nature of the infection. It has been noted that different areas of the lung are likely to have different oxygen levels, available nutrients, and varying microflora, and this may lead to large variations in both the phenotypic and genotypic characteristics of isolates collected from patients (Jorth *et al.* 2015).

Sample sizes were small for many of the NCFBr patients from whom multiple isolates were collected (mean isolates per patient: 5.2, median: 3) and so may still not necessarily reflect the true diversity of the *P. aeruginosa* populations within the lungs of individual patients. In the majority of patients (16 of 23) isolates collected in a single visit were closely related, clustering together on the phylogenetic tree (figure 4.9) and sharing a single ST. Of the seven patients whose isolates were not closely related, four had isolates present in both group 1 and group 2 clades indicating a large amount of genetic diversity between these isolates. Seven isolates were collected from patient 72 (Chertsey, Surrey) and six of these clustered closely together as part of the group 2 clade in the phylogenetic tree (figure 4.9). Five of these isolates were assigned ST620 and the remaining member of this cluster of isolates (C83) was unable to be assigned a ST. The final isolate collected from patient 72 falls into the group 1 clade and was assigned ST840. Both ST620 and ST840 were relatively uncommon in the PubMLST database (http://pubmlst.org) and both have previously been identified in veterinary isolates from Australia. Only one previous example of ST840 was recorded as an Australian canine wound isolate, however this ST has also been assigned to two isolates from two patients attending the Swansea bronchiectasis centre. Aside from these, the majority of STs assigned to isolates which did not cluster together were STs that were relatively common in the PubMLST database, many having been identified worldwide, which may indicate that NCFBr patients are capable of harbouring more than one environmentally-acquired strain of *P. aeruginosa* at any given time.

4.4.2.1 Diversity of multiple *Pseudomonas aeruginosa* isolates from three non-cystic fibrosis bronchiectasis patients attending the Liverpool bronchiectasis service

Preliminary genomic analysis carried out on multiple isolates from three patients attending the Liverpool bronchiectasis service was carried out to investigate the diversity of *P. aeruginosa* isolates within NCFBr patients. Upon initial selection of the isolates details of the colony morphology were recorded (table 4.1). Variations in the colony morphology of isolates was then compared alongside the ST assigned to each isolate by MLST, The variation in colony morphology between these isolates did not seem to be influenced by the relatedness of the isolates as all 15 isolates from patient 147 were assigned ST17 and had a branch length of 0 (figure 4.14). Four isolates collected from patient 148 were also assigned ST17 and the remaining 11 isolates were assigned ST175. ST17 has previously been associated with clone C strains of *P. aeruginosa* and so it is unlikely that the presence of isolates in two patients with this ST is due to interpatient transmission; the high prevalence of clone C in the environment implicates independent acquisition of the strain by both patients. Isolates previously assigned ST175 have mainly been recovered from blood isolates from France and Spain, as well as from other human infections in both of these countries (http://pubmlst.org). Although ST17 and ST175 are both part of the group 1 clade (figure 4.6) they are only very distantly related, falling virtually as far away from one another as possible on the phylogenetic tree. All 14 isolates from patient 149 cluster in the group 2 clade (figure 4.6) and were assigned ST667. This ST has not previously been identified in the PubMLST database (http://pubmlst.org). No other isolates collected from NCFBr patients clustered closely with the 14 isolates from patient 149. Given the versatility of *P. aeruginosa* and its adaptability it is likely that this strain is environmental and has been acquired independently by the patient. Further examination of the genomes of these isolates would allow us to compare it to chronic lung infection isolates and investigate the presence of mutations and adaptations associated with chronic lung infection isolates.

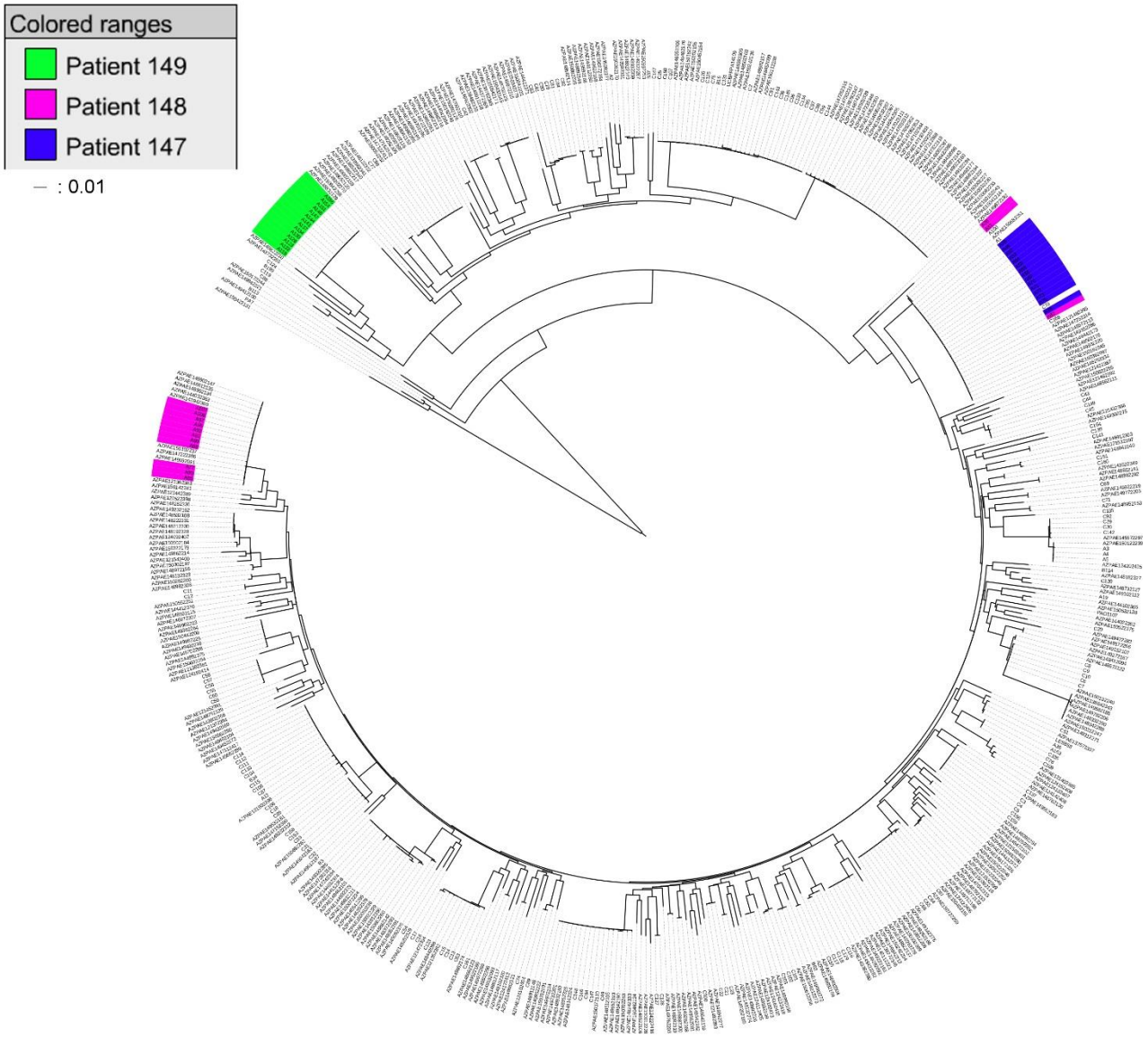

Figure 4.14: Phylogenetic tree showing the distribution of multiple isolates taken from 3 patients in a single attending the Liverpool bronchiectasis service. Tree labelled using iTOL software (Letunic & Bork 2007; Letunic & Bork 2011).

4.5 Conclusions

The main conclusions that can be drawn from this study are:

1. **In bronchiectasis centres throughout England and Wales there is little evidence of geographical clustering or transmissible epidemic strains of *P. aeruginosa*;**
2. **In the majority of NCFBr patients there is only one infecting strain of *P. aeruginosa* at a given time, though there is evidence for multiple strain infections in some cases.**

Chapter Five

General Discussion

The previous chapters have discussed in detail the current understanding of non-cystic fibrosis bronchiectasis (NCFBr) as a disease and the implications of *P. aeruginosa* infections in patients suffering the condition. It is clear that there is a fundamental lack of focused research into NCFBr both in terms of the disease itself and the microbial infections associated with it, particularly when compared with cystic fibrosis (CF).

Bronchiectasis has been considered in recent years to be easily treatable and of little clinical significance, possibly due to a belief that the disease would gradually fall into decline in developing countries alongside other pulmonary diseases, such as tuberculosis (TB) (Chalmers 2015). There are several known causes of bronchiectasis including: CF; childhood infection, most commonly pneumonia leading to post-infective bronchiectasis; and particle inhalation (NHS Choices 2015). It is estimated, however, that in 25 – 50% of NCFBr cases a cause is never identified and this is known as idiopathic bronchiectasis (http://www.blf.org.uk/page/bronchiectasis). Conversely, CF is an easily defined disease due to its genetic aetiology and has rightfully garnered public and scientific attention due to the significantly reduced quality of life and life expectancy experienced by sufferers. In the UK more than 10 000 people are affected by CF (http://www.cysticfibrosis.org.uk/about-cf). However, in just nine centres in the UK there are 5000 patients undergoing regular follow-up treatment for NCFBr (De Soyza *et al.* 2013) suggesting a much higher prevalence than CF, and higher incidence than previously believed. In CF patients chronic lung infection with *P. aeruginosa* is the leading cause of morbidity and mortality (Fothergill *et al.* 2010a) and many studies have demonstrated an association between chronic *P. aeruginosa* infection and increased mortality, lowered lung function, and worsened quality of life in NCFBr patients (Evans

et al. 1996; Davies *et al.* 2006; King *et al.* 2007; Loebinger *et al.* 2009). However it has not been conclusively shown that *P. aeruginosa* is the cause of a worsening prognosis in NCFBr patients instead of merely a marker of existing disease severity (Chalmers 2015). The uncertainty surrounding the role of *P. aeruginosa* in NCFBr has been the basis for this study.

The main focus of this work has been to reveal more information about the population structure of *P. aeruginosa* in NCFBr patients in a manner similar to work that has already been undertaken with regards to CF. Once colonised with *P. aeruginosa* CF patients generally carry the infection for life and often maintain the same strain of the bacteria throughout the course of infection (Şener *et al.* 2001; Leone *et al.* 2008) although more recently it has been acknowledged that transmissible "epidemic" strains can superinfect patients and displace the established strain (McCallum *et al.* 2001). Previously there has only been a very small amount of work undertaken to investigate the maintenance of strains of *P. aeruginosa* in the lungs of NCFBr patients and the possibility of transmissible strains. The work undertaken by De Soyza *et al.* (2014) investigated the incidence of transmission of *P. aeruginosa* between NCFBr patients in a single centre, as well as including paired longitudinal isolates from 10 patients. The majority of patients were shown to harbour unique strains of *P. aeruginosa* and both isolates from nine of the 10 patients with paired isolates were found to be indistinguishable by both array tube (AT) and variable number tandem repeat (VNTR) typing. The work described in chapter three aimed to build on the previous research by De Soyza *et al.* (2014) through the use of AT genotyping of longitudinal NCFBr isolates. In the majority of the 20 patients from whom isolates were collected the strain type was maintained for the period spanned by this study. Use of the AT system allows for rapid identification of isolates and provides information regarding the accessory genome. Assigning each isolate a four digit AT code also allows for the use of the eBURST algorithm (Feil *et al.* 2004; Spratt *et al.* 2004) to examine the distribution and population structure of *P. aeruginosa* isolates from NCFBr patients. There was shown to be no clustering of isolates collected from NCFBr patients and the strains identified were

widely distributed throughout the general population, although common clones were found to be more prevalent. This is in line with work carried out previously which has defined the population structure of *P. aeruginosa* as nonclonal though punctuated by closely related clonal complexes, such as clone C (Pirnay *et al.* 2002; Pirnay *et al.* 2009; Curran *et al.* 2004). The main limitation of the AT system is its lack of depth. The immobilised oligonucleotides in the AT chip only represent a very limited number of sequences from the large and versatile genome of *P. aeruginosa* and the dichotomy of PAO1- vs. non-PAO1-type sequences necessary for analysis of the AT data does not accurately portray the diversity of *P. aeruginosa* genotypes. Whilst some information regarding the accessory genome of isolates can be shown, it mainly concerns the presence or absence of mobile genetic elements and cannot provide information regarding single nucleotide polymorphisms (SNPs) or indels which can differentiate closely related isolates. With the ever-decreasing cost of whole genome sequencing it is likely that the AT system will cease to be used in research in favour of the increased the depth of information acquired by whole genome sequencing.

The work undertaken in chapter three focused only on patients from one bronchiectasis centre in the north-east of England, and so to achieve a broader understanding of the population of *P. aeruginosa* among NCFBr patients the work described in chapter four included isolates from 16 bronchiectasis services in England and Wales. Whole genome sequencing provides a much greater depth of study and has previously been used in studies of *P. aeruginosa* to investigate virulence mechanisms, including antibiotic resistance genes and virulence factors (Lee *et al.* 2006; Boyle *et al.* 2012); the relatedness of isolates and possible transmission events (Snyder *et al.* 2013; Jeukens *et al.* 2014; Quick *et al.* 2014); and the evolution of infectious bacterial strains (Wong *et al.* 2012; Dettman *et al.* 2013; Marvig *et al.* 2014). The collection of isolates from this study were compiled into a phylogenetic tree with isolates from work by Kos *et al.* (2015) in order to place them in the context of the general *P. aeruginosa* population. Similarly to the results in chapter three, the NCFBr isolates were not found to cluster extensively and were distributed throughout the phylogenetic tree. The results

reflected work by Stewart *et al.* (2014) and Freschi *et al.* (2015) which showed two distinct subgroups of *P. aeruginosa* within the population, termed group 1 and group 2, with a third sparsely populated group containing taxonomic outlier PA7 (Roy *et al.* 2010). Strains from group 1, including PAO1, clone C strains, LESB58, and PAK, have been suggested to be naturally more abundant than strains from group 2, which includes PA14. However, it has also been suggested that this apparent disparity between group 1 and group 2 isolates may be introduced due to selection bias, given that the data used to compile these phylogenetic trees are largely from clinical sources (Freschi *et al.* 2015). The International *Pseudomonas aeruginosa* Consortium (IPC) aims to compile a database of over 1000 *P. aeruginosa* genomes to allow easily accessible and user-friendly comparisons of genomic data and so as the number of environmental isolates in the dataset increases there may be a shift in the abundance of group 2 isolates. Chapter four also included work undertaken to investigate the possible heterogeneity of multiple isolates taken from NCFBr patients in a single visit to a bronchiectasis centre. In the majority of patients all isolates taken in a single visit were found to cluster closely together and shared a single sequence type (ST) assigned by multilocus sequence typing (MLST), indicating that typically NCFBr patients do not experience multi-strain infections of *P. aeruginosa*. Further analysis of the STs assigned to isolates showed that many were strains commonly identified worldwide, both in the environment and in clinical isolates, demonstrating that there are not strains directly associated with NCFBr pulmonary infections. The sample sizes for multiple isolates from NCFBr patients were small (mean isolates per patient: 5.2, median: 3) and so any further research to confirm that these results are typical would be better served by larger sample sizes. It has been shown in CF patients that coexisting, divergent strains of *P. aeruginosa* can infect the lungs (Williams *et al.* 2015) but sample sizes of 40 isolates per sputum sample from each patient were used in this work and so further investigation into heterogeneity of *P. aeruginosa* infections in NCFBr patients would be better served on this scale. On a smaller scale, it would be beneficial to further investigate the isolates collected from patients 147 and 149 (patients attending Liverpool bronchiectasis service) from whom a

larger number of isolates were collected from a single visit. In the case of both patients all isolates clustered closely together on the larger phylogenetic tree but construction of a core genome from the isolates from each patient would allow for a higher resolution investigation of relatedness between the isolates. Patient 147 has been known to have been colonised with *P. aeruginosa* for >10 years, whereas the isolates collected from patient 149 are the first *Pseudomonas*-positive culture from this patient. Given the known diversification of *P. aeruginosa* within the lungs of CF patients over time (Williams *et al.* 2015) it would be expected that there would be greater heterogeneity between the isolates collected from patient 147 than in patient 149. Further analysis of these isolates would allow for a greater comparison between the behaviour of *P. aeruginosa* in the lungs of CF patients vs. NCFBr.

There was also very little evidence of transmissible strains of *P. aeruginosa* among NCFBr patients. After the emergence of epidemic strains of *P. aeruginosa* among CF patients work was undertaken to investigate the prevalence of these strains in the UK. The Liverpool Epidemic Strain (LES) was found to account for 11% of the patient isolates collected from CF sufferers in the UK (Scott & Pitt 2004). Among this cohort of NCFBr patients only one patient was found to be infected with the LES. The patient was attending the Liverpool bronchiectasis service and no other epidemic strains were identified in this or any other centre. Although the lungs of NCFBr and CF patients provide similar conditions for bacterial growth the disparity between the prevalence of transmissible strains among CF patients and NCFBr patients may imply that there are subtle differences in the host environment which mean that these epidemic strains are more suited to life in the CF lung as opposed to in the lungs of those suffering other chronic lung conditions. Further work should continue to be undertaken using larger sample sizes across UK centres to investigate evidence of transmissible strains of *P. aeruginosa* among the NCFBr population, as only a small sample of the predicted NCFBr patients in the UK have been included in this work.

The genomic analysis undertaken in this work has largely been preliminary, and has not fully explored the fine detail offered by the availability of whole genomes. With

regards to CF there have been large amounts of work undertaken to investigate the specific mutations acquired by *P. aeruginosa* in chronic lung infections, including changes in *lasR*, *mucA*, and *mutS*, (Marvig et al. 2013; Jeukens et al. 2014; Marvig et al. 2014) and so further investigation into the presence of similar mutations in isolates from NCFBr patients would allow a more direct comparison between infections in both diseases. Although the isolates collected from NCFBr patients have been compared alongside a panel of other *P. aeruginosa* isolates (Kos *et al.* 2015) it is also important to understand their place within the larger, general *P. aeruginosa* population. Further work to place NCFBr isolates in the context of the wider population would allow any common strains among NCFBr patients to be identified and observed with regards to patient health.

It is only recently that the world's first national guidelines for the diagnosis and treatment of NCFBr were developed in the UK by the British Thoracic Society (BTS) (Pasteur *et al.* 2010) and so the amount of research being undertaken to better understand the disease and its implications on both patients and health services is gradually increasing. It is hoped by many that the development of cheaper, quicker, and more user-friendly sequencing platforms will allow whole genome sequencing to play a role in the future of diagnostic microbiology (Török & Peacock 2012) as well as bringing greater depth to clinical research. As well as striving to develop a better understanding of NCFBr as a disease, research must continue to progress into the implications of bacterial infections in chronic lung conditions.

Appendix

Table A1: Complete list of all isolates, and accompanying information, collected as part of this study.

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	COPD	09/10/2014	1	A1	N	N	N	C4AA	Y		
Liverpool	COPD	16/10/2014	2	A2	N	N	N	EC29	Y		
Liverpool	4B	10/10/2014	3	A3	N	N	N	049A	Y		Spreading
Liverpool	4B	10/10/2014	3	A4	N	N	N		Y		Smooth
Liverpool	4B	10/10/2014	3	A5	N	N	N		Y		Rough
Liverpool	GP	09/10/2014	4	A6	N	N	N	C408			
Liverpool	GP	10/10/2014	5	A7	N	N	N	2CAA			
Liverpool	GP	09/11/2014	6	A8	N	N	N				
Liverpool	GP	09/11/2014	7	A9	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	GP	11/11/2014	33	A13	N	N	N				
Liverpool	GP	13/11/2014	34	A11	N	N	N				
Liverpool	CPOCCU	14/11/2014	35	A12	N	N	N		Y		Smooth
Liverpool	CPOCCU	14/11/2014	35	A10	N	N	N				Rough
Liverpool	6Y	16/12/2014	116	A14	N	N	N				Pip/Tazo Sens
Liverpool	GP	18/12/2014	117	A15	N	N	N				
Liverpool	GP	31/12/2014	118	A16	N	N	N				
Liverpool	Link 6Z	16/12/2014	119	A17	N	N	N				Small
Liverpool	Link 6Z	16/12/2014	119	A18	N	N	N				Large
Liverpool	COPD	16/12/2014	120	A19	N	N	N	6C2A	Y		
Liverpool	6Y	15/12/2014	121	A20	N	N	N				Pip/Tazo Res
Liverpool	GP	11/11/2014	122	A21	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	Link 6Z	13/01/2015	123	A2	N	N	N				
Liverpool	GP	20/01/2015	124	A3	N	N	N				
Liverpool	COPD	13/01/2015	125	A4	N	N	N				
Liverpool	Link 6Z	22/01/2015	126	A5	N	N	N				
Liverpool	6X	22/01/2015	127	A6	N	N	N				
Liverpool	GP	26/01/2015	128	A7	N	N	N				
Liverpool	GP	26/01/2015	129	A8	N	N	N				
Liverpool	GP	26/01/2015	130	A9	N	N	N				
Liverpool	AMAU	02/02/2015	131	A30	N	N	N				
Liverpool	GP Aintree	08/02/2015	132	A31	N	N	N				
Liverpool	GP Aintree	09/02/2015	133	A32	N	N	N				
Liverpool	GP Aintree	12/02/2015	134	A33	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	GP Aintree	16/02/2015	135	A34	N	N	N				
Liverpool	CTC	19/02/2015	136	A35	N	N	N				
Liverpool	GP	17/02/2015	137	A36	Y	N	N		Y		
Liverpool	GP	18/02/2015	138	A37	N	N	N				
Liverpool	GP Aintree	19/02/2015	139	A38	N	N	N				
Liverpool	Aintree OP	18/02/2015	140	A39	N	N	N				
Liverpool	GP Aintree	13/02/2015	141	A40	N	N	N				
Liverpool	Ward 23 (Aintree)	25/02/2015	142	A41	N	N	N				
Liverpool	Ward 23 (Aintree)	27/02/2015	143	A42	N	N	N				
Liverpool	Aintree Outpatient Unit	04/03/2015	144	A43	N	N	N				
Liverpool	COPD	05/03/2015	145	A44	N	N	N				
Liverpool	GP Aintree	07/03/2015	146	A45	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	Link 6Z	07/04/2015	147	A46	N	N	N		Y	Earliest known: 2004	Mucoid
Liverpool	Link 6Z	07/04/2015	147	A47	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A48	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A49	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A50	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A51	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A52	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A53	N	N	N		Y		White
Liverpool	Link 6Z	07/04/2015	147	A54	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A55	N	N	N		Y		White
Liverpool	Link 6Z	07/04/2015	147	A56	N	N	N		Y		White
Liverpool	Link 6Z	07/04/2015	147	A57	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	Link 6Z	07/04/2015	147	A58	N	N	N		Y		White
Liverpool	Link 6Z	07/04/2015	147	A59	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A60	N	N	N		Y		White
Liverpool	Link 6Z	07/04/2015	147	A61	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A62	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A63	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A64	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A65	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A66	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A67	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A68	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A69	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	Link 6Z	07/04/2015	147	A70	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A71	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A72	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A73	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A74	N	N	N				
Liverpool	Link 6Z	07/04/2015	147	A75	N	N	N		Y		Mucoid
Liverpool	Link 6Z	07/04/2015	147	A76	N	N	N		Y		Mucoid
Liverpool	BGH Alexandra Wing	07/04/2015	148	A77	N	N	N		Y	Earliest known: 2010	Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A78	N	N	N		Y		Mucoid
Liverpool	BGH Alexandra Wing	07/04/2015	148	A79	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A80	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A81	N	N	N		Y		Mucoid

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	BGH Alexandra Wing	07/04/2015	148	A82	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A83	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A84	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A85	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A86	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A87	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A88	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A89	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A90	N	N	N		Y		Mucoid
Liverpool	BGH Alexandra Wing	07/04/2015	148	A91	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A92	N	N	N		Y		Mucoid
Liverpool	BGH Alexandra Wing	07/04/2015	148	A93	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	BGH Alexandra Wing	07/04/2015	148	A94	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A95	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A96	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A97	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A98	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A99	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A100	N	N	N		Y		Mucoid
Liverpool	BGH Alexandra Wing	07/04/2015	148	A101	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A102	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A103	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A104	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A105	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 06	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 07	N	N	N		Y		Brown
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 08	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 09	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 10	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 11	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 12	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 13	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 14	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 15	N	N	N				
Liverpool	BGH Alexandra Wing	07/04/2015	148	A1 16	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 17	N	N	N			Earliest known: 2015	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	LHCH Maple Suite	15/05/2015	149	A118	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A119	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A120	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A121	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A122	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A123	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A124	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A125	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A126	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A127	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A128	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A129	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	LHCH Maple Suite	15/05/2015	149	A130	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A131	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A132	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A133	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A134	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A135	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A136	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A137	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A138	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A139	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A140	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A141	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 42	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 43	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 44	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 45	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 46	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 47	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 48	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 49	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 50	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 51	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 52	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A1 53	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Liverpool	LHCH Maple Suite	15/05/2015	149	A154	N	N	N		Y		
Liverpool	LHCH Maple Suite	15/05/2015	149	A155	N	N	N				
Liverpool	LHCH Maple Suite	15/05/2015	149	A156	N	N	N		Y		
Liverpool	GP (Aintree)	17/04/2015	150	A157	N	N	N				
Liverpool	GP (Aintree)	07/05/2015	152	A158	N	N	N				
Liverpool	22 (Aintree)	09/05/2015	153	A159	N	N	N				
Liverpool	GP (Aintree)	14/05/2015	154	A160	N	N	N				
Liverpool	6Y	15/05/2015	155	A161	N	N	N				
Liverpool	Link 6Z	15/05/2015	156	A162	N	N	N				
Liverpool	6Y	19/05/2015	137	A163	Y	N	N		Y		
Liverpool	6Y	19/05/2015	158	A164	N	N	N				
Newcastle		01/07/2008	8	B1	N	N	N	0C1A		10	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		01/11/2008	8	B3	N	N	N	0C1A	Y	10	
Newcastle		23/10/2014	8	B7	N	N	N	0C1A		10	
Newcastle		26/10/2011	9	B10	N	N	N	D421		New patient	Non-mucoid
Newcastle		18/12/2011	9	B12	N	N	N			New patient	
Newcastle		24/05/2013	9	B15	N	N	N			New patient	
Newcastle		25/11/2013	9	B16	N	N	N	D421	Y	New patient	
Newcastle		14/07/2011	10	B17	N	N	N	741E		26	Non-mucoid
Newcastle		21/03/2012	10	B18	N	N	N			26	Non-mucoid
Newcastle		19/09/2012	10	B19	N	N	N			26	
Newcastle		24/05/2013	10	B20	N	N	N	741E		26	
Newcastle		22/07/2008	11	B21	N	N	N	C40A		11	
Newcastle		01/01/2009	11	B22	N	N	N	C40A		11	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		27/07/2011	11	B24	N	N	N	AC2E		11	Mucoid
Newcastle		03/09/2014	11	B34	N	N	N		Y	11	
Newcastle		23/10/2014	11	B35	N	N	N	3C2A		11	
Newcastle		20/07/2011	12	B36	N	N	N	F42A		31	Non-mucoid
Newcastle		18/01/2012	12	B37	N	N	N		Y	31	
Newcastle		24/05/2013	12	B38	N	N	N	F42A		31	Mucoid
Newcastle		01/10/2007	13	B40	N	N	N	3C52		New patient	Mucoid
Newcastle		04/11/2011	13	B45	N	N	N	3C52		New patient	
Newcastle		12/10/2012	14	B46	N	N	N	3C28		New patient	
Newcastle		23/10/2014	14	B49	N	N	N	D421		New patient	
Newcastle		01/03/2011	15	B50	N	N	N	AC2A		24	
Newcastle		06/11/2012	15	B58	N	N	N			24	Mucoid

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		03/09/2014	15	B62	N	N	N	AC2A	Y	24	
Newcastle		09/07/2011	16	B63	N	N	N	F469		New patient	Mucoid
Newcastle		11/07/2014	16	B71	N	N	N	F469		New patient	
Newcastle		01/06/2008	17	B72	N	N	N	239A		35	
Newcastle		27/07/2011	17	B74	N	N	N	239A		35	
Newcastle		20/08/2012	17	B75	N	N	N			35	
Newcastle		04/09/2013	17	B77	N	N	N	239A		35	
Newcastle		01/10/2007	18	B79	N	N	N	B420		8	Mucoid
Newcastle		01/10/2007	18	B80	N	N	N			8	
Newcastle		01/12/2007	18	B81	N	N	N			8	
Newcastle		01/08/2008	18	B82	N	N	N			8	
Newcastle		01/10/2008	18	B84	N	N	N	B420		8	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		01/05/2010	18	B85	N	N	N			8	
Newcastle		01/11/2010	18	B87	N	N	N			8	
Newcastle		25/07/2011	18	B90	N	N	N	B420		8	Non-mucoid
Newcastle		22/03/2013	18	B99	N	N	N			8	
Newcastle		22/03/2013	18	B100	N	N	N			8	
Newcastle		23/10/2014	18	B112	N	N	N			8	
Newcastle		23/10/2014	18	B113	N	N	N	B420	Y	8	
Newcastle		11/04/2012	19	B114	N	N	N	0C4A	Y	New patient	Non-mucoid
Newcastle		18/02/2014	19	B125	N	N	N			New patient	
Newcastle		19/02/2014	19	B126	N	N	N	0C4A		New patient	
Newcastle		09/10/2013	20	B127	N	N	N	6852		New patient	
Newcastle		19/02/2014	20	B132	N	N	N			New patient	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		22/10/2014	20	B139	N	N	N	6852		New patient	Mucoid
Newcastle		12/07/2011	21	B141	N	N	N	D421		New patient	Mucoid
Newcastle		06/03/2012	21	B145	N	N	N			New patient	Mucoid
Newcastle		03/04/2014	21	B150	N	N	N	D421		New patient	
Newcastle		01/11/2010	22	B151	N	N	N	1BAE		17	
Newcastle		18/04/2012	22	B152	N	N	N	2C12		17	Mucoid
Newcastle		01/03/2009	23	B156	N	N	N	059A		New patient	
Newcastle		03/09/2014	23	B162	N	N	N	059A		New patient	
Newcastle		29/11/2012	24	B163	N	N	N			New patient	
Newcastle		20/03/2012	25	B164	N	N	N	CC60		New patient	Mucoid
Newcastle		23/10/2014	25	B169	N	N	N	CC62		New patient	
Newcastle		10/06/2014	26	B170	N	N	N			New patient	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		29/08/2014	26	B174	N	N	N			New patient	
Newcastle		08/07/2008	27	B179	N	N	N			New patient	
Newcastle		07/11/2013	28	B183	N	N	N			New patient	
Newcastle		29/11/2014	28	B184	N	N	N			New patient	
Newcastle		23/09/2011	29	B185	N	N	N	4C8A		New patient	Mucoid
Newcastle		24/05/2013	29	B186	N	N	N	4C8A		New patient	Mucoid
Newcastle		01/12/2007	30	B187	N	N	N	682A		14	
Newcastle		01/07/2010	30	B190	N	N	N	682E		14	
Newcastle		05/07/2011	30	B191	N	N	N		Y	14	
Newcastle		05/07/2011	30	B192				682E		14	
Newcastle		04/09/2013	30	B194	N	N	N	682A		14	
Newcastle		14/03/2012	31	B196	N	N	N			New patient	

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Newcastle		04/09/2013	31	B198	N	N	N			New patient	
Newcastle		18/10/2011	32	B199	N	N	N	F429	Y	New patient	
Newcastle		23/08/2013	32	B202	N	N	N	0C2E		New patient	
Sheffield	4	14/10/2009	36	C1	N	N	N				
Sheffield	4	14/10/2009	36	C2	N	N	N		Y		
Sheffield	4	25/02/2010	37	C3	N	N	N		Y		
Sheffield	4	25/02/2010	37	C4	N	N	N		Y		
Sheffield	4	25/02/2010	37	C5	N	N	N		Y		
Sheffield	4	03/03/2010	38	C6	N	N	N		Y		
Sheffield	4	23/03/2010	39	C7	N	N	N		Y		
Sheffield	4	16/04/2010	40	C8	N	N	N		Y		
Sheffield	4	16/04/2010	40	C9	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Sheffield	4	16/04/2010	40	C10	N	N	N		Y		
Sheffield	4	19/08/2010	41	C11	N	N	N		Y		
Sheffield	4	20/08/2010	42	C12	N	N	N		Y		
Sheffield	4	20/08/2010	42	C13	N	N	N		Y		
Sheffield	4	20/08/2010	42	C14	N	N	N		Y		
Sheffield	4	20/08/2010	42	C15	N	N	N		Y		
Sheffield	4	20/08/2010	42	C16	N	N	N		Y		
Sheffield	4	20/08/2010	42	C17	N	N	N		Y		
Sheffield	4	15/04/2011	43	C18	N	N	N		Y		
Sheffield	4	01/07/2011	44	C19	N	N	N				
Sheffield	4	01/07/2011	44	C20	N	N	N		Y		
Papworth	6	14/04/2009	45	C21	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Papworth	6	14/04/2009	45	C22	N	N	N		Y		
Papworth	6	14/04/2009	45	C23	N	N	N		Y		
Papworth	6	20/05/2009	46	C24	N	N	N				
Papworth	6	20/05/2009	46	C25	N	N	N		Y		
Papworth	6	01/06/2009	47	C26	N	N	N				
Papworth	6	01/06/2009	47	C27	N	N	N				
Papworth	6	03/06/2009	48	C28	N	N	N				
Papworth	6	03/06/2009	48	C29	N	N	N		Y		
Papworth	6	04/06/2009	49	C30	N	N	N		Y		
Papworth	6	25/08/2009	50	C31	N	N	N		Y		
Papworth	6	25/08/2009	50	C32	N	N	N		Y		
Papworth	6	25/08/2009	50	C33	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Papworth	6	30/12/2009	51	C34	N	N	N				
Papworth	6	30/12/2009	51	C35	N	N	N				
Papworth	6	21/05/2010	52	C36	N	N	N		Y		
Papworth	6	21/05/2010	52	C37	N	N	N				
Papworth	6	30/06/2010	53	C38	N	N	N				
Papworth	6	30/06/2010	53	C39	N	N	N				
Papworth	6	30/06/2010	53	C40	N	N	N				
Papworth	6	12/07/2010	54	C41	N	N	N				
Papworth	6	12/07/2010	54	C42	N	N	N		Y		
Papworth	6	28/07/2010	55	C43	N	N	N		Y		
Papworth	6	28/07/2010	55	C44	N	N	N		Y		
Papworth	6	28/07/2010	55	C45	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Papworth	6	24/08/2010	56	C46	N	N	N				
Papworth	6	02/02/2011	57	C47	N	N	N				
Papworth	6	02/02/2011	57	C48	N	N	N				
Papworth	6	22/02/2011	58	C49	N	N	N		Y		
Papworth	6	22/02/2011	58	C50	N	N	N				
Papworth	6	23/02/2011	59	C51	Y	N	N	4C12	Y		
Papworth	6	16/03/2011	60	C52	N	N	N				
Papworth	6	16/03/2011	60	C53	N	N	N				
Papworth	6	15/06/2011	61	C54	N	N	N		Y		
London Chest Hospital	9	26/06/2009	62	C55	N	N	N		Y		
London Chest Hospital	9	26/06/2009	62	C56	N	N	N		Y		
London Chest Hospital	9	26/06/2009	62	C57	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
London Chest Hospital	9	26/06/2009	62	C58	N	N	N		Y		
London Chest Hospital	9	26/06/2009	62	C59	N	N	N		Y		
London Chest Hospital	9	26/06/2009	62	C60	N	N	N		Y		
London Chest Hospital	9	17/11/2009	63	C61	N	N	N		Y		
Norfolk & Norwich	10	02/09/2009	64	C62	N	N	N				
Norfolk & Norwich	10	02/09/2009	64	C63	N	N	N		Y		
Norfolk & Norwich	10	25/11/2009	65	C64	N	N	N		Y		
Norfolk & Norwich	10	25/11/2009	65	C65	N	N	N		Y		
Norfolk & Norwich	10	25/11/2009	65	C66	N	N	N		Y		
Norfolk & Norwich	10	25/11/2009	65	C67	N	N	N		Y		
Norfolk & Norwich	10	25/11/2009	65	C68	N	N	N		Y		
Norfolk & Norwich	10	12/05/2010	66	C69	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Reading	11	04/09/2009	67	C70	N	N	N				
Reading	11	04/09/2009	67	C71	N	N	N		Y		
Reading	11	05/11/2010	68	C72	N	N	N				
Reading	11	05/11/2010	68	C73	N	N	N		Y		
Reading	11	03/12/2010	69	C74	N	N	N		Y		
Reading	11	03/12/2010	69	C75	N	N	N				
Chertsey, Surrey	15	12/05/2009	70	C76	N	N	N		Y		
Chertsey, Surrey	15	03/07/2009	71	C77	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C78	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C79	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C80	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C81	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Chertsey, Surrey	15	14/07/2009	72	C82	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C83	N	N	N		Y		
Chertsey, Surrey	15	14/07/2009	72	C84	N	N	N		Y		
Chertsey, Surrey	15	06/08/2009	73	C85	N	N	N		Y		
Chertsey, Surrey	15	06/08/2009	73	C86	N	N	N		Y		
Chertsey, Surrey	15	06/08/2009	73	C87	N	N	N		Y		
Chertsey, Surrey	15	15/12/2009	74	C88	N	N	N		Y		
Chertsey, Surrey	15	25/03/2010	75	C89	N	N	N		Y		
Chertsey, Surrey	15	25/03/2010	75	C90	N	N	N				
Chertsey, Surrey	15	13/01/2011	76	C91	N	N	N		Y		
Chertsey, Surrey	15	01/02/2011	77	C92	N	N	N		Y		
Dorset County Hospital	16	05/06/2009	78	C93	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Dorset County Hospital	16	03/07/2009	78	C94	N	N	N		Y		
Dorset County Hospital	16	29/07/2009	79	C95	N	N	N		Y		
Dorset County Hospital	16	29/07/2009	79	C96	N	N	N		Y		
Dorset County Hospital	16	29/07/2009	79	C97	N	N	N		Y		
Dorset County Hospital	16	29/07/2009	79	C98	N	N	N		Y		
Dorset County Hospital	16	29/07/2009	79	C99	N	N	N		Y		
Dorset County Hospital	16	13/10/2009	80	C100	N	N	N		Y		
Dorset County Hospital	16	21/10/2009	81	C101	N	N	N		Y		
Dorset County Hospital	16	21/10/2009	81	C102	N	N	N		Y		
Dorset County Hospital	16	21/10/2009	81	C103	N	N	N		Y		
Swansea	17	16/05/2009	82	C104	N	N	N		Y		
Swansea	17	25/07/2009	83	C105	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Swansea	17	11/08/2009	84	C106	N	N	N		Y		
Swansea	17	11/08/2009	84	C107	N	N	N		Y		
Swansea	17	11/08/2009	84	C108	N	N	N		Y		
Swansea	17	11/08/2009	85	C109	N	N	N		Y		
Swansea	17	11/08/2009	85	C110	N	N	N		Y		
Swansea	17	11/08/2009	85	C111	N	N	N		Y		
Swansea	17	11/08/2009	85	C112	N	N	N		Y		
Swansea	17	05/12/2009	86	C113	N	N	N				
Swansea	17	05/12/2009	86	C114	N	N	N		Y		
Swansea	17	05/12/2009	86	C115	N	N	N		Y		
Swansea	17	04/06/2010	87	C116	N	N	N		Y		
Swansea	17	04/06/2010	87	C117	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Swansea	17	04/06/2010	87	C118	N	N	N		Y		
Hull	20	23/01/2010	88	C119	N	N	N		Y		
Hull	20	29/01/2010	89	C120	N	N	N		Y		
Hull	20	29/01/2010	89	C121	N	N	N				
Hull	20	02/04/2010	90	C122	N	N	N				
Hull	20	02/04/2010	90	C123	N	N	N		Y		
Hull	20	08/04/2010	91	C124	N	N	N		Y		
Hull	20	29/04/2010	92	C125	N	N	N		Y		
Hull	20	29/04/2010	92	C126	N	N	N		Y		
Hull	20	29/04/2010	92	C127	N	N	N		Y		
Hull	20	29/04/2010	92	C128	N	N	N		Y		
Hull	20	29/04/2010	92	C129	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Hull	20	08/05/2010	93	C130	N	N	N				
Hull	20	08/05/2010	93	C131	N	N	N		Y		
Hull	20	28/05/2010	94	C132	N	N	N				
Hull	20	28/05/2010	94	C133	N	N	N		Y		
Hull	20	19/12/2010	95	C134	N	N	N		Y		
Sunderland	21	02/11/2009	96	C135	N	N	N		Y		
Sunderland	21	16/04/2010	97	C136	N	N	N				
Sunderland	21	16/04/2010	97	C137	N	N	N		Y		
Sunderland	21	08/09/2010	98	C138	N	N	N				
Sunderland	21	08/09/2010	98	C139	N	N	N		Y		
Sunderland	21	01/10/2010	99	C140	N	N	N				
Sunderland	21	01/10/2010	99	C141	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Sunderland	21	11/02/2011	100	C142	N	N	N		Y		
Colchester	22	08/07/2010	101	C143	N	N	N		Y		
Colchester	22	08/07/2010	101	C144	N	N	N		Y		
Colchester	22	08/07/2010	101	C145	N	N	N		Y		
Colchester	23	25/06/2010	102	C146	N	N	N		Y		
Colchester	23	25/06/2010	102	C147	N	N	N		Y		
Colchester	23	25/06/2010	102	C148	N	N	N		Y		
Colchester	23	22/03/2011	103	C149	N	N	N		Y		
Portsmouth	25	27/08/2010	104	C150	N	N	N		Y		
Portsmouth	25	03/03/2011	105	C151	N	N	N		Y		
Portsmouth	25	03/03/2011	105	C152	N	N	N				
Portsmouth	25	07/04/2011	106	C153	N	N	N		Y		

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
Portsmouth	25	07/04/2011	106	C154	N	N	N				
Cardiff	26	04/12/2010	107	C155	N	N	N		Y		
West Wales G. Hospital	28	16/11/2010	108	C156	N	N	N		Y		
West Wales G. Hospital	28	03/12/2010	109	C157	N	N	N				
West Wales G. Hospital	28	03/12/2010	109	C158	N	N	N		Y		
West Wales G. Hospital	28	09/12/2010	110	C159	N	N	N		Y		
West Wales G. Hospital	28	09/12/2010	111	C160	N	N	N		Y		
West Wales G. Hospital	28	03/03/2011	112	C161	N	N	N		Y		
West Wales G. Hospital	28	03/03/2011	112	C162	N	N	N				
West Wales G. Hospital	28	03/03/2011	112	C163	N	N	N				
West Wales G. Hospital	29	08/12/2010	113	C164	N	N	N		Y		
West Wales G. Hospital	29	08/12/2010	113	C165	N	N	N				

Centre	Location	Date collected	Patient	Isolate	LES (PCR)	MID1 (PCR)	MA15 (PCR)	AT Type	Sequenced	Original patient info.	Notes
West Wales G. Hospital	29	21/04/2011	114	C166	N	N	N				
West Wales G. Hospital	29	21/04/2011	114	C167	N	N	N		Y		
West Wales G. Hospital	29	21/12/2010	115	C168	N	N	N		Y		

Table A2: List of probe sequences used in the Array Tube system. Adapted from supplementary information from Wiehlmann *et al*. (2007).

Probe name	**Reference**	**5'-3'-Sequence (probe)**
oriC PAO	PAO1- Sequence, Stover *et al.* (updated 2006)	GAAGCCCAGCAATTGCGTGTTTC
oriC non-PAO	UCBPP-PA14, complete genome, Lee *et al*. (2006)	GAAGCCCAGCAACTGCGTGTTTC
oprL (1) PAO	PAO1- Sequence, Stover *et al.* (updated 2006)	GGTGCTGCAGGGTGTTTCGCCGG
oprL (1) non-PAO	UCBPP-PA14, complete genome, Lee *et al.* (2006)	GGTGCTGCAGGGCGTTTCGCCGG
oprL (2) PAO	PAO1- Sequence, Stover et al. (updated 2006)	GTGCTGCAGGGTGTTTCGCCG
oprL (2) non-PAO	UCBPP-PA14, complete genome, Lee *et al.* (2006)	GCTGCAGGGCGTTTCGCCG
fliCa (1) PAK	PAK, Totten and Lory (1990), flagellin type a2, Giske et al. (2006)	CAAGATCGCCGCAGCGGTCAAC
fliCa (1) non-PAK	ATCC15691, Spangenberg et al. (1998), flagellin type a1, Giske et al. (2006)	CAAGATCGCCGCTGCGGTCAAC
fliCa (2) PAK	PAK, Totten and Lory (1990), flagellin type a2, Giske et al. (2006)	CAAGATCGCCGCAGCGGTCAACGAC
fliCa (2) non-PAK	ATCC15691, Spangenberg et al. (1998), flagellin type a1, Giske et al. (2006)	CAAGATCGCCGCTGCGGTCAACGAC
alkB2 PAO	PAO1- Sequence, Stover et al. (updated 2006)	CCTCGCCCTGTTCCCACCGCTCTGG
alkB2 non-PAO	ATCC 15691, Morales et al. (2004)	CTCGCCCTGTTCCCGCCGCTCTGG
citS-1 PAO	PAO1- Sequence, Stover et al. (updated 2006)	TCGAGCAACTGGCAGAGAAATCCG
citS-1 non-PAO	UCBPP-PA14, complete genome, Lee et al. (2006)	CGAGCAACTGGCGGAGAAATCCG
citS-2 PAO	PAO1- Sequence, Stover et al. (updated 2006)	GCGGAAAACTTCCTGCACATGATGTT
citS-2 non-PAO	Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	GCGGAAAACTTCCTCCACATGATGTT
oprI (1) PAO	PAO1- Sequence, Stover et al. (updated 2006)	AGCTCAGCAGACTGCTGACGAGG
oprI (1) non-PAO	UCBPP-PA14, complete genome, Lee et al. (2006)	AGCTCAGCAGACCGCTGACGAG
oprI (2) PAO	PAO1- Sequence, Stover et al. (updated 2006)	GCTCAGCAGACTGCTGACGAGGCTAACG
oprI (2) non-PAO	UCBPP-PA14, complete genome, Lee et al. (2006)	GCTCAGCAGACCGCTGACGAGGCTAAC
ampC-1 PAO	PAO1- Sequence, Stover et al. (updated 2006)	ACGGCCGCCGGGTGACGCC
ampC-1 non-PAO	De Champs et al. (2002), Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	ACGGCCGCCAGGTGACGCCG

Probe name	Reference	5'-3'-Sequence (probe)
ampC-3 PAO	PAO1- Sequence, Stover et al. (updated 2006)	CGACCTACGCGCCGGGCAG
ampC-3 non-PAO	De Champs et al. (2002), Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	CGACCTATGCGCCGGGCAGC
ampC-4 PAO	PAO1- Sequence, Stover et al. (updated 2006)	CGTTCGAACGGCTCATGGAGCAG
ampC-4 non-PAO	De Champs et al. (2002), Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	CGTTCGAACGACTCATGGAGCAGC
ampC-5 PAO	PAO1- Sequence, Stover et al. (updated 2006)	TGGAGCAGCAAGTGTTCCCGGC
ampC-5 non-PAO	De Champs et al. (2002), Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	TGGAGCAGCAACTGTTCCCGGC
ampC-6 PAO	PAO1- Sequence, Stover et al. (updated 2006)	GAACAAGACCGGTTCCACCAACGG
ampC-6 non-PAO	UCBPP-PA14, complete genome, Lee et al. (2006)	AACAAGACCGGCTCCACCAACGG
ampC-7 PAO	PAO1- Sequence, Stover et al. (updated 2006)	CGACCTGGGCCTGGTGATCCT
ampC-7 non-PAO	De Champs et al. (2002), Kiewitz and Tummler. J Bacteriol. (2000) 182:3125-35.	GCGACCTGGGACTGGTGATCCTGG
fliC a	ATCC15691, Spangenberg et al. (1998)	GTCGCTGAACGGCACCTACTTCA
fliC b	PAO1- Sequence, Stover et al. (updated 2006)	GCCGACCAACTGAACTCCAACTCG
exoS	PAO1- Sequence, Stover et al. (updated 2006)	CAGCCCAGTCAGGACGCGCA
exoU	UCBPP-PA14, complete genome, Lee et al. (2006)	CGCCAGTTTGAGAACGGAGTCACC
fpvA type I	PAO1- Sequence, Stover et al. (updated 2006)	CCTGAATCCGACCATTCGCGAGTC
fpvA type IIa	de Chial et al. (2003)	TCGGACTGTACTCCTACGAAGCAGC
fpvA type IIb	Spencer et al. (2003)	CCAATCCCTATCGCTGGAACCGTACC
fpvA type III	de Chial et al. (2003)	GCTCGGGACTCGCATTTCGTCC
fpvB	PAO1- Sequence, Stover et al. (updated 2006)	GCGTTATTGCTCGGTCTCTCCTCG
LES	LES400 (personal communication C. Winstanley)	TGCATAGGAGTCATGCCGACAGCA
PA0636	PAO1- Sequence, Stover et al. (updated 2006)	GCCAATTGGGTCAGCAAGCAACG
PA0722	PAO1- Sequence, Stover et al. (updated 2006)	CGTGTCGCGAACTCGCATGGC
PA0728	PAO1- Sequence, Stover et al. (updated 2006)	CTGGAGCCTGCGAAAGTGGCTC
PA2185	PAO1- Sequence, Stover et al. (updated 2006)	ACGAGGGTGATGGCTGGGAATACG

Probe name	Reference	5'-3'-Sequence (probe)
PA2221	PAO1- Sequence, Stover et al. (updated 2006)	CAGTTGTCGCCAGGTCTGGAGAATCC
PA3835	PAO1- Sequence, Stover et al. (updated 2006)	CACATCAATGTCAGCCCACGCCA
fla-island	Arora et al. (2001)	ACCTGTGTCGCTGGAGGGTATGTT
orfA	Arora et al. (2001)	CGCTGGAGGGTATGTTCCGCAAGG
orfI	Arora et al. (2001)	CCTGGACCTCTCCAAGGTTCGCCT
orfJ	Arora et al. (2001)	GCCATTCCGACGACCAAACAAGGC
PA0980	PAO1- Sequence, Stover et al. (updated 2006)	CGGTATGAAGATGGGTGGTTGGGTCG
XF1753	UCBPP-PA14, complete genome, Lee et al. (2006)	TGCGAGGACCAGAAACCTTGATGG
acetyltransferase	UCBPP-PA14, complete genome, Lee et al. (2006)	CGAAGCGTAGGGTCTTCGTAGCC
pKL-1	Klockgether et al. (2004)	CACCATGCAAATGCTCGATGGACTGC
pKL-3	Klockgether et al. (2004)	TCTGAACTGCGGCTATCACCTGGA
TB-C47-1	P.aeruginosa TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	GCAGGCGTCCAAGTTGGAGCTCTCC
TB-C47-2	P.aeruginosa TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	TCCAACAGGCAGGAGTACAGGGTG
PAPI-1 pili chaperone	UCBPP-PA14, complete genome, Lee et al. (2006)	GGAACACAACGTGGGGCGTGAC
PAPI-1 luminal binding protein	UCBPP-PA14, complete genome, Lee et al. (2006)	CCAGTTGGCACCACCATGCTTGC
pKLC conserved hypothetical	Klockgether et al. (2004)	GCCTGCCTACTTGTTCCCAACGC
pKLC adhesin	Klockgether et al. (2004)	GGCTGTATTGCCCGCCATTCTCC
pKLC fatty acid synthase	Klockgether et al. (2004)	CGACAGACAGAAAGGGTTCTTGCGC
PAGI-2/3-4	Larbig et al. (2002)	GCGCCTTCTCCTCTTTGCAGATGT
PAGI-2/3-5	Larbig et al. (2002)	CAGTATGGTACGGACACGAAGCGC
PAGI-2/3-6	Larbig et al. (2002)	CCATGGTCGGAACAGGCACGATATGC
C-45	Larbig et al. (2002)	CGAGGAGTTTCGGACCCGCTTTGA
C-46	Larbig et al. (2002)	CGAAGTCTGAGGTGTGGACCCGC
C-47	Larbig et al. (2002)	CCACTCGATCATGTTGAGCATCGGCTCC

Probe name	Reference	5'-3'-Sequence (probe)
PAGI-2	Larbig et al. (2002)	GCATCATTGCGCGTCACATCTGGT
PAGI-2/3-1	Larbig et al. (2002)	GACCGCAAGCAGAAACGGCATGC
PAGI-3-1	Larbig et al. (2002)	CCCGTTGCTCATAACCCGTTCCTG
PAGI-3-8	Larbig et al. (2002)	GGTTAGTCCCTTCTGCCCGCATCG
tRNA(Pro)- island 1	P.aeruginosa TB, gene island integrated into tRNA(Pro) PA2736.1	GTGTCACGGCCCATGTCTAGCAGC
tRNA(Pro)- island 2	P.aeruginosa TB, gene island integrated into tRNA(Pro) PA2736.1	AGGCCATGGGCTAGCCGGATGC
PAGI-1	Liang et al. (2001)	TTCTCGGTGTCGAGGGATTCTCGG

Table A3: SNP data from AT genotyping for all longitudinal isolates included in chapter 3.

Patient	Date collected	AT code	oriC	oprL	alkB2	citS-1	citS-2	oprI	ampC-1	ampC-3	ampC-4	ampC-5	ampC-6	ampC-7	fliCa	fliCa-SNP	exoS	exoU
8	01/07/2008	0C1A	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0
	01/11/2008	0C1A	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0
	23/10/2014	0C1A	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0
9	14/07/2011	D421	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
	24/05/2013	D421	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
10	14/07/2011	741E	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	0
	24/05/2013	741E	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	0
11	22/07/2008	C40A	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0
	01/01/2009	C40A	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0
	27/07/2011	AC2E	1	0	1	0	1	1	0	0	0	0	1	0	1	1	1	0
	23/10/2014	3C2A	0	0	1	1	1	1	0	0	0	0	1	0	1	0	1	0
12	20/07/2011	F42A	1	1	1	1	0	1	0	0	0	0	1	0	1	0	1	0
	24/05/2013	F42A	1	1	1	1	0	1	0	0	0	0	1	0	1	0	1	0
13	01/10/2007	3C52	0	0	1	1	1	1	0	0	0	1	0	1	0	0	1	0
	04/11/2011	3C52	0	0	1	1	1	1	0	0	0	1	0	1	0	0	1	0
14	12/10/2012	3C28	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0
	23/10/2014	D421	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
15	01/03/2011	AC2A	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0
	03/09/2014	AC2A	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0
16	09/07/2011	F469	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1
	11/07/2014	F469	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1

Patient	Date collected	AT code	oriC	oprL	alkB2	citS-1	citS-2	oprI	ampC-1	ampC-3	ampC-4	ampC-5	ampC-6	ampC-7	fliCa	fliCa-SNP	exoS	exoU
17	01/06/2008	239A	0	0	1	0	0	0	1	1	1	0	0	1	1	0	1	0
	27/07/2011	239A	0	0	1	0	0	0	1	1	1	0	0	1	1	0	1	0
	04/09/2013	239A	0	0	1	0	0	0	1	1	1	0	0	1	1	0	1	0
18	01/10/2007	B420	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0
	01/10/2008	B420	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0
	25/07/2011	B420	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0
	23/10/2014	B420	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0
19	11/04/2012	0C4A	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1	0
	19/02/2014	0C4A	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1	0
20	09/10/2013	6852	0	1	1	0	1	0	0	0	0	1	0	1	0	0	1	0
	22/10/2014	6852	0	1	1	0	1	0	0	0	0	1	0	1	0	0	1	0
21	12/07/2011	D421	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
	03/04/2014	D421	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
22	01/11/2010	1BAE	0	0	0	1	1	0	1	1	1	0	1	0	1	1	1	0
	18/04/2012	2C12	0	0	1	0	1	1	0	0	0	0	0	1	0	0	1	0
23	01/03/2009	059A	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	0
	03/09/2014	059A	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	0
25	20/03/2012	CC60	1	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0
	23/10/2014	CC60	1	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0
29	23/09/2011	4C8A	0	1	0	0	1	1	0	0	1	0	0	0	1	0	1	0
	24/05/2013	4C8A	0	1	0	0	1	1	0	0	1	0	0	0	1	0	1	0

Patient	Date collected	AT code	oriC	oprL	alkB2	citS-1	citS-2	oprI	ampC-1	ampC-3	ampC-4	ampC-5	ampC-6	ampC-7	fliCa	fliCa-SNP	exoS	exoU
30	01/12/2007	682A	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0
	01/07/2010	682E	0	1	1	0	1	0	0	0	0	0	1	0	1	1	1	0
	01/07/2011	682E	0	1	1	0	1	0	0	0	0	0	1	0	1	1	1	0
	04/09/2013	682A	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0
32	18/10/2011	F429	1	1	1	1	0	1	0	0	0	0	1	0	1	0	0	1
	23/08/2013	0C2E	0	0	0	0	1	1	0	0	0	0	1	0	1	1	1	0

Table A4: Accessory genome data from AT genotyping of all isolates in chapter 3.

Patient	AT code	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6	pKL-1	pKL-3	TB-C47-1	TB-C47-2
8	0C1A	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	0C1A	0	1	1	0	0	0	0	0	0	0	1	1	0	0
	0C1A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	D421	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D421	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	741E	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	741E	0	0	0	0	0	0	0	0	0	0	1	0	0	0
11	C40A	0	0	1	0	0	0	0	1	0	1	1	1	0	0
	C40A	0	0	1	0	0	0	0	1	0	1	1	1	0	0
	AC2E	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	3C2A	1	0	1	0	0	0	0	1	1	1	1	0	0	0
12	F42A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	F42A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	3C52	0	1	1	0	0	0	1	1	0	0	1	1	0	0
	3C52	0	1	1	0	0	0	1	0	0	0	1	1	0	0
14	3C28	1	0	1	0	0	0	0	1	1	1	0	1	0	0
	D421	0	0	1	0	0	0	0	0	0	1	1	0	0	0
15	AC2A	0	0	1	0	0	0	0	1	0	1	1	1	0	0
	C40A	0	1	0	0	0	0	1	0	0	0	1	0	0	0
	AC2A	0	1	0	0	0	0	0	0	0	0	1	0	0	0
16	F469	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	F469	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	239A	0	0	1	0	0	0	0	1	0	1	1	0	0	0
	239A	0	1	1	0	0	0	0	1	0	1	1	0	0	0
	239A	0	1	1	0	1	0	0	1	0	0	1	0	0	0
18	B420	0	1	1	0	0	0	0	0	0	0	0	0	0	0
	B420	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	B420	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B420	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0C4A	0	0	0	0	0	0	0	0	0	0	1	1	0	0
	0C4A	0	0	0	0	0	0	0	0	0	0	1	1	0	0
20	6852	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6852	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	D421	0	1	0	0	0	0	0	0	0	0	1	1	0	0
	D421	0	1	1	0	0	0	0	0	0	0	1	1	0	0
22	1BAE	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2C12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	059A	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	059A	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Patient	AT code	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6	pKL-1	pKL-3	TB-C47-1	TB-C47-2
25	CC60	1	0	1	0	0	0	0	1	1	1	0	0	0	0
	CC60	1	1	1	0	0	0	0	1	1	1	0	0	0	0
29	4C8A	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	4C8A	0	1	0	0	0	0	0	0	0	0	1	0	0	0
30	682A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	682E	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	682A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	F429	0	0	1	0	0	0	0	1	1	1	1	1	0	0
	0C2E	0	0	1	0	0	0	0	0	0	1	1	0	0	0

Patient	AT code	PAPI-1 Pili-Ch.	PAPI-1 LuBiPr.	pKLC-unkown	pKLC-adhesin	pKLC-metabol.	Pyov.Rec-1	Pyov.Rec-2a	Pyov.Rec-2b	Pyov.Rec-3	Pyov.Rec-B	LES	PA0636	PA0722
8	0C1A	0	0	1	1	0	0	1	0	0	1	0	0	1
	0C1A	0	1	0	0	0	0	1	0	1	1	0	0	1
	0C1A	0	0	0	0	0	0	1	0	0	1	0	0	1
9	D421	0	0	0	0	0	1	0	0	0	1	0	1	1
	D421	0	0	0	0	0	1	0	0	0	1	0	1	1
10	741E	0	0	1	0	0	0	1	0	0	1	0	1	0
	741E	0	0	1	0	0	0	1	0	0	1	0	1	0
11	C40A	0	0	1	1	1	0	0	1	0	1	0	1	1
	C40A	0	0	1	1	1	0	0	1	0	1	0	1	1
	AC2E	0	1	1	0	0	0	0	0	1	1	0	0	0
	3C2A	0	0	1	0	0	0	0	1	0	1	0	0	0
12	F42A	0	0	0	0	0	1	0	0	0	1	0	1	0
	F42A	0	0	0	0	0	1	0	0	0	1	0	1	0
13	3C52	0	0	0	0	0	0	0	0	1	1	0	0	0
	3C52	0	0	0	0	0	0	0	0	1	1	0	0	0
14	3C28	0	0	1	0	0	0	0	1	0	1	0	0	0
	D421	1	0	1	0	0	1	0	0	0	1	0	1	1
15	AC2A	0	0	1	1	1	0	0	1	1	1	0	1	1
	C40A	1	1	1	0	0	0	0	0	0	1	0	1	1
	AC2A	0	1	1	0	0	0	0	0	0	1	0	0	0
16	F469	0	0	0	0	0	0	0	0	0	1	0	1	1
	F469	0	0	0	0	0	0	0	0	0	1	0	1	1
17	239A	0	0	1	0	0	1	0	0	0	1	0	1	0
	239A	0	0	1	0	0	1	0	0	0	1	0	1	0
	239A	0	0	1	1	0	1	0	0	0	1	0	1	0
18	B420	0	0	0	0	0	0	0	1	0	0	0	1	1
	B420	0	0	0	0	0	0	0	1	0	0	0	1	1
	B420	0	0	0	0	0	0	0	1	0	0	0	1	1
	B420	0	0	0	0	0	0	0	1	0	0	0	1	1
19	0C4A	0	0	1	1	1	1	0	0	0	1	0	1	0
	0C4A	0	0	1	1	1	1	0	0	0	1	0	1	0
20	6852	0	0	0	0	0	0	0	1	0	1	0	0	0
	6852	0	0	0	0	0	0	0	1	0	1	0	0	0
21	D421	1	1	0	0	0	0	0	0	1	1	0	1	1
	D421	1	1	0	0	0	0	0	0	0	1	0	1	1
22	1BAE	0	0	0	0	0	0	0	0	1	1	0	1	1
	2C12	0	0	0	0	0	0	0	0	1	1	0	1	1
23	059A	0	0	0	0	1	0	1	0	0	1	0	1	0
	059A	0	0	0	0	0	0	1	0	0	1	0	1	0

Patient	AT code	PAPI-1 Pili-Ch.	PAPI-1 LuBiPr.	pKLC-unkown	pKLC-adhesin	pKLC-metabol.	Pyov.Rec-1	Pyov.Rec-2a	Pyov.Rec-2b	Pyov.Rec-3	Pyov.Rec-B	LES	PA0636	PA0722
25	CC60	0	0	0	0	0	0	0	0	1	1	0	1	1
	CC60	0	0	0	0	0	0	0	0	1	1	0	1	1
29	4C8A	0	0	1	0	0	0	0	1	0	1	0	1	0
	4C8A	0	0	1	0	0	0	0	1	0	1	0	1	0
30	682A	0	0	0	0	0	0	1	0	0	1	0	1	1
	682E	0	0	0	0	0	0	1	0	0	1	0	1	1
	682A	0	0	0	0	0	0	1	0	0	1	0	1	1
32	F429	0	0	0	0	0	0	0	0	1	1	1	1	0
	0C2E	0	0	1	0	0	1	0	0	0	1	0	0	1

Patient	AT code	PAGI-1	PAPI-2-PA0980	PA0728	PA2185	fla-island-1	fla-2 orfA	47D7-1	PAPI-2-AcTr.	PA2221	PA3835	fla-2 orfI	fla-2 orfJ
8	0C1A	1	1	0	1	1	1	0	1	0	1	1	1
	0C1A	1	1	1	1	1	1	0	1	0	1	1	1
	0C1A	1	0	0	1	1	1	0	1	0	1	1	1
9	D421	1	1	0	0	0	0	0	1	0	0	0	0
	D421	1	1	0	0	0	0	0	1	0	0	0	0
10	741E	1	0	0	1	1	1	0	0	0	0	1	1
	741E	1	0	0	1	1	1	0	0	0	0	1	1
11	C40A	1	1	0	1	1	1	1	1	0	0	1	1
	C40A	1	1	0	1	1	1	1	1	0	0	1	1
	AC2E	1	1	0	1	1	1	0	1	0	1	1	1
	3C2A	1	0	0	0	1	1	0	1	1	0	0	0
12	F42A	1	0	1	0	1	1	0	0	0	1	0	1
	F42A	1	0	1	0	1	1	0	0	0	1	0	0
13	3C52	1	1	0	1	1	0	1	1	0	1	0	0
	3C52	1	1	0	1	1	0	1	1	0	1	0	0
14	3C28	1	1	0	0	1	1	0	1	1	0	0	0
	D421	1	1	0	1	1	0	1	1	0	1	0	0
15	AC2A	1	1	0	1	1	1	1	1	0	0	1	1
	C40A	1	1	0	1	1	1	0	1	0	0	1	1
	AC2A	1	1	0	1	1	1	0	1	0	1	1	1
16	F469	1	1	0	1	1	0	0	1	0	0	0	1
	F469	1	1	0	0	0	0	0	1	0	0	0	0
17	239A	1	1	0	1	1	1	0	1	1	1	1	1
	239A	1	1	0	1	1	1	0	1	1	1	1	1
	239A	1	1	0	1	1	1	1	1	1	1	1	1
18	B420	1	0	0	1	1	0	1	0	0	0	0	0
	B420	1	0	0	0	1	0	0	0	0	0	0	0
	B420	1	0	0	0	1	0	0	0	0	0	0	0
	B420	1	0	0	0	0	0	0	0	0	0	0	0
19	0C4A	1	0	0	1	1	1	0	1	0	1	0	0
	0C4A	1	0	0	1	1	1	0	1	0	1	0	0
20	6852	1	0	0	1	0	0	0	0	0	1	0	0
	6852	1	1	0	1	1	0	1	0	0	1	0	0
21	D421	1	1	0	1	1	1	1	1	0	1	0	1
	D421	1	1	0	1	1	0	1	1	0	1	0	0
22	1BAE	1	0	0	1	1	0	0	0	0	1	0	0
	2C12	1	1	0	1	1	0	0	0	0	1	1	1
23	059A	1	1	0	1	1	1	1	0	0	1	1	0
	059A	1	1	0	1	1	1	0	0	0	1	1	1

Patient	AT code	PAGI-1	PAPI-2-PA0980	PA0728	PA2185	fla-island-1	fla-2 orfA	47D7-1	PAPI-2-AcTr.	PA2221	PA3835	fla-2 orfI	fla-2 orfJ
25	CC60	1	1	1	1	1	0	1	1	1	1	0	0
	CC60	1	1	1	1	1	0	1	1	1	1	0	0
29	4C8A	1	1	0	1	1	1	0	1	0	0	1	1
	4C8A	1	1	0	1	1	1	0	1	0	0	1	1
30	682A	1	1	0	1	1	1	1	0	0	1	1	1
	682E	0	0	1	1	1	1	0	0	0	1	1	1
	682A	1	0	0	1	1	1	0	0	0	1	1	1
32	F429	1	1	0	1	1	1	1	1	0	1	0	0
	0C2E	1	1	1	1	1	1	0	0	0	1	1	1

Patient	AT code	47D7-2	PAPI-2-XF1753	fliCa	fliCb	exoS	exoU
8	0C1A	0	0	1	0	1	0
	0C1A	0	0	1	0	1	0
	0C1A	0	0	1	0	1	0
9	D421	0	1	0	1	0	1
	D421	0	1	0	1	0	1
10	741E	0	0	1	0	1	0
	741E	0	0	1	0	1	0
11	C40A	1	0	1	0	1	0
	C40A	1	0	1	0	1	0
	AC2E	0	0	1	0	1	0
	3C2A	0	0	1	0	1	0
12	F42A	0	0	1	0	1	0
	F42A	0	0	1	0	1	0
13	3C52	1	0	0	1	1	0
	3C52	1	0	0	1	1	0
14	3C28	0	0	1	0	0	0
	D421	0	1	0	1	0	1
15	AC2A	1	0	1	0	1	0
	C40A	0	0	1	0	1	0
	AC2A	0	0	1	0	1	0
16	F469	0	0	1	0	0	1
	F469	0	0	1	0	0	1
17	239A	0	0	1	0	1	0
	239A	0	0	1	0	1	0
	239A	0	0	1	0	1	0
18	B420	0	0	0	1	0	0
	B420	0	0	0	1	0	0
	B420	0	0	0	1	0	0
	B420	0	0	0	1	0	0
19	0C4A	0	0	1	0	1	0
	0C4A	0	0	1	0	1	0
20	6852	0	0	0	1	1	0
	6852	0	0	0	1	1	0
21	D421	0	1	0	1	0	1
	D421	0	1	0	1	0	1
22	1BAE	0	0	1	0	1	0
	2C12	1	1	0	1	1	0
23	059A	0	0	1	0	1	0
	059A	0	0	1	0	1	0

Patient	AT code	47D7-2	PAPI-2-XF1753	fliCa	fliCb	exoS	exoU
25	CC60	1	0	0	1	0	0
	CC60	1	0	0	1	0	0
29	4C8A	0	0	1	0	1	0
	4C8A	0	0	1	0	1	0
30	682A	0	0	1	0	1	0
	682E	0	0	1	0	1	0
	682A	0	0	1	0	1	0
32	F429	0	1	1	0	0	1
	0C2E	0	0	1	0	1	0

Table A5: All isolates with complete genome sequences from chapter 4, including MLST sequence type

Isolate ID	Centre	Patient	ST
C155	Cardiff	107	1211
C76	Chertsey, Surrey	70	253
C77	Chertsey, Surrey	71	308
C78	Chertsey, Surrey	72	840
C79	Chertsey, Surrey	72	620
C80	Chertsey, Surrey	72	620
C81	Chertsey, Surrey	72	620
C82	Chertsey, Surrey	72	620
C83	Chertsey, Surrey	72	-
C84	Chertsey, Surrey	72	620
C85	Chertsey, Surrey	73	-
C86	Chertsey, Surrey	73	308
C87	Chertsey, Surrey	73	179
C88	Chertsey, Surrey	74	1251
C89	Chertsey, Surrey	75	1239
C91	Chertsey, Surrey	76	253
C92	Chertsey, Surrey	77	252
C143	Colchester	101	253
C144	Colchester	101	253
C145	Colchester	101	253
C146	Colchester	102	395
C147	Colchester	102	395
C148	Colchester	102	395
C149	Colchester	103	108
C100	Dorset County Hospital	80	612
C101	Dorset County Hospital	81	-
C102	Dorset County Hospital	81	-
C103	Dorset County Hospital	81	-
C94	Dorset County Hospital	78	395
C95	Dorset County Hospital	79	253
C96	Dorset County Hospital	79	253
C97	Dorset County Hospital	79	253
C98	Dorset County Hospital	79	253
C99	Dorset County Hospital	79	253
C119	Hull	88	-
C120	Hull	89	-
C123	Hull	90	27
C124	Hull	91	1753
C125	Hull	92	253
C126	Hull	92	253
C127	Hull	92	164

Isolate ID	Centre	Patient	ST
C128	Hull	92	164
C129	Hull	92	871
C131	Hull	93	253
C133	Hull	94	253
C134	Hull	95	253
A1	Liverpool	1	17
A12	Liverpool	35	179
A163	Liverpool	150	146
A19	Liverpool	120	-
A2	Liverpool	2	207
A3	Liverpool	3	252
A36	Liverpool	137	146
A4	Liverpool	3	252
A46	Liverpool	147	17
A48	Liverpool	147	17
A5	Liverpool	3	252
A52	Liverpool	147	17
A53	Liverpool	147	17
A54	Liverpool	147	17
A55	Liverpool	147	17
A56	Liverpool	147	17
A58	Liverpool	147	17
A60	Liverpool	147	17
A70	Liverpool	147	17
A71	Liverpool	147	17
A72	Liverpool	147	17
A73	Liverpool	147	17
A75	Liverpool	147	17
A76	Liverpool	147	17
A100	Liverpool (BGH)	148	17
A106	Liverpool (BGH)	148	175
A107	Liverpool (BGH)	148	175
A77	Liverpool (BGH)	148	175
A78	Liverpool (BGH)	148	17
A80	Liverpool (BGH)	148	175
A81	Liverpool (BGH)	148	17
A82	Liverpool (BGH)	148	17
A85	Liverpool (BGH)	148	175
A86	Liverpool (BGH)	148	175
A90	Liverpool (BGH)	148	175
A91	Liverpool (BGH)	148	175
A92	Liverpool (BGH)	148	175
A95	Liverpool (BGH)	148	175

Isolate ID	Centre	Patient	ST
A97	Liverpool (BGH)	148	175
A119	Liverpool (LHCH)	149	667
A122	Liverpool (LHCH)	149	667
A123	Liverpool (LHCH)	149	667
A126	Liverpool (LHCH)	149	667
A130	Liverpool (LHCH)	149	667
A134	Liverpool (LHCH)	149	667
A137	Liverpool (LHCH)	149	667
A141	Liverpool (LHCH)	149	667
A144	Liverpool (LHCH)	149	667
A147	Liverpool (LHCH)	149	667
A148	Liverpool (LHCH)	149	667
A151	Liverpool (LHCH)	149	667
A154	Liverpool (LHCH)	149	667
A156	Liverpool (LHCH)	149	667
C55	London Chest Hospital	62	-
C56	London Chest Hospital	62	-
C57	London Chest Hospital	62	-
C58	London Chest Hospital	62	-
C59	London Chest Hospital	62	-
C60	London Chest Hospital	62	-
C61	London Chest Hospital	63	620
B113	Newcastle	18	1328
B114	Newcastle	19	198
B16	Newcastle	9	253
B199	Newcastle	32	1182
B3	Newcastle	8	281
B34	Newcastle	11	179
B37	Newcastle	12	-
B62	Newcastle	15	-
C63	Norfolk & Norwich	64	27
C64	Norfolk & Norwich	65	274
C65	Norfolk & Norwich	65	274
C66	Norfolk & Norwich	65	274
C67	Norfolk & Norwich	65	274
C68	Norfolk & Norwich	65	274
C69	Norfolk & Norwich	66	-
C21	Papworth	45	-
C22	Papworth	45	-
C23	Papworth	45	-
C25	Papworth	46	253
C29	Papworth	48	252
C30	Papworth	49	252

Isolate ID	Centre	Patient	ST
C31	Papworth	50	-
C32	Papworth	50	-
C33	Papworth	50	-
C36	Papworth	52	253
C42	Papworth	54	309
C43	Papworth	55	108
C44	Papworth	55	108
C45	Papworth	55	108
C49	Papworth	58	395
C51	Papworth	59	683
C54	Papworth	61	1342
C150	Portsmouth	104	253
C151	Portsmouth	105	1244
C153	Portsmouth	106	155
C71	Reading	67	968
C73	Reading	68	17
C74	Reading	69	1202
C10	Sheffield	40	244
C11	Sheffield	41	282
C12	Sheffield	42	282
C13	Sheffield	42	27
C14	Sheffield	42	27
C15	Sheffield	42	27
C16	Sheffield	42	-
C17	Sheffield	42	-
C18	Sheffield	43	-
C2	Sheffield	36	253
C20	Sheffield	44	878
C3	Sheffield	37	260
C4	Sheffield	37	-
C5	Sheffield	37	260
C6	Sheffield	38	244
C7	Sheffield	39	244
C8	Sheffield	40	244
C9	Sheffield	40	244
C135	Sunderland	96	160
C137	Sunderland	97	260
C139	Sunderland	98	-
C141	Sunderland	99	-
C142	Sunderland	100	252
C104	Swansea	82	179
C105	Swansea	83	840
C106	Swansea	84	-

Isolate ID	Centre	Patient	ST
C107	Swansea	84	253
C108	Swansea	84	179
C109	Swansea	85	840
C110	Swansea	85	179
C111	Swansea	85	179
C112	Swansea	85	179
C114	Swansea	86	179
C115	Swansea	86	179
C116	Swansea	87	871
C117	Swansea	87	871
C118	Swansea	87	871
C156	West Wales G. Hospital	108	260
C158	West Wales G. Hospital	109	155
C159	West Wales G. Hospital	110	260
C160	West Wales G. Hospital	111	1244
C161	West Wales G. Hospital	112	110
C164	West Wales G. Hospital	113	-
C167	West Wales G. Hospital	114	296
C168	West Wales G. Hospital	115	17

References

Aaron, S.D. et al., 2010. Infection with transmissible strains of *Pseudomonas aeruginosa* and clinical outcomes in adults with cystic fibrosis. *The Journal of the American Medical Association*, 304(19), pp.2145 – 2153.

Agusti, A. et al., 2010. Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respiratory Research*, 11, p.122.

Ajayi, T. et al., 2003. Single-nucleotide-polymorphism mapping of the *Pseudomonas aeruginosa* type III secretion toxins for development of a diagnostic multiplex PCR system. *Journal of Clinical Microbiology*, 41(8), pp.3526–31.

Aksamit, T.R. et al., 2012. The Bronchiectasis Research Registry: A Collaborative Research Cohort For Non-Cystic Fibrosis Bronchiectasis (ATS Journals). *American Thoracic Society International Conference Meetings Abstracts*.

Al-Aloul, M. et al., 2003. Increased morbidity associated with chronic infection by an epidemic *Pseudomonas aeruginosa* strain in CF patients. *Thorax*, 59(4), pp.334–336.

Al-Aloul, M., Govin, B. & Stockton, P.A., 2002. Antibiotic related renal impairment in adult cystic fibrosis patients. *Thorax*.

Amiel, E. et al., 2010. *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infection and Immunity*, 78(7), pp.2937–45.

Angrill, J. et al., 2002. Bacterial colonisation in patients with bronchiectasis: microbiological pattern and risk factors. *Thorax*, 57(1), pp.15–9.

Anthony, M. et al., 2002. Genetic Analysis of *Pseudomonas aeruginosa* Isolates from the Sputa of Australian Adult Cystic Fibrosis Patients. *Journal of Clinical Microbiology*, 40(8), pp.2772–2778.

Armstrong, D. et al., 2003. Evidence for Spread of a Clonal Strain of *Pseudomonas aeruginosa* among Cystic Fibrosis Clinics. *Journal of Clinical Microbiology*, 41(5), pp.2266–2267.

Armstrong, D.S. et al., 2002. Detection of a widespread clone of *Pseudomonas aeruginosa* in a pediatric cystic fibrosis clinic. *American Journal of Respiratory and Critical Care Medicine*, 166(7), pp.983–7.

Arora, S.K. et al., 2004. Sequence Polymorphism in the Glycosylation Island and Flagellins of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 186(7), pp.2115–2122.

Ashish, A. et al., 2012. Increasing resistance of the Liverpool Epidemic Strain (LES) of *Pseudomonas aeruginosa* (Psa) to antibiotics in cystic fibrosis (CF)--a cause for concern? *Journal of Cystic Fibrosis*, 11(3), pp.173–9.

Bagge, N. et al., 2002. Constitutive High Expression of Chromosomal -Lactamase in *Pseudomonas aeruginosa* Caused by a New Insertion Sequence (IS1669) Located in *ampD*. *Antimicrobial Agents and Chemotherapy*, 46(11), pp.3406–3411.

Bagge, N. et al., 2004. *Pseudomonas aeruginosa* Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and -Lactamase and Alginate Production. *Antimicrobial Agents and Chemotherapy*, 48(4), pp.1175–1187.

Bankevich, A. et al., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, 19(5), pp.455–77.

Bardoel, B.W. et al., 2011. *Pseudomonas* evades immune recognition of flagellin in both mammals and plants. *PLoS Pathogens*, 7(8), p.e1002206.

de Bentzmann, S. & Plésiat, P., 2011. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environmental Microbiology*, 13(7), pp.1655–65.

Bjarnsholt, T. et al., 2010. Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. *PLoS ONE*, 5(4), p.e10115.

Bowen-Jones, J.R., Coovadia, Y.M. & Bowen-Jones, E.J., 1990. Infection control in a third world burn facility. *Burns*, 16(6), pp.445–448.

Boyle, B. et al., 2012. Complete genome sequences of three *Pseudomonas aeruginosa* isolates with phenotypes of polymyxin B adaptation and inducible resistance. *Journal of Bacteriology*, 194(2), pp.529–30.

Bradbury, R., Champion, A. & Reid, D.W., 2008. Poor clinical outcomes associated with a multi-drug resistant clonal strain of *Pseudomonas aeruginosa* in the Tasmanian cystic fibrosis population. *Respirology*, 13(6), pp.886–92.

British Thoracic Society, 2012. *Quality Standards for clinically significant bronchiectasis in adults*,

Brusasco, V., Crapo, R. & Viegi, G., 2005. Coming together: the ATS/ERS consensus on clinical pulmonary function testing. *European Respiratory Journal*, 26(1), pp.1–2.

Bryant, J.M. et al., 2013. Whole-genome sequencing to identify transmission of *Mycobacterium abscessus* between patients with cystic fibrosis: a retrospective cohort study. *Lancet*, 381(9877), pp.1551–60.

Burns, J.L. et al., 2001. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. *The Journal of Infectious Diseases*, 183(3), pp.444–52.

Calligaro, G.L. & Gray, D.M., 2015. Lung function abnormalities in HIV-infected adults and children. *Respirology*, 20(1), pp.24–32.

Carraro, D.S. et al., 2014. Study of *Burkholderia Cepacia* Complex Strains in Lung Transplant Patients: Analysis of Genomovar and Mortality Impact. *The Journal of Heart and Lung Transplantation*, 33(4), p.S182.

Chalmers, J.D., 2015. Bronchiectasis in adults: epidemiology, assessment of severity and prognosis. *Current Pulmonology Reports*, 4(3), pp.142–151.

Chalmers, J.D. et al., 2014. The bronchiectasis severity index. An international derivation and validation study. *American Journal of Respiratory and Critical Care Medicine*, 189(5), pp.576–85.

Cheng, K. et al., 1996. Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet*, 348(9028), pp.639–42.

de Chial, M. et al., 2003. Identification of type II and type III pyoverdine receptors from *Pseudomonas aeruginosa*. *Microbiology*, 149(4), pp.821–831.

Chitkara, Y.K. & Feierabend, T.C., 1981. Endogenous and exogenous infection with *Pseudomonas aeruginosa* in a burns unit. *International Surgery*, 66(3), pp.237–40.

Ciofu, O. et al., 2005. Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrobial Agents and Chemotherapy*, 49(6), pp.2276–82.

Copley, S.J. et al., 2009. Lung morphology in the elderly: comparative CT study of subjects over 75 years old versus those under 55 years old. *Radiology*, 251(2), pp.566–73.

Cramer, N. et al., 2012. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. *PLoS ONE*, 7(11), p.e50731.

Curran, B. et al., 2004. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology*, 42(12), pp.5644–9.

Cystic Fibrosis Trust, 2014. *UK Cystic Fibrosis Registry Annual data report 2013*,

Van daele, S. et al., 2006. Survey of *Pseudomonas aeruginosa* genotypes in colonised cystic fibrosis patients. *European Respiratory Journal*, 28(4), pp.740 – 747.

Daniels, R. et al., 2006. Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(40), pp.14965–70.

Darch, S.E. et al., 2015. Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. *Scientific Reports*, 5, p.7649.

Darling, A.C.E. et al., 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Research*, 14(7), pp.1394–403.

Davey, M.E., Caiazza, N.C. & O'Toole, G.A., 2003. Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology*, 185(3), pp.1027–1036.

Davies, G. et al., 2006. The effect of *Pseudomonas aeruginosa* on pulmonary function in patients with bronchiectasis. *European Respiratory Journal*, 28(5), pp.974–9.

Dettman, J.R. et al., 2013. Evolutionary genomics of epidemic and nonepidemic strains of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, 110(52), pp.21065–70.

Diaz Caballero, J. et al., 2015. Selective Sweeps and Parallel Pathoadaptation Drive *Pseudomonas aeruginosa* Evolution in the Cystic Fibrosis Lung. *mBio*, 6(5), pp.e00981–15–.

Dinesh, S.D. et al., 2003. European-wide distribution of *Pseudomonas aeruginosa* clone C. *Clinical Microbiology and Infection*, 9(12), pp.1228 – 1233.

Evans, S.A. et al., 1996. Lung function in bronchiectasis: the influence of *Pseudomonas aeruginosa*. *European Respiratory Journal*, 9(8), pp.1601–1604.

Feil, E.J. et al., 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *Journal of Bacteriology*, 186(5), pp.1518–30.

Feil, E.J. et al., 2001. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proceedings of the National Academy of Sciences of the United States of America*, 98(1), pp.182–7.

Le Fleche, P. et al., 2001. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BMC Microbiology*, 1(1), p.2.

Le Fleche, P. et al., 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BMC Microbiology*, 2(1), p.37.

Fluge, G. et al., 2001. Typing of *Pseudomonas aeruginosa* strains in Norwegian cystic fibrosis patients. *Clinical Microbiology and Infection*, 7(5), pp.238–243.

Fothergill, J.L. et al., 2008. Diagnostic multiplex PCR assay for the identification of the Liverpool, Midlands 1 and Manchester CF epidemic strains of *Pseudomonas aeruginosa*. *Journal of Cystic Fibrosis*, 7(3), pp.258–61.

Fothergill, J.L., Mowat, E., et al., 2010. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. *Journal of Medical Microbiology*, 59(Pt 4), pp.472–81.

Fothergill, J.L., White, J., et al., 2010. Impact of *Pseudomonas aeruginosa* genomic instability on the application of typing methods for chronic cystic fibrosis infections. *Journal of Clinical Microbiology*, 48(6), pp.2053–9.

Fothergill, J.L. et al., 2007. Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. *BMC Microbiology*, 7(1), p.45.

Fothergill, J.L., Walshaw, M.J. & Winstanley, C., 2012. Transmissible strains of *Pseudomonas aeruginosa* in cystic fibrosis lung infections. *European Respiratory Journal*, 40(1), pp.227–38.

Freschi, L. et al., 2015. Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Frontiers in Microbiology*, 6.

Galle, M. et al., 2012. The *Pseudomonas aeruginosa* type III secretion system has an exotoxin S/T/Y independent pathogenic role during acute lung infection. *PLoS ONE*, 7(7), p.e41547.

Gao, Y. et al., 2015. The role of viral infection in pulmonary exacerbations of bronchiectasis in adults: a prospective study. *Chest*, 147(6), pp.1635–43.

Garmendia, J. et al., 2014. Characterization of nontypable *Haemophilus influenzae* isolates recovered from adult patients with underlying chronic lung disease reveals genotypic and phenotypic traits associated with persistent infection. *PLoS ONE*, 9(5), p.e97020.

Gellatly, S.L. & Hancock, R.E.W., 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathogens and Disease*, 67(3), pp.159–73.

Girard, G. & Bloemberg, G. V., 2008. Central role of quorum sensing in regulating the production of pathogenicity factors in *Pseudomonas aeruginosa*. *Future Microbiology*, 3(1), pp.97–106.

Golovkine, G. et al., 2014. VE-cadherin cleavage by LasB protease from *Pseudomonas aeruginosa* facilitates type III secretion system toxicity in endothelial cells. *PLoS Pathogens*, 10(3), p.e1003939.

Goodman, A.L. et al., 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Developmental Cell*, 7(5), pp.745–54.

Govan, J.R.W. et al., 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet*, 342(8862), pp.15–19.

Govan, J.R.W., Brown, A.R. & Jones, A.M., 2007. Evolving epidemiology of *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex in cystic fibrosis lung infection. *Future Microbiology*, 2(2), pp.153–64.

Govan, J.R.W. & Deretic, V., 1996. Microbial Pathogenesis in Cystic Fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews*.

Grothues, D. et al., 1988. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. *Journal of Clinical Microbiology*, 26(10), pp.1973–1977.

Gurevich, A. et al., 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics*, 29(8), pp.1072–5.

Hall, A.J. et al., 2013. Intraclonal genetic diversity amongst cystic fibrosis and keratitis isolates of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*, 62(Pt 2), pp.208–16.

Hall, A.J. et al., 2014. Turnover of strains and intraclonal variation amongst *Pseudomonas aeruginosa* isolates from paediatric CF patients. *Diagnostic Microbiology and Infectious Disease*, 80(4), pp.324–6.

Hocquet, D. et al., 2003. Genetic and Phenotypic Variations of a Resistant *Pseudomonas aeruginosa* Epidemic Clone. *Antimicrobial Agents and Chemotherapy*, 47(6), pp.1887–1894.

Hoffman, L.R. et al., 2010. Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway. *PLoS Pathogens*, 6(1), p.e1000712.

Hogan, D.A. & Kolter, R., 2002. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science*, 296(5576), pp.2229–32.

Hogan, D.A., Vik, Å. & Kolter, R., 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Molecular Microbiology*, 54(5), pp.1212 – 1223.

Hoogkamp-Korstanje, J.A. et al., 1995. Risk of cross-colonization and infection by *Pseudomonas aeruginosa* in a holiday camp for cystic fibrosis patients. *Journal of Clinical Microbiology*, 33(3), pp.572–575.

Hurst, J.R., Elborn, J.S. & De Soyza, A., 2015. COPD-bronchiectasis overlap syndrome. *The European Respiratory Journal*, 45(2), pp.310–3.

Jain, M. et al., 2004. Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. *Journal of Clinical Microbiology*, 42(11), pp.5229–37.

Jairam, P.M. et al., 2015. Incidental findings on chest CT imaging are associated with increased COPD exacerbations and mortality. *Thorax*, 70(8), pp.725–31.

Jeffreys, A.J., Wilson, V. & Thein, S.L., 1985. Individual-specific “fingerprints” of human DNA. *Nature*, 316(6023), pp.76–79.

Jelsbak, L. et al., 2007. Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. *Infection and Immunity*, 75(5), pp.2214–24.

Jeukens, J. et al., 2014. Comparative genomics of isolates of a *Pseudomonas aeruginosa* epidemic strain associated with chronic lung infections of cystic fibrosis patients. *PLoS ONE*, 9(2), p.e87611.

Jimenez, P.N. et al., 2012. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiology and Molecular Biology Reviews*, 76(1), pp.46–65.

Jones, A.M. et al., 2002. Increased treatment requirements of patients with cystic fibrosis who harbour a highly transmissible strain of *Pseudomonas aeruginosa*. *Thorax*, 57(11), pp.924–925.

Jones, A.M. et al., 2001. Spread of a multiresistant strain of *Pseudomonas aeruginosa* in an adult cystic fibrosis clinic. *Lancet*, 358(9281), pp.557–558.

Jorth, P. et al., 2015. Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. *Cell Host & Microbe*, 18(3), pp.307–319.

Kaufmann, G.F. et al., 2005. Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones. *Proceedings of the National Academy of Sciences of the United States of America*, 102(2), pp.309–14.

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

Kidd, T.J. et al., 2013. Shared *Pseudomonas aeruginosa* genotypes are common in Australian cystic fibrosis centres. *European Respiratory Journal*, 41(5), pp.1091 – 1100.

Kiewitz, C. & Tummler, B., 2000. Sequence Diversity of *Pseudomonas aeruginosa*: Impact on Population Structure and Genome Evolution. *Journal of Bacteriology*, 182(11), pp.3125–3135.

King, P.T. et al., 2007. Microbiologic follow-up study in adult bronchiectasis. *Respiratory Medicine*, 101(8), pp.1633–8.

King, P.T. et al., 2005. Outcome in adult bronchiectasis. *COPD*, 2(1), pp.27–34.

Kos, V.N. et al., 2015. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrobial Agents and Chemotherapy*, 59(1), pp.427–36.

Kung, V.L., Ozer, E.A. & Hauser, A.R., 2010. The accessory genome of *Pseudomonas aeruginosa*. *Microbiology and Molecular Biology Reviews*, 74(4), pp.621–41.

Laarman, A.J. et al., 2012. *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the classical and lectin pathways. *Journal of Immunology*, 188(1), pp.386–93.

Laing, C. et al., 2010. Pan-genome sequence analysis using Panseq: an online tool for the rapid analysis of core and accessory genomic regions. *BMC Bioinformatics*, 11(1), p.461.

Langaee, T.Y., Gagnon, L. & Huletsky, A., 2000. Inactivation of the ampD Gene in *Pseudomonas aeruginosa* Leads to Moderate-Basal-Level and Hyperinducible AmpC beta -Lactamase Expression. *Antimicrobial Agents and Chemotherapy*, 44(3), pp.583–589.

Larbig, K.D. et al., 2002. Gene Islands Integrated into tRNAGly Genes Confer Genome Diversity on a *Pseudomonas aeruginosa* Clone. *Journal of Bacteriology*, 184(23), pp.6665–6680.

Larché, J. et al., 2012. Rapid identification of international multidrug-resistant *Pseudomonas aeruginosa* clones by multiple-locus variable number of tandem repeats analysis and investigation of their susceptibility to lytic bacteriophages. *Antimicrobial Agents and Chemotherapy*, 56(12), pp.6175–80.

Lau, G.W. et al., 2004. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infection and Immunity*, 72(7), pp.4275–8.

Ledson, M.J., Gallagher, M.J. & Walshaw, M.J., 1998. Chronic *Burkholderia cepacia* bronchiectasis in a non-cystic fibrosis individual. *Thorax*, 53(5), pp.430–432.

Lee, D.G. et al., 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biology*, 7(10), p.R90.

Leone, I. et al., 2008. Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* from cystic fibrosis patients. *European Journal of Clinical Microbiology & Infectious Diseases*, 27(11), pp.1093–9.

Letunic, I. & Bork, P., 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, 23(1), pp.127–8.

Letunic, I. & Bork, P., 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Research*, 39(Web Server issue), pp.W475–8.

Lewis, D.A. et al., 2005. Identification of DNA markers for a transmissible *Pseudomonas aeruginosa* cystic fibrosis strain. *American Journal of Respiratory Cell and Molecular Biology*, 33(1), pp.56–64.

Li, H. & Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25(14), pp.1754–60.

LiPuma, J.J. et al., 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 164(1), pp.92–6.

LiPuma, J.J. et al., 1990. Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. *Lancet*, 336(8723), pp.1094–1096.

Loebinger, M.R. et al., 2009. Mortality in bronchiectasis: a long-term study assessing the factors influencing survival. *European Respiratory Journal*, 34(4), pp.843–9.

López-Causapé, C. et al., 2013. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. *PLoS ONE*, 8(8), p.e71001.

Lyczak, J.B., Cannon, C.L. & Pier, G.B., 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and Infection*, 2(9), pp.1051–1060.

Maatallah, M. et al., 2011. Population Structure of *Pseudomonas aeruginosa* from Five Mediterranean Countries: Evidence for Frequent Recombination and Epidemic Occurrence of CC235 R. J. Redfield, ed. *PLoS ONE*, 6(10), p.e25617.

Maciá, M.D. et al., 2005. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrobial Agents and Chemotherapy*, 49(8), pp.3382–6.

Mahenthiralingam, E. et al., 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology*, 34(5), pp.1129–1135.

Maiden, M.C. et al., 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America*, 95(6), pp.3140–5.

Máiz, L. et al., 2015. Prevalence and factors associated with isolation of *Aspergillus* and *Candida* from sputum in patients with non-cystic fibrosis bronchiectasis. *Respiration; International Review of Thoracic Diseases*, 89(5), pp.396–403.

Malloy, J.L. et al., 2005. *Pseudomonas aeruginosa* protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 288(2), pp.L409–18.

Manos, J. et al., 2013. Virulence factor expression patterns in *Pseudomonas aeruginosa* strains from infants with cystic fibrosis. *European Journal of Clinical Microbiology & Infectious Diseases*, 32(12), pp.1583–92.

van Mansfeld, R. et al., 2009. *Pseudomonas aeruginosa* genotype prevalence in Dutch cystic fibrosis patients and age dependency of colonization by various P. aeruginosa sequence types. *Journal of Clinical Microbiology*, 47(12), pp.4096 – 4101.

Martin, K. et al., 2013. Clusters of genetically similar isolates of *Pseudomonas aeruginosa* from multiple hospitals in the UK. *Journal of Medical Microbiology*, 62(Pt 7), pp.988–1000.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17(1), p.10.

Martínez-García, M. et al., 2013. Prognostic value of bronchiectasis in patients with moderate-to-severe chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine*, 187(8), pp.823–31.

Martínez-García, M.A. et al., 2007. Factors associated with lung function decline in adult patients with stable non-cystic fibrosis bronchiectasis. *Chest*, 132(5), pp.1565–72.

Martínez-Solano, L. et al., 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. *Clinical Infectious Diseases*, 47(12), pp.1526–33.

Marvig, R.L. et al., 2014. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nature Genetics*, 47(1), pp.57–64.

Marvig, R.L. et al., 2013. Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genetics*, 9(9), p.e1003741.

Mathee, K. et al., 2008. Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proceedings of the National Academy of Sciences of the United States of America*, 105(8), pp.3100–5.

Matsumoto, K., 2004. Role of bacterial proteases in pseudomonal and serratial keratitis. *Biological Chemistry*, 385(11), pp.1007–16.

McCallum, S.J. et al., 2001. Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by P aeruginosa. *Lancet*, 358(9281), pp.558–560.

McDonnell, M.J. et al., 2015. Non cystic fibrosis bronchiectasis: A longitudinal retrospective observational cohort study of *Pseudomonas* persistence and resistance. *Respiratory Medicine*, 109(6), pp.716–26.

McKenna, A. et al., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, 20(9), pp.1297–303.

Mellmann, A. et al., 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS ONE*, 6(7), p.e22751.

Miao, E.A. et al., 2007. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Seminars in Immunopathology*, 29(3), pp.275–88.

Miller, J.H., 1996. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. *Annual Review of Microbiology*, 50, pp.625–43.

Mitchell, A. et al., 2015. Respiratory viruses can be isolated and identified from exhaled breath. *European Respiratory Journal*, 46(59), p.PA5033.

Morales, G. et al., 2004. Structure of *Pseudomonas aeruginosa* populations analyzed by single nucleotide polymorphism and pulsed-field gel electrophoresis genotyping. *Journal of Bacteriology*, 186(13), pp.4228–37.

Mowat, E. et al., 2011. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. *American Journal of Respiratory and Critical Care Medicine*, 183(12), pp.1674–9.

Neeld, D. et al., 2014. *Pseudomonas aeruginosa* injects NDK into host cells through a type III secretion system. *Microbiology*, 160(Pt 7), pp.1417–26.

NHS Choices, 2015. Bronchiectasis - Causes - NHS Choices.

Nicotra, M.B. et al., 1995. Clinical, pathophysiologic, and microbiologic characterization of bronchiectasis in an aging cohort. *Chest*, 108(4), pp.955–61.

O'Carroll, M.R. et al., 2004. Clonal strains of *Pseudomonas aeruginosa* in paediatric and adult cystic fibrosis units. *European Respiratory Journal*, 24(1), pp.101–106.

Ojeniyi, B., Frederiksen, B. & Høiby, N., 2000. *Pseudomonas aeruginosa* cross-infection among patients with cystic fibrosis during a winter camp. *Pediatric Pulmonology*, 29(3), pp.177–81.

Oliver, A., 2000. High Frequency of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Lung Infection. *Science*, 288(5469), pp.1251–1253.

Oliver, A., Baquero, F. & Blazquez, J., 2002. The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Molecular Microbiology*, 43(6), pp.1641–1650.

Onteniente, L. et al., 2003. Evaluation of the Polymorphisms Associated with Tandem Repeats for *Pseudomonas aeruginosa* Strain Typing. *Journal of Clinical Microbiology*, 41(11), pp.4991–4997.

Pai, H. et al., 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. *Antimicrobial Agents and Chemotherapy*, 45(2), pp.480–4.

Panagea, S. et al., 2003. PCR-Based Detection of a Cystic Fibrosis Epidemic Strain of *Pseudomonas aeruginosa*. *Molecular Diagnosis*, 7(3), pp.195–200.

Parkins, M.D. et al., 2014. Twenty-five-year outbreak of *Pseudomonas aeruginosa* infecting individuals with cystic fibrosis: identification of the prairie epidemic strain. *Journal of Clinical Microbiology*, 52(4), pp.1127–35.

Pasteur, M.C. et al., 2000. An investigation into causative factors in patients with bronchiectasis. *American Journal of Respiratory and Critical Care Medicine*, 162(4 Pt 1), pp.1277–84.

Pasteur, M.C., Bilton, D. & Hill, A.T., 2010. British Thoracic Society guideline for non-CF bronchiectasis. *Thorax*, 65 Suppl 1(Suppl_1), pp.i1–58.

Pauwels, A., Sifrim, D. & Dupont, L.J., 2012. Gastroesophageal Reflux in Cystic Fibrosis and Non-CF Bronchiectasis. In K. C. Meyer & G. Raghu, eds. *Gastroesophageal Reflux and the Lung*. Springer, pp. 153 – 174.

Pedersen, S.S. et al., 1986. An epidemic spread of multiresistant *Pseudomonas aeruginosa* in a cystic fibrosis centre. *Journal of Antimicrobial Chemotherapy*, 17(4), pp.505–516.

Persat, A. et al., 2015. Type IV pili mechanochemically regulate virulence factors in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, 112(24), pp.7563–8.

Pirnay, J.P. et al., 2002. *Pseudomonas aeruginosa* displays an epidemic population structure. *Environmental Microbiology*, 4(12), pp.898–911.

Pirnay, J.P. et al., 2009. *Pseudomonas aeruginosa* population structure revisited. *PLoS ONE*, 4(11), p.e7740.

Polverino, E. et al., 2015. Microbiology and outcomes of community acquired pneumonia in non cystic-fibrosis bronchiectasis patients. *Journal of Infection*, 71(1), pp.28–36.

Pujana, I. et al., 1999. Epidemiological Analysis of Sequential *Pseudomonas aeruginosa* Isolates from Chronic Bronchiectasis Patients without Cystic Fibrosis. *Journal of Clinical Microbiology*, 37(6), pp.2071–2073.

Quick, J. et al., 2014. Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing. *BMJ Open*, 4(11), pp.e006278–e006278.

Quint, J.K. et al., 2012. Time Trends in Incidence and Prevalence of Bronchiectasis in the UK. *Thorax*, 67.

Rakhimova, E. et al., 2009. *Pseudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. *The Journal of Infectious Diseases*, 200(12), pp.1928–35.

Rau, M.H. et al., 2010. Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environmental Microbiology*, 12(6), pp.1643–58.

Renders, N.H. et al., 1997. Exchange of *Pseudomonas aeruginosa* strains among cystic fibrosis siblings. *Research in Microbiology*, 148(5), pp.447–54.

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

Römling, U. et al., 1994. A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Applied and Environmental Microbiology*, 60(6), pp.1734–8.

Römling, U. et al., 2005. Worldwide distribution of *Pseudomonas aeruginosa* clone C strains in the aquatic environment and cystic fibrosis patients. *Environmental Microbiology*, 7(7), pp.1029–38.

Rommens, J.M. et al., 1989. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, 245(4922), pp.1059–65.

Roy, P.H. et al., 2010. Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. *PLoS ONE*, 5(1), p.e8842.

Salunkhe, P. et al., 2005. A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. *Journal of Bacteriology*, 187(14), pp.4908–20.

Sawa, T. et al., 1999. Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. *Nature Medicine*, 5(4), pp.392–398.

Schmid, J. et al., 2008. *Pseudomonas aeruginosa* transmission is infrequent in New Zealand cystic fibrosis clinics. *European Respiratory Journal*, 32(6), pp.1583 – 1590.

Schwartz, D.C. & Cantor, C.R., 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, 37(1), pp.67–75.

Scott, F.W. & Pitt, T.L., 2004. Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. *Journal of Medical Microbiology*, 53(7), pp.609–615.

Sener, B. et al., 2001. Epidemiology of chronic infections in cystic fibrosis. *International Journal of Medical Microbiology*, 291(5), pp.387–393.

Shankar, J. et al., 2012. Genotypic analysis of UK keratitis-associated *Pseudomonas aeruginosa* suggests adaptation to environmental water as a key component in the development of eye infections. *FEMS Microbiology Letters*, 334(2), pp.79–86.

Silby, M.W. et al., 2011. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiology Reviews*, 35(4), pp.652–80.

Slekovec, C. et al., 2012. Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. *PLoS ONE*, 7(12), p.e49300.

Smart, C.H.M., Scott, F.W., et al., 2006. Development of a diagnostic test for the Midlands 1 cystic fibrosis epidemic strain of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*, 55(Pt 8), pp.1085–91.

Smart, C.H.M., 2007. *Genetic studies of cystic fibrosis epidemic strains of Pseudomonas aeruginosa*. University of Liverpool.

Smart, C.H.M., Walshaw, M.J., et al., 2006. Use of suppression subtractive hybridization to examine the accessory genome of the Liverpool cystic fibrosis epidemic strain of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*, 55(Pt 6), pp.677–88.

Smith, A.L. et al., 2003. Susceptibility Testing of *Pseudomonas aeruginosa* Isolates and Clinical Response to Parenteral Antibiotic Administration. *Chest*, 123(5), p.1495.

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.

Snyder, L.A. et al., 2013. Epidemiological investigation of *Pseudomonas aeruginosa* isolates from a six-year-long hospital outbreak using high-throughput whole genome sequencing. *Eurosurveillance*, 18(42).

De Soyza, A. et al., 2014. Molecular epidemiological analysis suggests cross-infection with *Pseudomonas aeruginosa* is rare in non-cystic fibrosis bronchiectasis. *European Respiratory Journal*, 43(3), pp.900–3.

De Soyza, A., Brown, J. & Loebinger, M.R., 2013. Research priorities in bronchiectasis. *Thorax*, 68(7), pp.695–6.

Speert, D.P. et al., 2002. Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis in British Columbia, Canada. *American Journal of Respiratory and Critical Care Medicine*, 166(7).

Speert, D.P. & Campbell, M.E., 1987. Hospital epidemiology of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *Journal of Hospital Infection*, 9(1), pp.11–21.

Spencer, D.H. et al., 2003. Whole-Genome Sequence Variation among Multiple Isolates of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 185(4), pp.1316–1325.

Spiers, A.J., Buckling, A. & Rainey, P.B., 2000. The causes of Pseudomonas diversity. *Microbiology*, 146(10), pp.2345–50.

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.

Spratt, B.G. et al., 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. *FEMS Microbiology Letters*, 241(2), pp.129–34.

Stenton, C., 2008. The MRC breathlessness scale. *Occupational Medicine*, 58(3), pp.226–7.

Stephenson, A.L. et al., 2015. A contemporary survival analysis of individuals with cystic fibrosis: a cohort study. *The European Respiratory Journal*, 45(3), pp.670–9.

Stewart, J.L. et al., 2012. Clinical impact of CT radiological feature of bronchiectasis in the COPDgene cohort. *American Journal of Respiratory and Critical Care Medicine*, 185.

Stewart, L. et al., 2014. Draft genomes of 12 host-adapted and environmental isolates of *Pseudomonas aeruginosa* and their positions in the core genome phylogeny. *Pathogens and Disease*, 71(1), pp.20–5.

Stewart, P.S. & Costerton, W.J., 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet*, 358(9276), pp.135–138.

Stewart, R.M.K. et al., 2011. Genetic characterization indicates that a specific subpopulation of *Pseudomonas aeruginosa* is associated with keratitis infections. *Journal of Clinical Microbiology*, 49(3), pp.993–1003.

Stover, C.K. et al., 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406(6799), pp.959–64.

Syrmis, M.W. et al., 2004. Rapid genotyping of *Pseudomonas aeruginosa* isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. *Journal of Medical Microbiology*, 53(Pt 11), pp.1089–96.

Tamura, K. et al., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30(12), pp.2725–9.

Tenover, F.C. et al., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33(9), pp.2233–9.

The UK Cystic Fibrosis Trust Infection Control Group, 2004. *Pseudomonas aeruginosa infection in people with cystic fibrosis. Suggestions for Prevention and Infection Control*,

Thomas, S.R. et al., 2000. Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. *Thorax*, 55(9), pp.795–797.

Toft, C. & Andersson, S.G.E., 2010. Evolutionary microbial genomics: insights into bacterial host adaptation. *Nature Reviews Genetics*, 11(7), pp.465–75.

Török, M.E. & Peacock, S.J., 2012. Rapid whole-genome sequencing of bacterial pathogens in the clinical microbiology laboratory--pipe dream or reality? *The Journal of Antimicrobial Chemotherapy*, 67(10), pp.2307–8.

Tümmler, B., 2006. Clonal variations in *Pseudomonas aeruginosa*. In *Pseudomonas*. pp. 35 – 68.

Uzun, S. et al., 2014. Azithromycin maintenance treatment in patients with frequent exacerbations of chronic obstructive pulmonary disease (COLUMBUS): a randomised, double-blind, placebo-controlled trial. *The Lancet Respiratory Medicine*, 2(5), pp.361–8.

Vandamme, P. et al., 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *International Journal of Systematic Bacteriology*, 47(4), pp.1188–200.

Versura, P. et al., 1987. Detection of mucus glycoconjugates in human conjunctiva by using the lectin colloidal gold technique in TEM. *Acta Ophthalmologica*, 64(4), pp.445–450.

Vogne, C. et al., 2004. Role of the Multidrug Efflux System MexXY in the Emergence of Moderate Resistance to Aminoglycosides among *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis. *Antimicrobial Agents and Chemotherapy*, 48(5), pp.1676–1680.

De Vos, D. et al., 1997. Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, oprI and oprL. *Journal of Clinical Microbiology*, 35(6), pp.1295–9.

Vu-Thien, H. et al., 2007. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Journal of Clinical Microbiology*, 45(10), pp.3175–83.

Weycker, D. et al., 2005. Prevalence and Economic Burden of Bronchiectasis. *Clinical Pulmonary Medicine*, 12(4), pp.205–209.

Wiehlmann, L. et al., 2007. Population structure of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, 104(19), pp.8101–6.

Wilder, C.N., Allada, G. & Schuster, M., 2009. Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. *Infection and Immunity*, 77(12), pp.5631–9.

Williams, D. et al., 2015. Divergent, Coexisting *Pseudomonas aeruginosa* Lineages in Chronic Cystic Fibrosis Lung Infections. *American Journal of Respiratory and Critical Care Medicine*, 191(7), pp.775–85.

Williams, J.G.K. et al., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18(22), pp.6531–6535.

Wilson, C.B. et al., 1997. Effect of sputum bacteriology on the quality of life of patients with bronchiectasis. *European Respiratory Journal*, 10(8), pp.1754–1760.

Wilson, C.B. et al., 1998. Systemic markers of inflammation in stable bronchiectasis. *European Respiratory Journal*, 12(4), pp.820–824.

Winsor, G.L. et al., 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic Acids Research*, 39(Database issue), pp.D596–600.

Winstanley, C. et al., 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Research*, 19(1), pp.12–23.

Winstanley, C. & Fothergill, J.L., 2009. The role of quorum sensing in chronic cystic fibrosis *Pseudomonas aeruginosa* infections. *FEMS Microbiology Letters*, 290(1), pp.1–9.

Winter, D.H. et al., 2015. Aging of the lungs in asymptomatic lifelong nonsmokers: findings on HRCT. *Lung*, 193(2), pp.283–90.

Wolfgang, M.C. et al., 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, 100(14), pp.8484–9.

Wong, A., Rodrigue, N. & Kassen, R., 2012. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genetics*, 8(9), p.e1002928.

Woodford, N., Turton, J.F. & Livermore, D.M., 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews*, 35(5), pp.736–55.

Worby, C.J., Lipsitch, M. & Hanage, W.P., 2014. Within-host bacterial diversity hinders accurate reconstruction of transmission networks from genomic distance data. *PLoS Computational Biology*, 10(3), p.e1003549.

Workentine, M.L. et al., 2013. Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. *PLoS ONE*, 8(4), p.e60225.

Yang, L. et al., 2011. Evolutionary dynamics of bacteria in a human host environment. *Proceedings of the National Academy of Sciences of the United States of America*, 108(18), pp.7481–6.