THE ROLE OF ANTIBODY IN THE KILLING OF *PSEUDOMONAS AERUGINOSA* BY HUMAN SERA

By

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A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSPHY

College of Medical and Dental Sciences

Institute of Microbiology and Infection

University of Birmingham

September 2019

ABSTRACT

Antibody plays an important role in the protection against many different infections. Here the role of antibody in the protection against *P aeruginosa* infection was further investigated. Patients suffering from chronic lung infection with *P. aeruginosa* can produce antibodies that protect the bacterium from the bactericidal activity of serum. The effect of the removal of these antibodies on the *P. aeruginosa* population was investigated. One clonal lineage appeared to be eradicated from a patient with a multi-lineage infection. However, the treatment did not promote strain replacement in either patient suggesting that a single strain was able to persist within a specific niche in the lung. The role of antibody in the protection against *P. aeruginosa* infection by sera from healthy individuals was further investigated. Killing of *P. aeruginosa* was complement-dependent and in some instances, complement alone was sufficient to elicit killing. *P. aeruginosa*-specific antibodies enhanced the killing of some strains and blocked the killing of others. Inhibitory antibody was identified in healthy serum, suggesting that healthy individuals can produce inhibitory antibodies without an active infection. The mechanism of inhibitory antibodies is not fully understood. A transposon library was constructed in a strain of *P. aeruginosa* isolated from a non-cystic fibrosis bronchiectasis patient and used to define the essential genome. This was the first library to be constructed in a multi-drug resistant respiratory isolate and consisted of 577,494 unique mutants. The library can be used as a tool to provide further insight into the mechanism of inhibitory antibodies and has the potential to lead to the development of new treatments and diagnostics for patients with inhibitory antibody.

ACKNOWLEDGEMENTS

Ian Henderson, thank you for giving me the opportunity to be part of your lab. It has consisted of an amazing group of people over the years. Thank you for your guidance and the lab trips. I am also grateful for all the travel opportunities that working with you has provided, both within and outside the UK.

Tim Wells, thank you for persuading me to do a PhD and then leaving…
But seriously, thank you for introducing me to this project, your guidance and teaching me all things *Pseudomonas* and inhibitory antibody. It has been awesome working with you over the last 5 years and I thank you for giving me the opportunity to experience life in Australia!

Jeff Cole, thank you for all the time and effort you have put in to teaching me the ins and outs of scientific writing and following the "golden rules".

Thank you to the BBSRC MIBTP for giving me the opportunity to do my PhD and for funding my work.

Mat, the fellow northerner. Thank you for all your help with all things TraDIS. I honestly don't think I would have remained sane during TraDIS analysis if it wasn't for your help with learning the command line and answering my stupid questions. And for always being up for a pint down t'pub.

Sammi, I don't think my time here would have been half as awesome if hadn't been around. You've been amazing to work and live with. Thank you for all your support and for being my rosé buddy.

Henderson group, Amanda, Jack, Georgia, Jess, Chris, Emily, JG, Kara and Camila I couldn't have asked for a better group to work with. You are all amazing and I have learnt so much from you all. Thank you for making the lab such a fun environment to work in.

To the "Twellettes", Carrie, Josh, Emma, Amy, Johannes, Rachel, Niamh and Jeimy. Thank you for making my 6 months in Australia amazing! Thank you for making me feel like part of the group instantly, I loved every minute working with you guys! Josh, thank you for all your help in the lab and for offering to be a taxi for experiencing Oz.

Scott, thank you for being there throughout the highs and lows of PhD life. Thank you for always supporting me and making me believe in myself. Thank you for not getting annoyed with me when I'd be in a bad mood because of thesis stress and for just always being there when I needed someone to talk to.

Mum, thank you for always supporting me throughout. Thank you for listening to my rants about my work even though you had no idea what I was talking about. Thank you for giving me the push to keep going. If you hadn't convinced me to carry on and to go and do my Masters at UoB, I might not have even got the stage of doing my PhD.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
AUC	Area under the curve
bp	Base pair
CDS	Coding sequence
CF	Cystic fibrosis
CFU	Colony forming units
Cm	Chloramphenicol
CPA	Core polysaccharide antigen
DNA	Deoxynucleic acid
dNTP	Deoxyribonucleotide triphosphate
EB	Elution buffer
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HCS	Healthy control serum
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
Il	Interleukin
IM	Inner membrane
Kb	Kilobase
kDa	Kilodalton
Kdo	3-deoxy-D-manno-octulosonic acid
LB	Luria Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
Mbp	Mega base pair
MLST	Multi-locus sequence type
nIg	Natural immunoglobulin
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OSA	O-specific antigen
PAGE	Polyacrylamide gel electrophoresis
PAL	Pooled amplified library
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
RT	Room temperature
SBA	Serum bactericidal assay
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOC	Super Optimal broth with Catabolite repression
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween20
TLR	Toll-like receptor
Tm	Trimethoprim
Tn	Transposon
TnCm	Chloramphenicol resistance transposon
TnTm	Trimethoprim resistance transposon
TraDIS	Transposon-directed insertion-site sequencing
UIP	Unique insertion point
UPEC	Uropathogenic *E. coli*

CHAPTER 1

GENERAL INTRODUCTION

1.1 Bronchiectasis

1.1.1 Aetiology and disease progression

Bronchiectasis is a progressive and debilitating lung condition where the bronchi become irreversibly widened. The condition was first recognized in 1819 and symptoms often include: a persistent productive cough, muco-purulent sputum, fatigue, chronic rhinosinusitis, and shortness of breath (King *et al.,* 2006; Grimwood, 2010). A cycle of impaired mucus clearance, chronic infection, inflammation, lung damage and widening of the airways is characteristic of the disease. However, the point of entry into this cycle is dependent on the underlying cause of the disease (Figure 1.1) (Stafler and Carr, 2010; Fuschillo *et al.,* 2008). Cystic fibrosis (CF) bronchiectasis has been well characterised and results from the increased production of viscous mucus caused by the genetic impairment of the cystic fibrosis transmembrane conductance regulator protein. This defective ion channel dysregulates the normal transport of chloride and bicarbonate across cells resulting in the secretion of sticky mucus that lines the airway (Cantin, 2016). Non-CF bronchiectasis has been less well studied. However, there are multiple known causes of non-CF bronchiectasis including, but not limited to: damage caused by childhood respiratory infections, the presence of foreign bodies in the airways, lung damage resulting from smoking, immune deficiencies, autoimmune diseases, and defects in mucociliary clearance mechanisms (McShane *et al.,* 2013). It has been estimated that in 20 to 60% of bronchiectasis patients, the underlying cause of disease is unknown (Redondo *et al.,* 2016).

1.1.2 Prevalence and clinical impact of non-cystic fibrosis bronchiectasis

The prevalence of bronchiectasis in the UK is increasing; it has been estimated that 1% of the population over the age of 70 have bronchiectasis (Quint *et al*., 2016). The disease is generally more prevalent in women and prevalence increases with age (Quint *et al*., 2016). Disease

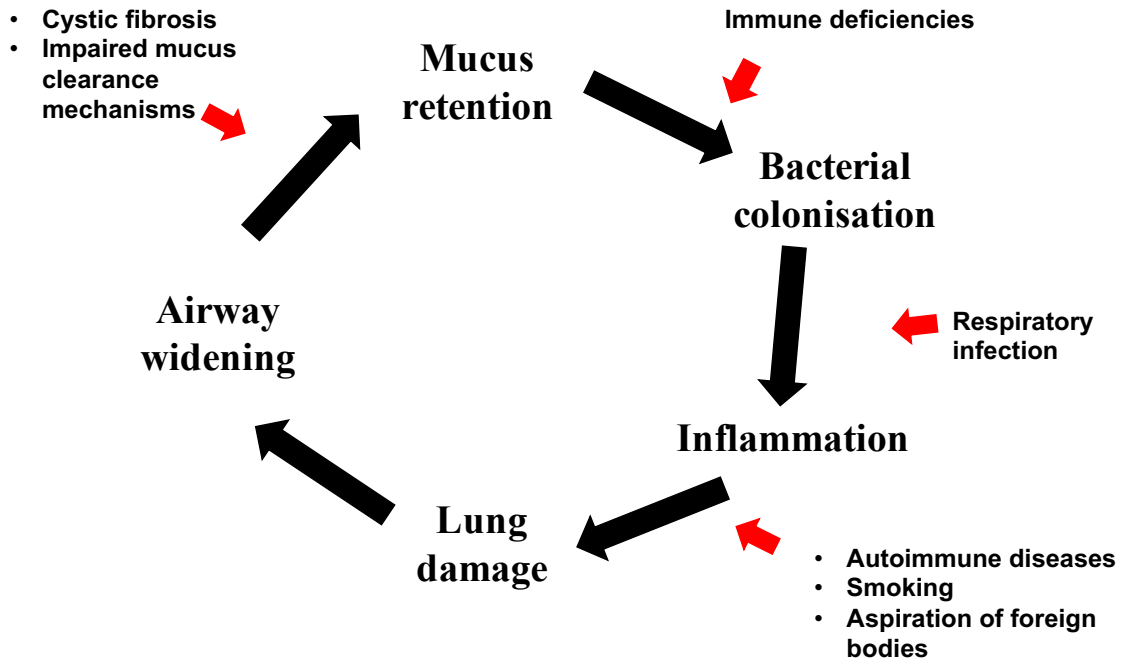


Figure 1.1 The cycle of disease associated with bronchiectasis

There are several underlying causes of bronchiectasis, which can be genetic and/or environmental in origin. A progressive and chronic cycle of infection, inflammation and lung damage is established, which leads to the permanent widening and thickening of the airways. The point of entry into this cycle varies depending on the nature of the underlying cause of bronchiectasis and is represented by the red arrows.

severity is believed to be greater in females as the airways are generally smaller than those in males (Vidaillac *et al*., 2018). Female sex hormones such as oestrogen are known to upregulate the secretion of mucin, whereas progesterone can reduce the cilia beat frequency resulting in hindered mucocilliary clearance (Jain *et al*., 2012). The prevalence of bronchiectasis is believed to be underreported as the disease is often misdiagnosed as other respiratory conditions including chronic obstructive pulmonary disease or asthma; or it may also co-exist with other diagnosed conditions (Chang *et al.,* 2010). A study of the mortality rates of bronchiectasis in England and Wales found that approximately 1,000 people die annually from the disease with the rate increasing on average by 3% each year (Roberts and Hubbard, 2010). Life expectancy in more severe cases of the disease is reduced and approximately 50% of patients will die as a result of respiratory complications (Redondo *et al.,* 2016).

The financial and healthcare burden of bronchiectasis due to both inpatient and outpatient care in the UK is also increasing, with a 9% annual rise in hospital admissions (Navaratnam *et al.,* 2014). In the US, annual medical costs associated with the disease have been estimated at $630 million (Weycker *et al.,* 2005). Many of the costs associated with the disease can be attributed to severe exacerbations requiring hospitilisation. On average, patients experience between 1 and 3 exacerbations each year. However, in severe cases patients can experience up to 12 exacerbations a year (Redondo *et al.,* 2016).

1.1.3 Microbiology of the non-cystic fibrosis bronchiectasis lung

The lung was once considered to be a sterile environment. However, microbial communities are now known to reside within the lungs (Mathieu *et al*., 2018). The lung microbiome varies in composition between healthy and diseased states where the microbial community shifts from

primarily consisting of Bacteroidetes to mainly comprising of Proteobacteria (Dickinson and Huffnagle, 2015). The most common bacterial species isolated from non-CF bronchiectasis patients can be found in Table 1.1. Analysis of sputum samples by 16S ribosomal RNA sequencing has detected the presence of multiple bacterial species in individual patients. *H. influenza* or *P. aeruginosa* are often identified as the dominant species and do not co-colonise the lung with each other (Rogers *et al*., 2015). Dominant colonisation with *H. influenza* or *P. aeruginosa* has been associated with an increase in exacerbation frequency and disease severity and a decline in lung function (Richardson *et al*., 2019). In the absence of these two species, an increase in the abundance of other bacterial species including *Prevotella*, *Viellonella* and *Streptococcus* has been observed (Rogers *et al*., 2015). Recurrent and chronic infection with these bacteria results in further inflammation and lung damage, contributing to the 'vicious cycle' development of the disease (Cole, 1986; King, 2009).

1.1.4 Current management techniques for non-CF bronchiectasis

1.1.4.1 Control of chronic lung infections

Patients with non-CF bronchiectasis are prone to chronic lung infections. As such, management of the disease often includes short and long-term antibiotic therapy (Aliberti *et al*., 2016). Studies have revealed that antibiotics capable of effectively lowering or eradicating the bacterial load of *P. aeruginosa* in the lung include: nebulised tobramycin (Barker *et al*., 2000), inhaled ciprofloxacin (Serisier *et al*., 2013a), nebulised gentamicin (Murray *et al*., 2010), and colistin (Dhar *et al*., 2010). Long-term antibiotic administration has been also associated with a decrease in the exacerbation frequency in bronchiectasis patients (Chalmers *et* al., 2015). Such antibiotics include: nebulised colistin (Dhar *et al*., 2010), azithromycin (Wong *et* al., 2012) and low-dose erythromycin (Serisier *et al*., 2013b). Although azithromycin has no effect on

Table 1.1 Bacterial species commonly isolated from patients with non-CF bronchiectasis (data obtained from Foweraker and Wat, 2011).

Bacterial species	Patient colonisation (%)
Haemophilus influenzae	14-52
Pseudomonas aeruginosa	12-43
Streptococcus pneumoniae	Up to 37
***Moraxella* catarrhalis**	Up to 27

P. aeruginosa, it has been linked to a reduction in neutrophil recruitment during infection resulting in less airway inflammation and damage (Tsai *et* al., 2004). The accumulation of azithromycin within host phagocytes deregulates toll-like receptor 4 signalling, resulting in the inhibition of pro-inflammatory cytokine release and neutrophil recruitment (Vrančić *et al*., 2012). A reduction in sputum production has been observed after the administration of colistin (Dhar *et al*., 2010) and long-term, low-dose (400 mg) erythromycin (Serisier *et al*., 2013b). Erythromycin can suppress neutrophil recruitment by inhibiting the release of interleukin-8 and reduces sputum production by inhibiting the expression of the *MUC5AC* gene, which results in a decrease in mucin secretion by goblet cells (Shimizu *et al*., 2003). High bacterial loads have been associated with an increase in airway inflammation and exacerbation frequency. A reduction in airway inflammation can be achieved by reducing the bacterial load though the short-term administration of intravenous antibiotics or through long-term treatment with nebulised gentamicin (Chalmers *et al*., 2012).

1.1.4.2 Mucus clearance from the airways

Mucus retention caused by the widening of the airways or by hindered mucocilliary clearance and an increase in mucus viscosity makes it difficult for patients to expel these secretions. This results in an increased susceptibility to bacterial colonisation of the lung. (Shen *et al*., 2018). Therefore, management of bronchiectasis often involves the implementation of a variety of mucus clearance techniques to try and limit disease progression (McShane *et al*., 2013).

Drugs that promote the clearance of mucus from the lungs (mucoactive drugs) are available. These drugs have four main mechanisms of action: 1) The breakdown of mucus reducing the viscosity (mucolytics); 2) The improvement of cilia function or the prevention of mucus

adhesion (mucokinetics); 3) The downregulation of mucus secretion (mucoregulators); and 4) The induction of mucus secretion to ease the release by cough (expectorants) (Balsamo *et al*., 2010).

Manual chest physiotherapy can be an effective technique for mucus clearance and several techniques have been described. The percussion technique involves the release of mucus through cycles of chest/back clapping with a cupped hand followed by vibrations or shaking during exhalation to aid expectoration (Main *et al*., 2015). This can be combined with the postural drainage technique, using gravity to increase the amount of sputum released by the patient (Eaton *et al*., 2007). Autogenic drainage utilizes cycles of set breathing techniques with variations in lung volume to remove mucus from the airways (Poncin *et al*., 2017).

Devices designed to help patients expel mucus from their lungs and to improve ventilation are also available. Positive expiratory pressure devices come in both mask and mouthpiece forms and use expiratory resistors to increase the pressure in the airways. This increased pressure is able to temporarily stent the airways while the patient exhales and aids mucus clearance (Myers, 2007). Adaptations to these devices have been made to incorporate air oscillation, which is achieved by the regular opening and closing of the resistor, as the patient exhales (Olsén *et al*., 2015). The resulting air oscillation in the airways loosens mucus making it easier to clear (Manor *et al*., 2017).

High frequency chest wall oscillation vests are also available and either use mechanical oscillation or come in an inflatable form that can be mechanically compressed (O'Brien *et al*., 2018). Compressible vests force air towards the mouth in pulses and create a shearing effect

against the mucus. They are also believed to have a potential effect on the viscoelasticity of the mucus (Hansen *et al*., 1994). Mechanical oscillation vests work in a similar way to manual chest physiotherapy, utilising chest vibrations to remove mucus from the airways (O'Brien *et al*., 2018).

1.1.4.3 Management of lung inflammation

Evidence to support the use of antibiotics to reduce inflammation in non-CF bronchiectasis patients is available (Chalmers *et al*., 2012). However, other anti-inflammatory therapies are available for these patients. Inflammation in the lung has been strongly linked with neutrophil activity (Martínez-García *et* al., 2015). During inflammation, neutrophil elastases become unregulated resulting in an increase in lung damage and elevated levels of proinflammatory cytokines (Kawabata *et al*., 2002). Neutrophil elastases are serine-protease enzymes that are located in the granules of neutrophils. Following activation of the neutrophils, the granules fuse with the phagosomes containing the ingested bacteria and the neutrophil elastases contribute to the killing of the bacteria (Gramegna *et al*., 2017). Neutrophil elastases are also released into the extracellular space. When present in high concentrations, the elastases can cause structural damage to the host and upregulate the secretion of proinflammatory cytokines (Hirche *et al*., 2008; Gramegna *et al*., 2017). The use of inhibitors of these elastases has provided promising results in bronchiectasis patients, with a reduction in inflammatory marker concentration in sputum and a significant improvement in lung function observed after 28 days of treatment (Stockley *et al*., 2013). Significant reductions in neutrophil counts in sputum have also been achieved through the administration of chemokine receptor-2 antagonists, which block the recruitment of neutrophils (De Soyza *et al*., 2015). The inhibition of cathepsin C, which in turn

inhibits neutrophil serine protease activity, has also been suggested as a treatment option for reducing inflammation in the lung (Chalmers *et al*., 2018).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is described as a rod-shaped, Gram-negative facultative anaerobe that belongs to the bacterial class of *Gammaproteobacteria* that can occupy a variety of niches within the environment (Williams *et al*., 2006; Curran *et al*., 2018; Bel Hadj Ahmed *et al*., 2019). Although renowned as an opportunistic pathogen, the bacterium has a broad host range and is able to cause disease in humans, plants and animals (He *et al*., 2004).

1.2.1 *P. aeruginosa* as a nosocomial pathogen

Community-acquired *P. aeruginosa* infections in healthy individuals do occur although much less frequently than nosocomial infections. These can include: community-acquired pneumonia, commonly following the use of contaminated hot tubs (Wang *et al*., 2019); contact lens-associated keratitis (Willcox, 2012); otitis externa (Hui, 2013) and folliculitis, following contaminated swimming pool use (Roser *et al*., 2014).

P. aeruginosa is commonly isolated within the hospital setting and several reservoirs of infection have been identified. These include: shower, sinks, taps, medical and cleaning equipment and through skin carriage by hospital staff and visitors. This has made infection control particularly problematic (Kerr and Snelling, 2009). *P. aeruginosa* is currently regarded as an important pathogen within healthcare settings (Garvey *et al.,* 2016) and is responsible for approximately 11% of all hospital-acquired infections (Lila *et al*., 2018).

Causing opportunistic infections in humans, *P. aeruginosa* is commonly isolated from immunocompromised patients (Migiyama *et al*., 2016). High mortality and morbidity rates have been associated with *P. aeruginosa* infection of burn and surgical wounds (Khan *et al*., 2015; Fournier *et al*., 2016). *P. aeruginosa* readily forms biofilms on invasive medical devices. Patients requiring ventilator assistance can develop ventilator-assisted pneumonia as the result of biofilm formation on endotracheal tubes (Perocolini *et al*., 2018). Catheterised patients are susceptible to urinary tract infections as a result of *P. aeruginosa* biofilm formation on catheters (Cole *et al*., 2014). Bloodstream infections with *P. aeruginosa* can occur in hospitalised patients and they have been associated with *P. aeruginosa* originating from the respiratory tract, the urinary tract and the biliary tract (Shi *et al*., 2019).

Complications following prosthetic joint replacements, such as knee and hip replacements, can be attributed to *P. aeruginosa* infection (Shah *et al*., 2016). Although relatively uncommon, patients receiving cardiac implants or prosthetic valves are at risk of developing infectious endocarditis caused by *P. aeruginosa* (Gürtler *et al*., 2019). *P. aeruginosa* infection following lung (Zeglen *et al*., 2009), abdominal organ (Su *et al*., 2016; Liu *et al*., 2018) and allogenic hematopoietic stem cell transplants (Kossow *et al*., 2017) can result in post-operative complications and are associated with increased rates of morbidity and mortality (Liu *et al*., 2018).

Although the source of acquisition can be environmental (Woo *et al*., 2018), *P. aeruginosa* commonly infects patients suffering from chronic lung diseases such as: chronic obstructive pulmonary disease, cystic fibrosis and non-CF bronchiectasis. Acquisition of *P. aeruginosa* due to cross-infection between non-CF bronchiectasis patients in medical facilities is relatively

uncommon (De Soyza *et al*., 2014). However, there is evidence indicating that cross-infection between CF and non-CF bronchiectasis patients can occur when patients attend the same medical facilities (Robinson *et al*., 2003). Cross-infection between CF patients is more prominent and has therefore prompted the implication of patient contact restrictions in healthcare settings (Chalmers *et al*., 2018).

1.2.2 *P. aeruginosa* pathogenesis

P. aeruginosa is able to colonise and survive within a wide range of environments and can utilize a variety of nutritional sources (Silby *et al.,* 2011). These abilities can be attributed to the large (5.5 to 7 Mbp), flexible genome, which allows the bacterium to readily adapt to changes within the environment (Klockgether *et al*., 2011). Adapting quickly to changes in the environment is achieved through the tight regulation of gene expression by two-component systems (Francis *et al*., 2017). Two-component systems consist of a sensor kinase, which is often localised in the inner membrane and responsible for detecting changes in the environment and then transmitting this signal to a response regulator. The response regulator controls gene expression in a phosphorylation-dependent or independent manner by binding to the promotor region of a gene, regulating the transcription of the gene (Gao *et al*., 2007; Francis *et al*., 2017). The success of *P. aeruginosa* as a human pathogen is largely due to the ability to employ a range of virulence factors in order to colonise and cause disease in a susceptible host (Qadri *et al*., 2016). These are described in more detail in the subsequent sections and summarised in Table 1.2 and Figure 1.2. Expression of such virulence factors can be controlled by a process known as quorum sensing (Rajkumari *et al*., 2018). Bacteria are able to communicate through the release chemical signal molecules (Miller and Bassler, 2001). *P. aeruginosa* produces four chemical signals for this process: integrated quorum sensing signal, *Pseudomonas* quinolone

Table 1.2 Virulence factors associated with *P. aeruginosa* pathogenesis

Virulence factor	Function	Reference
Flagellum	Attachment and colonisation of host tissues Swimming/swarming motility	Köhler *et al*, 2000; Gellatly and Hancock, 2013
Type IV pili	Attachment and colonisation of host tissues Twitching motility Biofilm formation	Marko *et al*., 2018; Craig *et al*., 2019; Persat *et al*., 2015
LPS	O-antigen stimulates the host inflammatory response and inhibits complement-mediated killing O-antigen mediated swimming and swarming motility Resistance to antibiotics (modification to LPS charge to inhibit cationic compounds/ modifications to lipid A to prevent penetration of the outer membrane) Lipid A-mediated cytotoxicity Biofilm formation (particularly CPA)	Huszczynski *et al*., 2019
Secretion systems (ss) Type 1 ss	**Secreted protein functions** Alkaline protease, protease	Filloux, 2011

Type 2 ss Type 3 ss Type 5 a, b and d ss Type 6 ss	Heme acquisition, lipase, alkaline phosphatase, protease, elastase, chitin binding, aminopeptidase, lipoxygenase, phospholipase GTPase activating protein and adenosine diphosphate ribosyltransferase, phospholipase, adenylate cyclase Lipase, adhesin protease, lipolytic enzyme Toxin	
Hydrogen cyanide	Colonisation and persistence – to kill competitive microorganisms	Lauridsen *et al*., 2017
Pyocyanin	Toxin - damage to the host caused by reactive oxygen species Proinflammatory effect Reduced ciliary clearance	Hall *et al*., 2016
Pyoverdine	Siderophore (Iron transport across the membrane) – establishment of infection Biofilm formation	Peek *et al*., 2012
Exoenzyme S Exoenzyme T Exoenzyme U Exoenzyme Y	Colonisation, invasion and dissemination, immune evasion Impair wound healing and inhibition of phagocytosis Tissue damage and modulation of inflammation Dissemination	Khodayary *et al*., 2019

Exotoxin A	Inhibits host protein synthesis, induced host cell apoptosis, reduces cytokine release	Gellatly and Hancock, 2013
Elastase A/B	Degrade host structural fibres, basement membranes, IgG/ IgA antibodies and cytokines	Caballero *et al*., 2001
Protease IV	Degrades host serine proteases, complement proteins and IgG antibodies	Caballero *et al*., 2001
Alkaline proteases (AprA)	Inhibition of host immune cell signalling e.g. TLR5 signalling Blocks classical and lectin pathway-mediated complement activation by cleaving complement protein C2	Bardoel *et al*., 2011 Laarman *et al*., 2012
Hemolysins (hemolytic phospholipase C and rhamnolipid)	Degrade lipids and lecithin Solubilise lung surfactants SP-A and SP-D Inhibition of mucociliary transport	Strateva and Mitov, 2011
Alginate secretion	Immune evasion and biofilm formation	Chanasit *et al*., 2020
Biofilm formation	Resistance to antibiotics Immune evasion Bacterial persistence	Moradali *et al*., 2017
Efflux pumps	Antibiotic resistance – broad class range Removal of toxic compounds	Blair *et al*., 2015; Moradali *et al*., 2017

Quorum sensing	Establish infections by controlled production of virulence factors, control of biofilm formation	Mukherjee *et al*., 2017
Two-component systems	Allows *P. aeruginosa* to adapt to changes in the environment: Transition between acute and chronic infection regulated by the GacS-GacA system Roc system regulates expression of fimbriae required for attachment and biofilm formation and inhibits the expression of efflux pumps PhoP-PhoQ system regulates LPS modifications leading to increased resistance to antimicrobial peptides	Francis *et al*., 2017

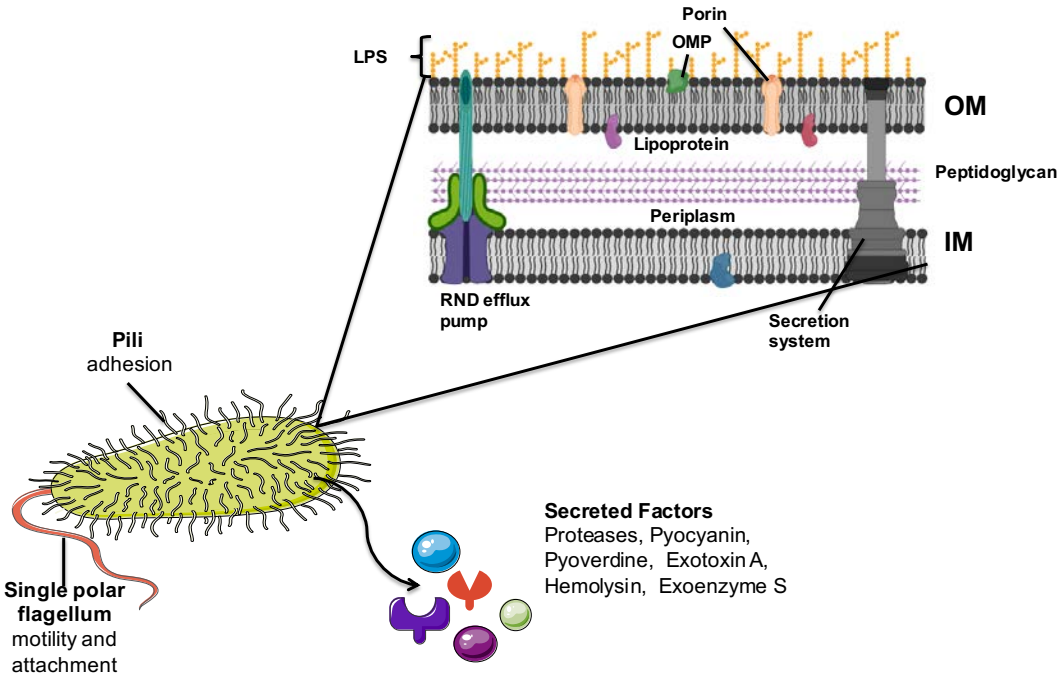


Figure 1.2 Virulence factors utilized by *P. aeruginosa* to colonise host

P. aeruginosa motility can be attributed to the production of the flagellum and type IV pili. Movement within a liquid or through a semi-solid environment requires the flagellum whereas movement across a solid surface is achieved through the use of type IV pili, which extend and attach to the surface and then retract to pull the cell across the surface. Initial attachment to the host cell can also be facilitated by these appendages. *P. aeruginosa* secretes a range of factors that play roles in the defence against the host immune system (proteases, pyocyanin, exoenzymes), the acquisition of nutrients from the environment (pyoverdine) and colonisation of the host (exoenzymes, hemolysins, pyocyanin, pyoverdine). *P. aeruginosa* has both an inner membrane (IM), and an outer membrane (OM) that exclude antibiotics and antimicrobial peptides from the cell. Lipopolysaccharide constitutes a large portion of the OM. The O-antigen region can confer resistance to complement-mediated killing whereas the lipid A is responsible for the cytotoxic effect of lipopolysaccharide. Resistance to antibiotics can also be attributed to the expression of efflux pumps. (Figure created using https://smart.servier.com/image-set-download/ and Biorender.com).

signal, *N*-(3-oxododecanoyl)-homoserine lactone and *N*-butyrylhomoserine lactone. The production of these molecules is controlled by the *regulatory* systems: *rhl, iqs, pqs,* and *las* (Lee and Zhang, 2015; Rajkumari *et al*., 2018).

1.2.2.1 Type IV pili and the flagellum

Type IV pili are filamentous surface located structures that contribute to the motility and virulence of bacteria. These appendages are able to extend, attach and retract enabling bacteria to move across surfaces in a process known as twitching motility (Craig *et al*., 2019). They also facilitate the adhesion and colonisation processes during the initial stages of host infection (Marko *et al*., 2018). Type IV pili also have roles in the sensing of surface contact and in the regulation of gene expression associated with surface motility and biofilm formation (Persat *et al*., 2015).

Swimming and swarming motility can be attributed to a single polar flagellum. This appendage also facilitates adhesion to host surfaces, the initiation of biofilm formation and the stimulation of the host immune response (Bucior *et al*., 2012). There is also evidence to support the role of the flagellum in the increased resistance to surfactant protein A in the lung (Zhang *et al*., 2007).

1.2.2.2 Enzymes and toxins

P. aeruginosa is able to secrete a variety of exotoxins and cytotoxins that promote bacterial adherence to host-tissues and host-cell lysis (Michalska and Wolf, 2015). Immune evasion can be achieved through the production of elastases, which are capable of degrading host mucins and surfactant proteins, and proteases, which are able to degrade host complement system proteins (Casilag *et al*., 2016). *P. aeruginosa* produces rhamnolipids, which have roles in:

metabolism, the establishment of infection, the inhibition of ciliary clearance, modulation of immune responses, motility and biofilm formation (Abdel-Mawgoud *et al*., 2010). Host tissue damage is also achieved through the secretion of pyocyanin, which mediates the induction of reactive oxygen species production (Mortzfeld *et al*., 2019) and hemolysins (Pollack, 1986).

1.2.2.3 Multidrug resistance

The emergence of multidrug resistance in *P. aeruginosa* is currently a cause for concern and has been classified as a serious threat by the Centers for Disease Control and Prevention (CDC, 2018). The bacterium has intrinsic resistance to many classes of antibiotics and is able to exploit several resistance mechanisms (Table 1.3) (Lambert, 2002).

P. aeruginosa has both an inner and outer membrane (Figure 1.2). The outer membrane (OM), is an asymmetric hydrophobic lipid bilayer containing glycerophospholipids and lipopolysaccharide (LPS), which is decorated with proteins and polysaccharide antigens. It acts as a physical barrier that prevents the access of many antimicrobials to their internal target sites (Delcour, 2009; Miller, 2016). Reduced expression of outer membrane porins and the overexpression of efflux pumps prevent the accumulation of antibiotics within the cell (Lambert, 2002). Porins allow small hydrophilic antimicrobial compounds, such as β-lactams, to cross the OM and gain access to the cell. Therefore, changes in the levels of expression, structure and/or function of these porins can result in resistance as these compounds are no longer able to access their targets within the cell (Delcour, 2009). *P. aeruginosa* can also expel antibiotics from the cell through efflux pumps. The most commonly described class of efflux pumps associated with the multi-drug resistance phenotype of *P. aeruginosa* is the resistance-nodulation-division (RND) class, which can transport antibiotics from the cytoplasm or

Table 1.3 Mechanisms of drug resistance employed by *P. aeruginosa*

Mechanism	Gene/gene product	Example antibiotic class	Reference
Decreased cell wall permeability	Outer membrane porin proteins: OprD, OprH	Aminoglycosides, quinolones, β-lactams, polymyxin	Lambert, 2002
	LPS	Hydrophobic antibiotics	Delcour, 2009
Enzymatic inactivation	Chromosomally encoded β-lactamases - *BlaI*	β-lactams	Hancock, 1998
Altered target site/ chromosomal mutations	Penicillin binding proteins *aphA* (activated by chromosomal mutation)	β-lactams kanamycin	Hancock, 1998
	DNA gyrase (*gyrA/B*) Topoisomerase IV (*parC/E*)	Quinolones	Lomovskaya *et al*., 2001
Drug efflux	Resistance-nodulation-division (RND) multidrug resistance efflux pumps	Fluoroquinolones Aminoglycosides β-lactams carbapenems tetracyclines macrolides	Dreier and Ruggerone, 2015
Acquired resistance	Plasmid encoded enzymes Chromosomal mutations	Aminoglycosides β-lactams	Henrichfreise *et al*., 2007

periplasm out of the cell (Sun *et al*., 2014). Twelve efflux pumps belonging to this family have been described in *P. aeruginosa* strain PAO1, 4 of which are commonly found in clinical isolates and are strongly associated with multi-drug resistance. These systems are: mexAB-oprM, mexCD-oprJ, mexEF-oprN and mexXY (-oprA) (Dreier and Ruggerone, 2015).

P. aeruginosa produce β-lactamases, which cleave and inactivate β-lactams, and aminoglycoside modifying enzymes, which inactivate aminoglycosides through phosphorylation, acetylation or adenylation. (Poole, 2011). Antibiotic resistance can also be conferred through the modification of target sites that arise due to spontaneous chromosomal mutations (Poole, 2011). This is commonly observed in hypermutator strains of *P. aeruginosa* isolated from chronic lung infections (Marciá *et al.,* 2005). Biofilm formation is also associated with an increase in resistance or tolerance to antibiotics. The restricted diffusion of aminoglycoside antibiotics has been associated with the overproduction of the biofilm associated exopolysaccharides: alginate, Pel and Psl (Germoni *et al*., 2016). The presence of negatively charged extracellular DNA (eDNA) within the extracellular matrix also hinders biofilm penetration of positively charged compounds leading to antibiotic tolerance (Ciofu and Tolker-Nielsen, 2019).

Multi-drug resistant strains of *P. aeruginosa* have become an emerging problem for CF patients. Resistance is believed to result from the prolonged exposure to multiple antibiotic classes during treatment and the occurrence of horizontal gene transfer within the hospital setting (Aloush *et al.,* 2006; Mesaros *et al.*, 2007).

1.2.2.4 Lipopolysaccharide (LPS)

LPS is the dominant constituent of the Gram-negative OM and is a key contributor to the virulence of *P. aeruginosa* (Pier, 2007). LPS is generally considered to be essential for the viability of Gram-negative bacteria due to its role in maintaining the stability of the outer membrane (Klein and Raina, 2019). The production of LPS has been associated with the resistance to both serum-mediated killing and antibiotics (Walsh *et al*., 2000). The negatively-charged LPS creates physical barrier through the association with divalent cations resulting in LPS bridging and also reduces outer membrane permeability (Lam *et al*., 2014; May and Grabowicz, 2018). *P. aeruginosa* produces two forms of LPS: common polysaccharide antigen (CPA) LPS, formerly known as A-band LPS (Figure 1.3A); and O-specific LPS, formerly known as B-band LPS (Figure 1.3B) (Lam *et al*., 2011).

O-specific LPS consists of three main domains: lipid A, core-antigen, and O-antigen (Maldonado *et al*., 2016). Lipid A anchors the LPS to the outer membrane and is responsible for the toxicity associated with LPS due to the recognition by Toll-like receptor 4, which stimulates an inflammatory response (Hajjar *et* al., 2002; Sampath, 2018). Acylation of lipid A varies between strains of *P. aeruginosa* and penta-, hexa- and hepta-acylated forms of lipid-A have been identified (Pier, 2007; SenGupta *et al*., 2016). Penta-acylated lipid A has been suggested to elicit a weaker inflammatory response compared to lipid A with higher levels of acylation (Cigana *et al*., 2009) and this structure has been found in lab adapted and non-CF bronchiectasis strains of *P. aeruginosa* (Hajjar *et* al., 2002). Strains isolated from early-stage CF patients have been associated with the production of hexa-acylated lipid A, whereas strains isolated from late-stage CF can produce hepta-acylated lipid A (SenGupta *et al*., 2016).

The core antigen can be further divided into the inner-core and outer-core regions (Figure 1.3).

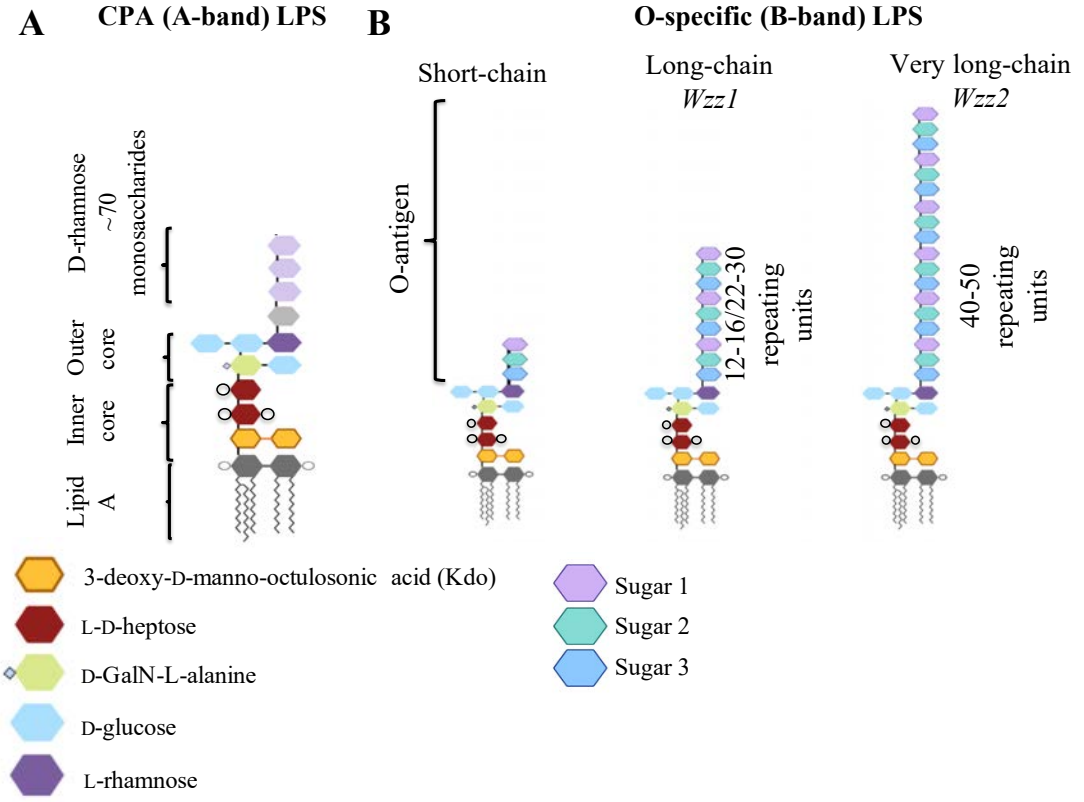


Figure 1.3 Representation of the two forms of LPS produced by *P. aeruginosa*

A) Core polysaccharide antigen (CPA) LPS is produced by most strains of *P. aeruginosa* and consists of: the membrane anchor, lipid A; a core region comprised of Kdo, phosphorylated L-D-heptose (phosphates represented by circles), D-glucose, D-rhamnose and D-galactosamine (GalN) which can be modified at position 2 with an alanine and finally a homopolymer of D-rhamnose monosaccharides. **B)** O-specific LPS is produced by most strains of *P. aeruginosa* and consists of lipid A, a core region and variable polysaccharide O-antigen region made up of repeating units consisting of 3-5 sugars. The variation in sugar composition forms the basis of *P. aeruginosa* serotyping. The length of the O-antigen is controlled by the chain length regulator Wzz, with Wzz1 modulating the production of long O-antigen and Wzz2 modulating the production of very long O-antigen. Strains producing O-antigen exhibit the smooth LPS phenotype, whereas strains lacking O-antigen exhibit the rough LPS phenotype (Figure adapted from Lam *et al*., 2011 and created with Biorender.com).

The inner-core is composed of two 3-deoxy-D-manno-octulosonic acid residues (Kdo) and 2 residues of highly phosphorylated L-glycero-D-manno-heptose. The outer-core consists of D-glucose, L-Rhamnose and D-Galactosamine, which can be modified through the addition of L-alanine (Lam *et al*., 2011). Modification of the core antigen has been associated with the increased resistance to antibiotics and serum-mediated killing (Walsh *et al*., 2000).

The O-antigen is highly variable between *P. aeruginosa* strains and is formed of repeating units containing 3-5 sugars (Figure 1.3B). The production of the O-antigen is dependent on the *wzy* pathway (Figure 1.4A) (King *et al*., 2009). Serotyping of *P. aeruginosa* by the International Antigenic Typing Scheme is based on the variation in the sugar composition of the repeating units (Lam *et al.,* 2011). The sugar composition of serotypes commonly isolated from patients infected with *P. aeruginosa* can be found in Figure 1.5. *P. aeruginosa* can produce long-chain O-antigen, length of which is controlled by the chain length regulator Wzz1 (up to 30 repeating units), or the very long-chain O-antigen (usually up to 50 repeating units), which is controlled by Wzz2 (Figure 1.4A). The production of long-chain O-antigen rather than very-long-chain O-antigen has been associated with increased resistance to complement-mediated killing (Kintz *et al*., 2008).

The structure of the common polysaccharide form of LPS contains the lipid A and core regions. However, the specific O-antigen is replaced by a homopolymer repeating unit consisting on average of 70 D-rhamnose monosaccharides (Figure 1.3A) (Lam *et al*., 2011). CPA is produced by most strains of *P. aeruginosa* simultaneously with O-specific LPS. However, the biosynthesis of CPA is dependent on an ABC-transporter pathway consisting of a cluster of 8 genes, *rmd-wbpM* (Figure 1.4B) (King *et al*., 2009; Lam *et al*., 2011). Although this gene

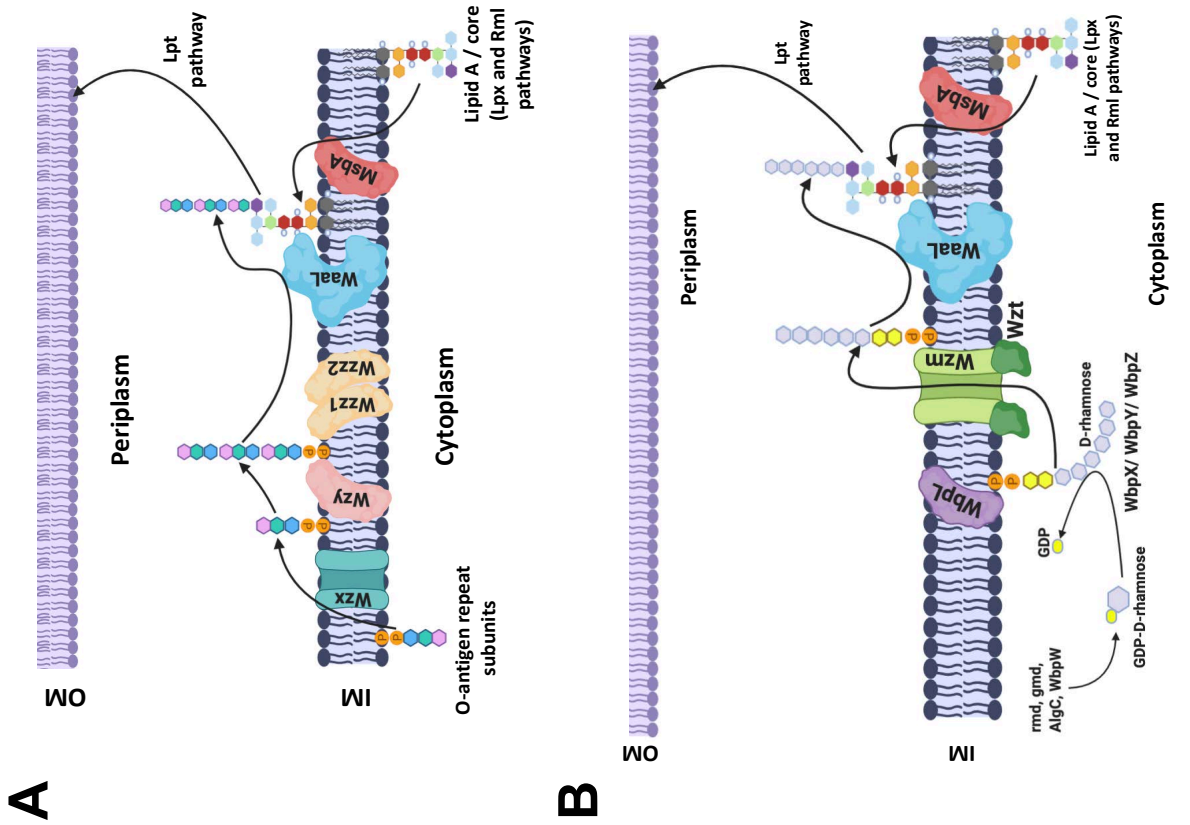


Figure 1.4 LPS biosynthetic pathways utilized by *P. aeruginosa*. Lipid A and the core antigen are synthesised on the cytoplasmic leaflet of the inner membrane (IM) by the Lpx and Rml pathways, respectively. The lipid A-core structure is then flipped across the IM by the flippase, MsbA. The O-antigen or common polysaccharide antigen (CPA) is ligated to the lipid A-core by WaaL and the finished LPS molecule is transported through the periplasm to the outer leaflet of the outer membrane (OM) by the Lpt pathway. **A) *wzy*-dependent synthesis of O-specific antigen.** O-antigen subunits are assembled on the cytoplasmic leaflet of the IM by glycosyltransferase enzymes. These are transported across the IM by the flippase Wzx. The O-antigen is polymerised by Wzy to a chain length regulated by Wzz. **B) ABC-transporter dependent synthesis of CPA.** Synthesis of CPA is initiated by the glycosyltransferase WbpL. D-rhamnose precursor sugars GDP-D-rhamnose are synthesised by the enzymes rmd, gmd, AlgC and WbpW. The D-rhamnose polymer is then synthesised from these precursors by the glycosyltransferase enzymes WbpX, Y and Z. The D-rhamnose polymer is transported across the IM by the ABC-transporter system Wzm-Wzt. (Islam and Lam, 2014; Maldonado *et al*., 2016; Mccarthy *et al*., 2017) (Figure created with Biorender.com).

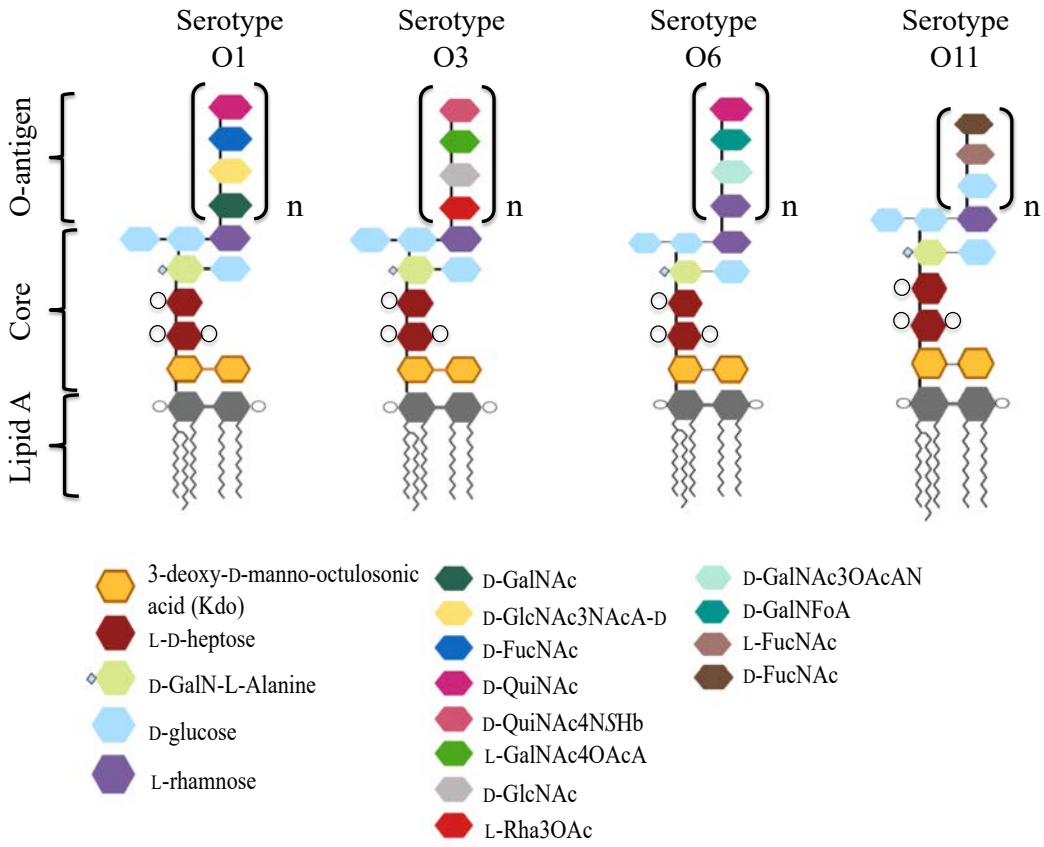


Figure 1.5 A schematic of the O-antigen composition of common *P. aeruginosa* serotypes isolated from patients

The O-antigen structures from *P. aeruginosa* serotypes O1, O3, O6 and O11 have been depicted. The O-antigen region illustrates the sugars that make up the repeating unit. The number of repeating units (n) differ between strains. O-antigen sugar compositions obtained from Lam *et al*., 2011; Knirel *et al*., 2006 (Figure created with Biorender.com).

cluster has been located in all 20 IATS reference strains, a lack of CPA expression has been identified in strains belonging to serogroups O7, O12, O13, O14, O15 and O16 (Lam *et al*., 2011). Analysis of the genomes of *P. aeruginosa* strains PAO1 (O5), PA14 (O10), PA7 (O12) and LESB58 (O6) led to the discovery of another gene cluster directly adjacent to the *rmd-wbpM* cluster on the opposite DNA strand that is required for CPA biosynthesis (Figure 1.4B) (Hao *et al.,* 2013). The lack of CPA expression by strain PA7 has been attributed to the potential promotor disruption caused by the second gene cluster being located on the same strand, 10 kb upstream of the *rmd-wbpM* cluster (Lam *et al.,* 2011). CPA has been suggested to play an important role in the adherence of *P. aeruginosa* to human tissues and the maturation of *P. aeruginosa* biofilms (Lam *et al.,* 2011; Murphy *et al*., 2014).

1.2.2.5 The acute to chronic switch

Once colonisation has been established, *P. aeruginosa* undergoes several genotypic and phenotypic changes as it switches from an acute to a chronic infecting strain (Table 1.4). Many of these changes allow the bacteria to adapt to the lung environment and to avoid clearance by the host immune system (Winstanley *et al.,* 2016). To survive within the host, *P. aeruginosa* downregulates the production of virulence factors and immunogenic surface antigens in order to minimise the inflammatory response and to prevent immune recognition. This is often associated with an increased production of alginate and a switch from planktonic to biofilm lifestyle (Cullen and McClean, 2015).

Biofilm formation plays an important role in *P. aeruginosa* pathogenesis and enables the bacterium to establish and sustain chronic infections in susceptible hosts (Maurice *et al*., 2018). Biofilm formation is initiated by bacterial attachment to a surface followed by the formation of

Table 1.4 *P. aeruginosa* adaptations during switch from acute to chronic infection (Cullen and McClean, 2015; Winstanley *et al.,* 2016).

Adaption	Effect
Loss of flagellum	Loss of motility Immune evasion
Non-mucoid to mucoid	Biofilm formation Decreased susceptibility to antibiotics Immune evasion
Loss of O-antigen	Immune evasion
Down-regulation of virulence factors	Reduced virulence Immune evasion

microcolonies, which then mature into a biofilm (Gnanadhas *et* al., 2015). Mature biofilms primarily consist of a matrix of exopolysaccharides, proteins, lipids and eDNA with bacterial cells only contributing to 10% of the dry weight (Moradali *et al*, 2017). The production of eDNA, rhamnolipids, lectins and swarming and twitching motility is controlled by the Las/Rhl quorum sensing systems, which in turn are regulated by the Gac, Ret and Lad two-component regulatory systems (Rasamiravaka *et al*., 2015). The production of the exopolysaccharide Psl is regulated by the two-component regulatory systems whereas the production of Pel and alginate are regulated by the aforementioned quorum sensing systems and by the intracellular levels of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Rasamiravaka *et al*., 2015).

P. aeruginosa can form biofilms on invasive medical devices and the resulting infections are often associated with a high mortality rate. Such infections include: ventilator associated pneumonia following the use of endotracheal or tracheostomy tubes (Diaconu *et al*., 2018) and endocarditis following prosthetic valve implantation (Gürtler *et al*., 2019). *P. aeruginosa* biofilms can also be problematic for patients requiring urinary catheters, central venous catheters, prosthetic joints and for burn or wound patients and contact lens users (Jamal *et al*, 2018).

Chronic colonisation of the lung is also facilitated by *P. aeruginosa* biofilm formation in patients with CF, bronchiectasis and COPD (Mulcahy *et al*., 2014). In the lung, *P. aeruginosa* commonly forms aggregate biofilms in sputum and the mucus that lines the airways (Høiby *et al*., 2017). The switch from planktonic to biofilm lifestyle results in physical, metabolic and growth changes and renders cells in the biofilm resistant to the antibiotics that target these

processes. The loss of immunogenic external appendages such as the flagellum and O-antigen also hinders the immune recognition of *P. aeruginosa* by the host (Maurice *et al*., 2018). The biofilm matrix also enables *P. aeruginosa* to evade the host immune response through a variety of mechanisms and can protect the cells from antimicrobial peptides, neutrophil oxidative burst, phagocytosis, opsonisation and complement-mediated killing (Maurice *et al*., 2018).

Chronic colonisation with *P. aeruginosa* in bronchiectasis patients correlates with poorer lung function and an increase in the rate of lung-function decline in patients with bronchiectasis (Evans *et al.,* 1996). Mucoid *P. aeruginosa* strains have been associated with increased sputum viscosity and a poor clinical outcome (Alcaraz-Serranoa *et al*., 2019). An increase in both the number of hospital admissions and mortality rate have also been observed in patients chronically infected with *P. aeruginosa* (Finch *et al.,* 2015).

Several longitudinal studies have genotypically assessed the *P. aeruginosa* population in the lungs of CF and non-CF bronchiectasis patients (Williams *et* al., 2015; Hilliam *et al*., 2017; Woo *et al*., 2018; Bianconi *et al*., 2019). There is a general consensus that the majority of non-CF bronchiectasis patients tend to be infected with a single clonal lineage of *P. aeruginosa.* However, co-infection with multiple strains of *P. aeruginosa* or strain displacement can also occur (Hilliam *et al*., 2017; Woo *et al*., 2018). Similarly, strains longitudinally isolated from CF patients often belong to a single clonal lineage (Aaron *et al*., 2004; Woo *et al*., 2018; Bianconi *et al*., 2019). Strains can be transmitted between CF patients who attend the same CF treatment centres (Wee *et al*., 2018). Epidemic strains, such as the Liverpool epidemic strain, have been identified and these strains tend to exhibit increased resistance to antibiotics and are often associated with increased morbidity (Fothergill *et al*., 2012).

1.3 The host response to lung infection

1.3.1 Conducting airway immunity

Lung epithelial cells are the initial point of contact and form the first line of defence against inhaled pathogens (Figure 1.6A). Goblet cells are responsible for the constant secretion of a thin layer of mucus into the airway lumen which forms a physical barrier and traps inhaled antigens (Eisele and Anderson, 2011). Mucus primarily consists of mucins, which are the heavily glycosylated carbohydrates responsible for forming the physical barrier against pathogens (Lamblin *et al*. 2001).

Membrane-bound mucins can also regulate the immune response (Zanin *et al*., 2016). Mucus also contains a variety of proteins and enzymes that have antimicrobial properties (Zanin *et al*., 2016). Ciliated cells are responsible for the mucocilliary clearance of inhaled particles and pathogens. This occurs through the regular mechanical beating of the cilia, which moves the mucus out of the airways to be removed through cough (Whitsett, 2018). The cilia on these cells also have a role in modulating immune responses by sensing the presence of pathogens in the airway lumen (Tilley *et al*., 2015).

Pulmonary neuroendocrine cells have an immunoregulatory role, controlling the inflammatory response and infiltration of immune cells (Branchfield *et al*., 2016). Basal cells are a form of stem cell responsible for repairing lung epithelial cell damage and also have a role in regulating the immune response through the increased secretion of cytokines and other immune response regulators (Shaykhiev, 2015). Club cells are responsible for lung epithelial cell repair and the neutralisation of oxidative compounds. These cells also protect against infection by secreting antimicrobial peptides and induce the mucociliary clearance of pathogens (Liu *et al*., 2019).

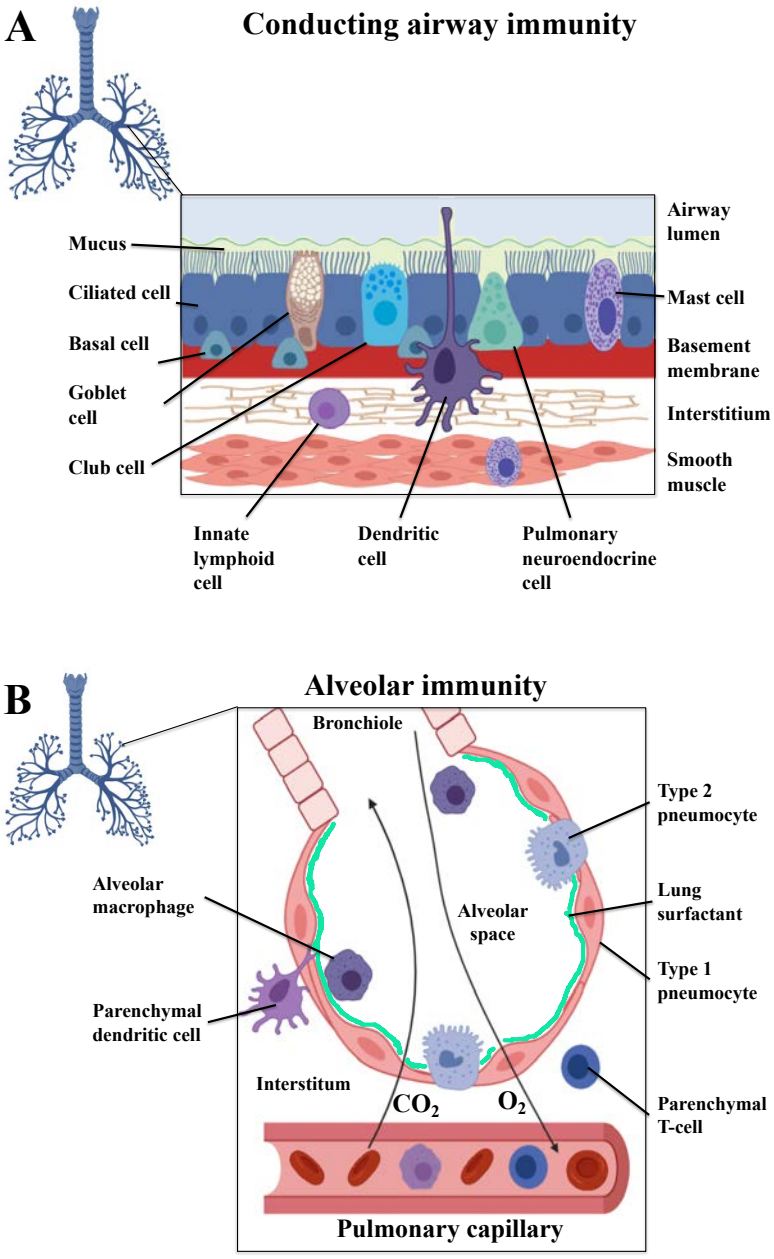


Figure 1.6 Cells involved in the innate lung immune response

A The conducting airway immunity. The lung epithelium forms an initial barrier against pathogens. A thin layer of mucus is secreted by epithelial goblet cells, which entraps pathogens and inhaled particles aiding the removal by mucociliary clearance by ciliated epithelial cells. Basal and club epithelial cells have roles in the repair of epithelial cell damage. Pulmonary neuroendocrine cells modulate the immune response and dendritic cells, innate lymphoid cells and mast cells are involved in the inflammatory response and the recruitment off neutrophils and macrophages. **B Alveolar immunity.** The alveolar epithelium consists of type 1 and type 2 pneumocytes. Type 1 cells allow gas exchange between the alveoli and the capillaries and type 2 cells release immune signalling molecules and secrete surfactant, which has roles in the structural integrity of the lungs and immunomodulation. Alveolar macrophages are the main cells involved in an innate immune response to invading pathogens (Figure created with Biorender.com).

1.3.2 Alveolar immunity

The alveolar epithelium consists of type 1 and type 2 cells known as pneumocytes (Figure 1.6B). Type 1 cells are long, thin cells that permit gas exchange between the alveolus and the surrounding capillaries. Type 2 cells are cuboidal cells with two main functions: 1) the secretion of lung surfactant, which reduces the lung surface tension and prevents the airways from collapsing; and 2) to differentiate into type 1 cells following alveolar epithelial cell damage (Guillot *et* al., 2013; Olajuyin *et al*., 2019). Type 2 cells also release signalling molecules to activate an immune response as well as antimicrobial molecules to help control infection (Chuquimia *et* al., 2012). Unlike the epithelial cells of the conducting airways, alveolar epithelial cells are not ciliated. In a healthy state, alveolar macrophages account for more than 90% of the immune cells in the alveolar space and are responsible for phagocytosis and immune signalling (Holt *et al*., 2008). Other immune cells such as dendritic cells and T-cells are found in the surrounding tissues. Complement proteins and antibodies are also present in the alveolar fluid lining the epithelium to prevent bacterial colonisation (Martin and Frevert, 2005; Holt *et al*., 2008).

1.3.3 Lung immune cell responses

Epithelial cells have pathogen-associated molecular pattern receptors, which upon stimulation, cause the release of cytokines followed by the recruitment of monocytes and dendritic cells and the initiation of an immune response (Papazian *et al*., 2015). Dendritic cells within the lung tissue are also able to sample the antigens present in the airway lumen (Cook and MacDonald, 2016) (Figure 1.6) and migrate to the lymph nodes where an adaptive T-cell immune response can be initiated (Nakano *et al*., 2013). Mast cells can also be activated when they come into contact with a pathogenic antigen or opsonised pathogen. This activation results in

degranulation of the cell and the release of antimicrobial peptides, proteases and inflammatory mediators. Mast cells can also prevent the invasion of lung tissue by engulfing pathogens (Virk *et al*., 2016). Innate lymphoid cells are activated by a range of interleukins and function as neutrophil and alveolar macrophage recruiters, tissue repair initiators and dendritic cell activators (Stehle *et al*., 2018).

1.3.4 The complement system

The complement system plays a crucial role in the innate clearance of invading pathogens. The complement system causes bacterial cell lysis, promotes inflammation, opsonises bacteria for clearance by phagocytes and promotes adaptive immune responses (Heesterbeek *et al*., 2018). The complement system can be activated via three main routes: the classical, alternative and lectin pathways (Merle *et al*., 2015). These pathways differ in their mechanism of activation. However, initiation of any of the pathways results in the formation of the C3 convertase and the proceeding cascade of reactions ultimately ending with bacterial cell lysis (Nesargikar *et al*., 2012).

1.3.4.1 The classical pathway

Activation of the classical pathway (Figure 1.7) is initiated following the binding of antibody or serum proteins to bacterial antigens (Merle *et al*., 2015; Thurman and Holers, 2006). The binding of complement protein C1 to the antibody-antigen complexes activates C1, resulting in the cleavage of proteins C4 and C2, and the formation of the C3 convertase (C4b2a). The C3 convertase cleaves protein C3 into C3a, which promotes inflammation and C3b, which then binds to C3 convertase forming the C5 convertase (C4b2a3b). The C5 convertase then cleaves C5 into C5a, which also promotes inflammation, and C5b. C5b sequentially recruits and binds

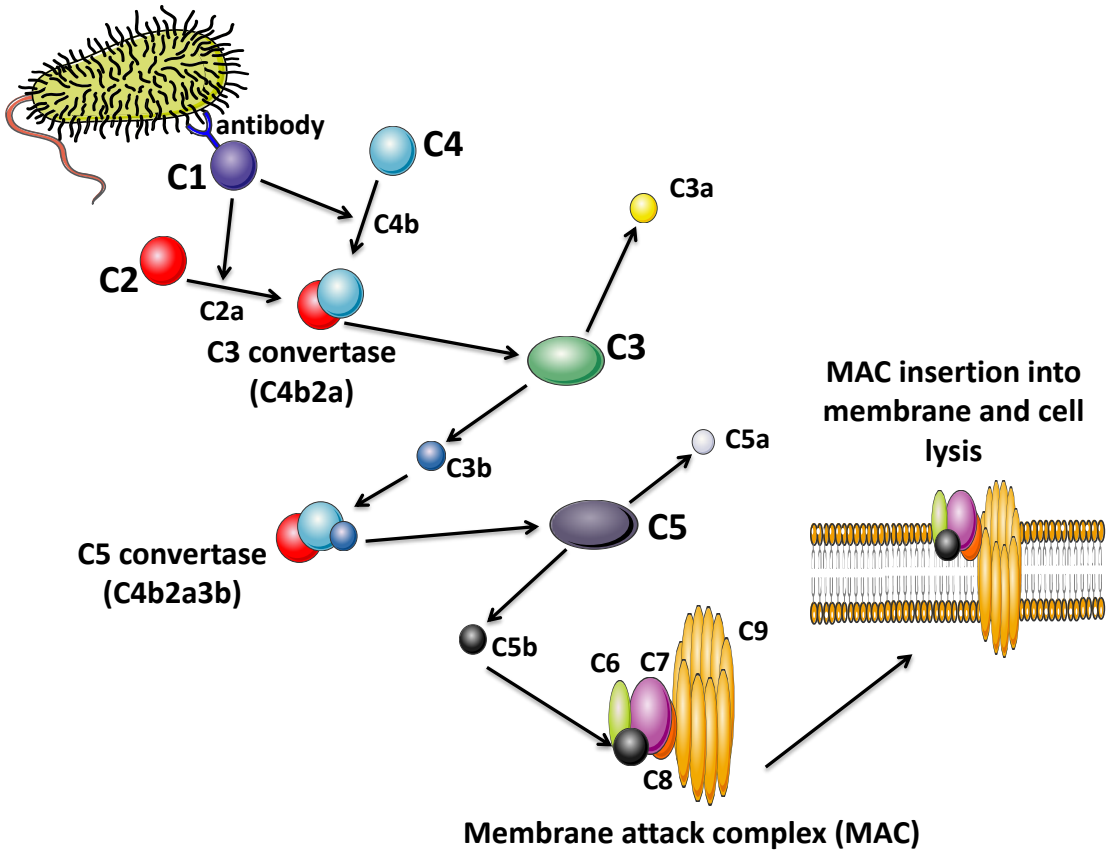


Figure 1.7 Activation of the complement system via the classical pathway

Antibody binding to bacterial antigens triggers the activation of complement protein C1 which causes a cascade of further complement protein activation, convertase production (C3 convertase and C5 convertase) and the release of proinflammatory molecules (C3a and C5a). This process terminates in the formation of the membrane attack complex. The membrane attack complex inserts into the bacterial cell membrane causing pore formation and leads to the loss of intracellular compounds and cell lysis. Figure created using images available from http://www.servier.com/Powerpoint-image-bank.

proteins C6,7,8 and multiple C9s resulting in the formation of the membrane attack complex (MAC). The MAC forms a pore structure and inserts into the membrane causing membrane damage and cell lysis (Merle *et al*., 2015).

1.3.4.2 The lectin pathway

The lectin pathway can be activated by mannose-binding lectins (MBLs) or ficolins. These molecules recognise and bind to mannose residues or *N*-acetylglucosamine (GlcNAc) and subsequently associate with MBL-associated serine proteases (MASPs) (Matsushita *et al*., 2001). MASP-2 is able to activate the complement system via the cleavage of complement proteins C4 and C2, which leads to the formation of the C3 convertase C4b2a and the subsequent cascade of reactions as described for the classical pathway (Figure 1.8) (Takahashi *et al*., 2008). MASP-1 has been identified as a MASP-2 activator (Héja *et al*., 2012), whereas MBL-MASP-3 complexes have been implicated in the activation of the alternative pathway through the activation of factor D (Iwaki *et al*., 2011; Dobó *et al*., 2016).

1.3.4.3 The alternative pathway

The alternative pathway is constitutively activated in a spontaneous manner due to the hydrolysis of a thioester bond in protein C3 (Pangburn and Müller-Eberhard, 1983). This results in the formation of a molecule similar to C3b known as C3(H_2O). An alternative C3 convertase (C3(H_2O)Bb) molecule can be formed from the interaction of C3(H_2O) and Factor B in a magnesium-dependent manner and its subsequent cleavage by Factor D (Figure 1.9) (Chen *et al*., 2016). C3 cleavage by this enzyme releases C3b, which is then available to opsonise foreign antigens (Harboe *et al*., 2017). The surface-bound C3b forms the C3 convertase C3bBb, again following interactions with Factor B and D (Chen *et al*., 2016; Harboe *et al*., 2017). This

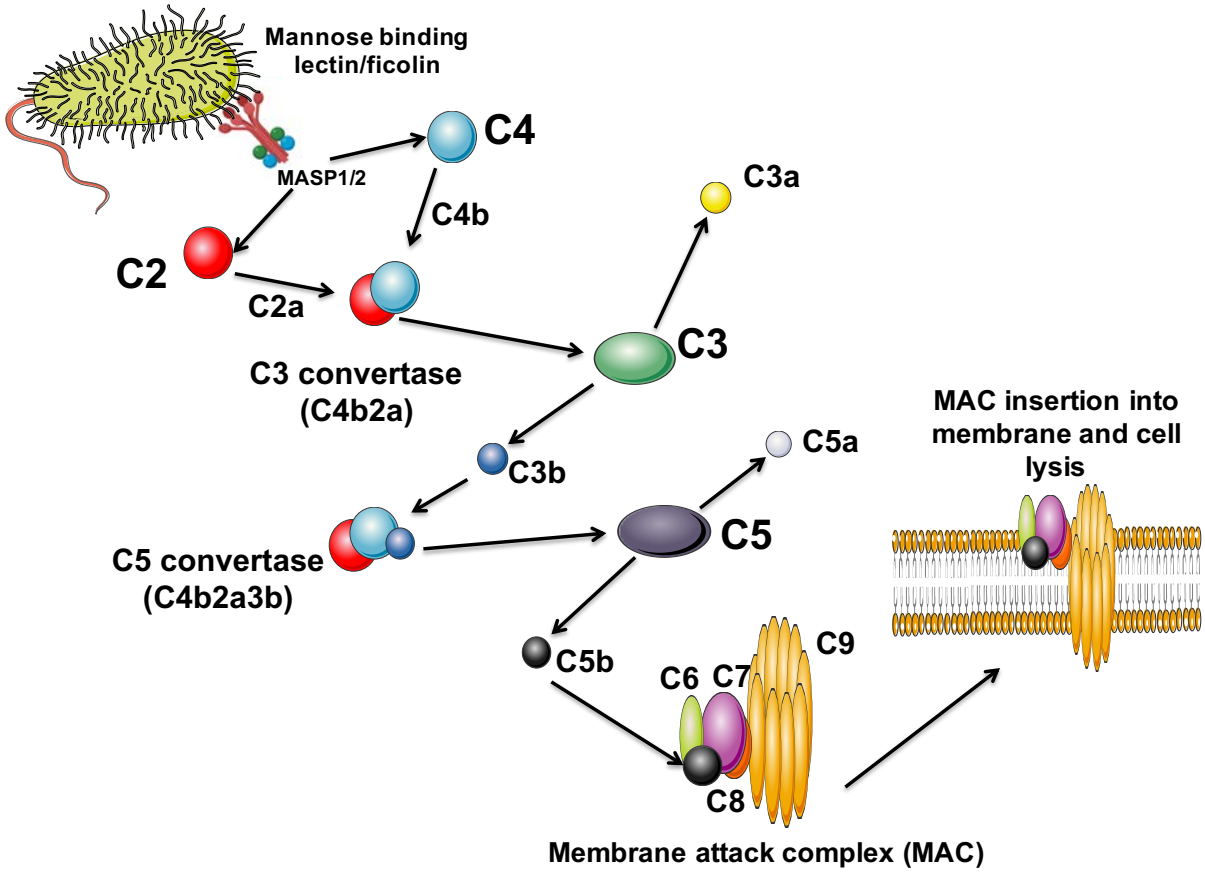


Figure 1.8 Activation of the complement system by the lectin pathway

The binding of lectins to bacterial surface mannose residues or ficolins to *N*-acetylglucosamine (GlcNAc) activates the associated serine protease enzymes (MASPs). These MASPs act in a similar fashion to the classical pathway C1 protein by cleaving complement proteins C2 and C4. The resulting C2a and C4b molecules combine to form the C3 convertase, C4b2a. The ensuing cascade of reactions occurs in the same manner as the classical pathway, terminating with the insertion of the membrane attack complex into the bacterial membrane and pore formation leading to cell lysis. Figure created using images available from http://www.servier.com/Powerpoint-image-bank and Biorender.com.

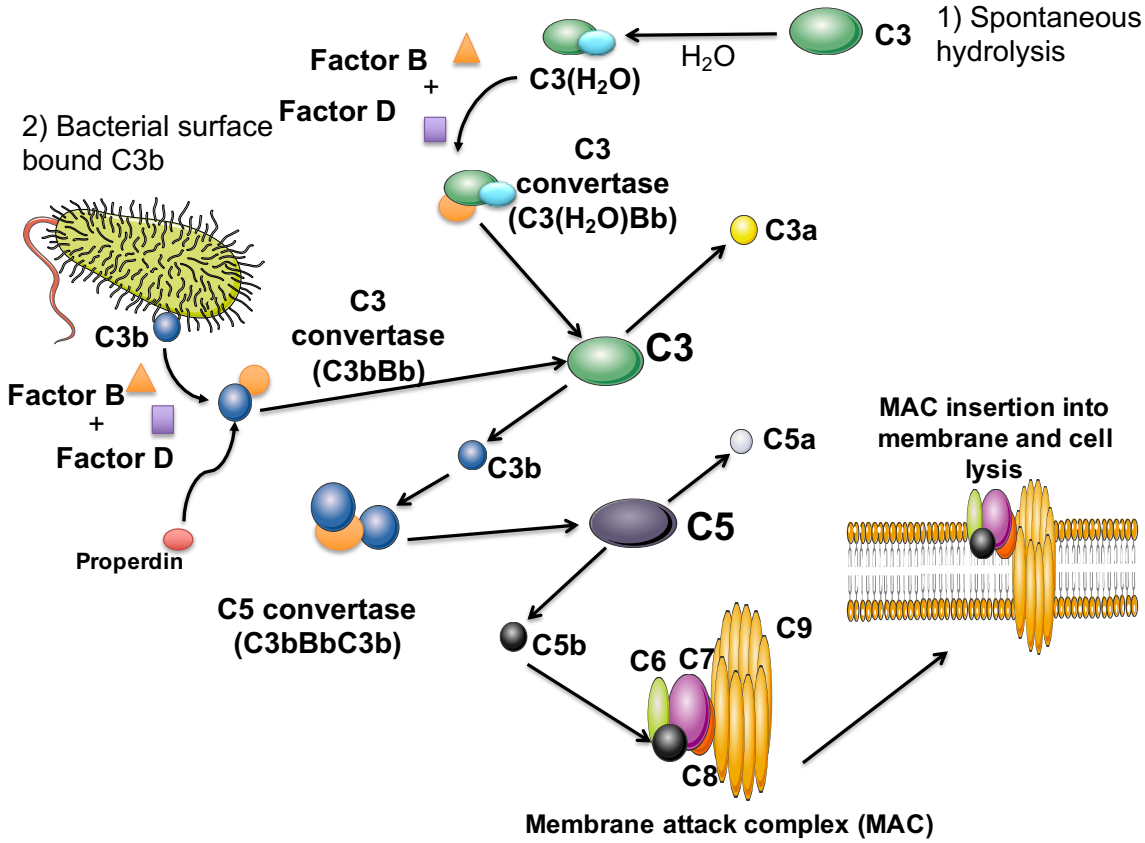


Figure 1.9 Activation of the complement system by the alternative pathway

The alternative pathway is constitutively activated by the spontaneous hydrolysis of the complement C3 protein. The hydrolysed C3 in association with factor B and modification by factor D forms the alternative C3 convertase enzyme, $C3(H_2O)Bb$. This enzyme cleaves C3 releasing C3b, which is then free to opsonise foreign antigens or form the C5 convertase. Activation of the alternative pathway also occurs following the binding of C3b to bacterial surfaces, which triggers the formation of the C3 convertase C3bBb through interactions with factor B and Factor D. This complex can be further stabilised in the presence of the protein properdin. The C3bBb convertase cleaves C3 and combines with the released C3b to form the C5 convertase, C3bBbC3b. The ensuing cascade of reactions occurs in the same manner as the classical pathway, terminating with the insertion of the membrane attack complex into the bacterial membrane and pore formation leading to cell lysis. Figure created using images available from http://www.servier.com/Powerpoint-image-bank and Biorender.com.

convertase can be further stabilised through the interaction with properdin and subsequently forms the C5 convertase C3bBbC3b following C3 cleavage. The C5 convertase cleaves C5 and leads to the formation of the MAC as described above (Figure 1.9) (Chen *et al*., 2016; Pederson *et al*., 2017).

1.3.5 Humoral responses

1.3.5.1 B lymphocytes

Antibodies are produced by a population of white blood cells known as B-lymphocytes (B-cells). In mice, B-lymphocytes are divided into B1, B2 and B-memory lymphocytes (Rothstein *et al*., 2013). B1 and B2 cells are further subdivided into B1a and B1b; and marginal zone B2 (spleen) and follicular B2 cells (spleen and lymph nodes), respectively (Montecino-Rodriguez and Dorshkind, 2012; Hoffman *et* al, 2016). B1 and marginal zone B lymphocytes have been identified in humans. However, these differ to murine B lymphocytes (Garaaud *et* al., 2012; Baumgarth, 2017). It has been suggested that human B-cells can be divided into B1, naïve, IgM memory and switched memory cells (Rothstein *et al*., 2013). However, a consensus has yet to be reached for defining human B-cell classes (Baumgarth, 2017). The antibodies produced by B-cells are involved in both the innate and adaptive immune response but differ in their specificity and affinity towards particular antigens (Montecino-Rodriguez and Dorshkind, 2012). Most research into understanding the role of B-lymphocytes in innate and adaptive immunity has been based on murine models. These functions are described in more detail below.

The primary cells responsible for the production of natural antibodies belong to the B1 class and develop from haematopoietic stem cells that are mainly derived from foetal liver or to a

lesser degree from adult bone marrow (Rodriguez-Zhurbenko *et al*., 2019). Marginal zone B2 cells derived from the bone marrow also secrete natural IgM antibodies (Hoffman *et* al, 2016). B1 cells are characterised by their ability to self-renew and the expression of CD11b, CD19 and IgM. Murine B1a and B1b cells are characterised based on the presence or absence of CD5, respectively (Deng *et al*., 2016). Natural antibodies are mainly produced by B1a cells whereas B1b cells can act as memory cells in T cell-independent IgM immunity (Palma *et al*., 2018; Alugupalli *et al*., 2004). B1-derived natural antibodies recognise self-antigens, which is important for the clearance of apoptotic cells and in the regulation of autoimmunity (Milner *et al*., 2005). These antibodies are also produced spontaneously in the absence of a foreign antigen and are polyspecific, allowing them to bind to bacterial antigens such as LPS (Montecino-Rodriguez and Dorshkind, 2012). The production of B1 cells in mice and humans declines with age, consequently resulting in reduced protection against autoimmune diseases and microbial infections (Rothstein *et al*., 2013).

Antibodies involved in the adaptive immune response are produced by the bone marrow-derived follicular B2 lymphocytes. These cells are the most abundant class of B-cells and produce high affinity, monospecific antibodies (Hoffman *et* al, 2016). These cells differentiate into B-memory cells and B-plasma cells, which are responsible for long-term immunity against foreign antigens (Hoffman *et* al, 2016). Marginal zone B2 cells also play a role in long-term immunity by transferring opsonised antigens to the follicular B2 cells (Arnon *et al*., 2012).

Natural antibodies are found in normal circulation and are produced irrespective of the presence of foreign antigens. These antibodies form part of the first line of defence against invading pathogens (Hernandez and Holodick, 2017). In humans, natural antibodies are mainly produced

by the B1 subclass of lymphocytes and belong to the IgM, IgA and IgG isotypes (Panda and Ding, 2015). Natural antibodies of the IgM isotype have been found in the highest abundance, however antibodies belonging to the IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4 isotypes have also been documented (Palma *et al*., 2018). When the innate response fails, the adaptive antibody response is triggered by the persistence of the foreign antigen and specific antibodies are produced by B2-lymphocytes. The production of these antibodies is induced by chemical signals, the binding of antigens to the B cell receptors or by the interaction with T-helper lymphocytes (Hoffman *et al*., 2016).

1.3.5.2 Natural IgM (nIgM)

nIgM antibodies are able to recognise a broad range of antigens, including self-antigens. Binding to these antigens is believed to be low affinity and high avidity due to the pentameric structure of most nIgM antibodies (Baumgarth *et al*., 2005; Manson *et al*., 2005). The pentameric structure enables nIgM to bind 10 antigens simultaneously, resulting in the molecule having strong complement activation capabilities by the interaction with C1q (Boes *et al*., 1998). Furthermore, nIgM has the ability to directly neutralise invading pathogens, promote phagocytosis and influence the adaptive immune response (Ochschenbein and Zinkernagel, 2000; Manson *et al*., 2005; Rapaka *et al*., 2010).

1.3.5.3 Natural IgG (nIgG)

nIgG was previously considered to be non-reactive. However, recent research identified that nIgG antibodies were able to bind to pathogen-bound lectins, such as ficolins and MBLs (Panda and Ding, 2015). This recognition was shown to enhance pathogen clearance by phagocytosis compared to clearance by lectin binding alone.

1.3.5.4 Natural IgA (nIgA)

Secretory nIgA antibodies have been isolated from human saliva. Quan *et al*. (1997) were able to confirm that these antibodies could recognise a variety of bacterial antigens and elucidated their protective role in innate immunity. These antibodies are believed to have proteolytic activity and have been implicated in the cleavage of HIV surface proteins and resistance to HIV infection (Planque *et* al., 2007; Lutz *et al*., 2009).

1.3.5.5 Adaptive IgM

Adaptive IgM antibodies are primarily produced during the initial stages of infection due to their high avidity towards antigens and ability to activate complement (Díaz-Zaragoza *et al*., 2015). These antibodies differ to nIgM in their increased specificity towards bacterial antigens (Kaveri *et al.,* 2012) and are produced by B2 subclass lymphocytes in the spleen and lymph nodes (Racine and Winslow, 2009). IgM exists mainly in the pentameric form, however monomeric IgM is present on the surface of B-cells (Schroeder and Cavacini, 2010). Following increased antigen stimulation, B-cells undergo a process known as class switching where the activated cells switch from producing the IgM isotype to the production of IgG and IgA. The basic characteristics of the antibody isotypes can be found in Table 1.5. These isotypes have increased antigen-specificity compared to IgM (Panda and Ding, 2015). Monospecific IgM molecules still remain in circulation, however, at much lower concentrations (Díaz-Zaragoza *et al*., 2015).

1.3.5.6 Adaptive IgA

Chronic colonisation with *P. aeruginosa* in CF patients has been associated with detection of elevated levels of secretory IgA in the mucosal secretions of the upper respiratory tract.

Table 1.5 Characteristics of the various antibody isotypes (adapted from Hoffman *et al*., 2016. Antibody structures created with Biorender.com)

Characteristic	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**IgM**	**IgA**
Size (kDa)	146	146	165	146	970	160
Structure						
Polysaccharide antigens	+	+++	+/-	+/-	++	++
Protein antigens	++	+/-	++	++	+	+
Classical pathway activation	++	+	+++	-	++++	-
Serum concentration mg/ml	9	3	1	0.5	1.5	2.1

IgM monomeric and pentameric structures and IgA monomeric and dimeric structures are presented.

+ relative recognition of antigen or response initiation

– unable to recognise antigen or initiate response

Secretory IgA is mainly present in the dimeric form (Table 1.5) (Breedveld and van Egmond, 2019). These antibodies do not trigger an inflammatory response; however, it has been hypothesised that IgA functions to prevent access to the epithelial cells lining the airway (Aanaes *et al*., 2013). High titres of serum IgA are also associated with chronic *P. aeruginosa* infection and are responsible for the protection of the upper airways (Brett *et al*., 1990). Specific serum IgA can be produced in response to infection and it has been implicated in the inflammatory and phagocytic processes (Wolfe *et al*., 2007). Unlike secretory IgA, serum IgA is mainly present in the monomeric form (Table 1.4) (Breedveld and van Egmond, 2019).

1.3.5.7 Adaptive IgG

Elevated specific IgG can be indicative of chronic infection (Stanley *et al*., 1984). IgG antibodies have been implicated in the protection of the lower airways (Brett *et al*., 1990). The IgG isotype consists of four subtypes: IgG1, IgG2, IgG3 and IgG4. The subtypes vary in their ability to activate the complement system, the recruitment of immune cells, their target antigens and the flexibility of their hinge regions (Table 1.4, Hoffman *et* al., 2016). Elevated levels of IgG1, 2 and 3 have been observed in bronchiectasis patients with chronic respiratory infection when compared to healthy individuals (Hill *et* al., 1998). Increased susceptibility to respiratory infections is often associated with IgG subclass deficiencies (Cinetto *et al*., 2018). Deficiencies in IgG3 and IgG4 are most commonly associated with chronic airways diseases and correlate with an increased incidence of pneumonia and bronchitis, worse lung function and an increase in exacerbation frequency (Kim *et al*., 2016).

1.4 Inhibitory antibodies

1.4.1 Resistance to serum-mediated killing

Several mechanisms employed by serum resistant bacteria to evade killing by the complement system have been identified. Such mechanisms include: binding complement system regulators, which can inhibit activation of proteins at several stages of the complement cascade; alteration of surface antigen structures, preventing antibody binding and the production of a capsule or long O-antigen chains, preventing MAC access to the cell surface (Rautemaa and Meri, 1999). *P. aeruginosa* is known to produce elastases and alkaline proteases, which are able to degrade host immunoglobulins and other complement proteins, hindering direct killing of the bacteria and reducing opsonisation and phagocytic killing (Lambris *et al*., 2008; Laarman *et al*., 2012).

1.4.2 The history of inhibitory antibodies

Serum factors with the ability to inhibit the bactericidal effect of complement and thus promoting bacterial survival during infection have been described since the early 1900's. In 1901, working with *Vibrio metschnikovii*, Neisser and Wechsberg observed that high concentrations of immune sera were unable to elicit a bactericidal effect against the homologous strain whereas in smaller doses, complete killing was achieved. This later became known as the Neisser-Wechsberg phenomenon (Pandit, 1923). The authors hypothesised that the excess antibody present within the serum reduces bactericidal activity due to insufficient complement ligands for the antibody (Thjøtta, 1920).

Waisbren and Brown (1966) also discovered the presence of a blocking factor in chronically infected patient sera. This blocking factor had the ability to inhibit serum mediated killing by normal healthy sera. The authors identified that the factor was strain specific, heat stable, able to persist within the patients and only found in patients with chronic infections. The inhibition of bactericidal activity was reported against several Gram-negative bacteria including: *P.*

aeruginosa, Aerobacter aerogenes, Escherichia coli, Salmonella Typhimurium, *Salmonella java* Group B and *Proteus mirabilis.* The authors hypothesised that the blocking factor may be an antibody due to its heat-stable and specific nature.

1.4.3 The role of IgG

In the early 1970's, the same phenomenon was observed for 2 patients presenting with upper urinary tract infections (Taylor, 1972). An anti-bactericidal factor specific for the infecting Gram-negative isolate was detected in patient sera and was subsequently identified as IgG. An antagonistic role was proposed for the inhibitory IgG whereby the IgG antibodies outcompete bactericidal IgM for bacterial surface antigens (Taylor, 1972). Further work by Guttman and Waisbren (1975) also confirmed IgG as the inhibitory factor observed in chronic *P. aeruginosa* infections.

More recently, an increased concentration of IgG2 in both chronically infected non-CF bronchiectasis patient sera and urosepsis patient sera has been associated with the inhibition of serum-mediated killing of *P. aeruginosa* and uropathogenic *E. coli* (UPEC), respectively (Wells *et al.,* 2014; Coggan *et al*., 2018). IgG3 has been implicated in the blocking of the fungicidal activity of protective IgG1 and IgG2a against the fungus *Cryptococcus neoformans* (Nussbaum *et al*., 1996). A study by Clerc *et al.* (2017) reported that elevated IgG4 titres in CF patients correlated with increased *Staphylococcus aureus* colonisation. A link between increased IgG4 and hypergammaglobulinemia was suggested, however the inhibitory potential of this elevated IgG4 was not examined.

1.4.4 The role of other immunoglobulin isotypes

Inhibitory antibodies belonging to other isotypes have also been observed. Griffiss and Bertram (1977) established that IgA could inhibit the bactericidal effect of IgG and IgM in infections caused by *Neisseria meningitidis*. IgA inhibitory antibodies have also been detected against *Pasteurella haemolytica* (now *Mannheimia haemolytica*) in cattle (MacDonald *et al.*, 1983). IgM has also been implicated in the inhibition of serum-mediated killing of *Salmonella enterica* Serovar Typhimurium (Trebicka *et al.,* 2013).

1.4.5 Targets of inhibitory antibodies

Work by Goh *et al.* (2016) demonstrated that high titres of LPS-specific IgG, IgA and IgM can all inhibit serum-mediated killing of *S.* Typhimurium even though anti-LPS IgG and IgM are bactericidal. LPS, specifically the O-antigen, has been identified as the target of inhibitory IgG2 in non-CF patients chronically infected with *P. aeruginosa* (Wells *et al*., 2014). Similarly, O-antigen specific inhibitory IgG2 has been detected in sera from urosepsis patients infected with UPEC (Coggan *et al.*, 2018). Glucuronoxylomannan, which is the major constituent of capsular polysaccharide, has been identified as the target of the inhibitory IgG3 targeting *C. neoformans* (Nussbaum *et al*., 1996).

Unlike *E. coli*, *S.* Typhimurium and *P. aeruginosa*, members of the genus *Neisseria* produce lipooligosaccharide (LOS) rather than LPS. There is currently no literature confirming LOS as a target of inhibitory antibodies. However, outer membrane proteins, protein III (Rmp) and protein IV (RmpM), have been identified as targets for *N. gonorrhoeae* and *N. meningitidis*, respectively (Rice *et al*., 1986; Munkley *et al*., 1991). More recently, inhibitory IgG targeting the lipoproteins Lip H:8 and Laz of *N. meningitidis* have been identified (Ray *et al.*, 2011).

1.4.6 Current proposed mechanism for *P. aeruginosa* inhibitory antibodies in chronic lung infections

The current proposed mechanism of action of inhibitory antibodies in chronic *P. aeruginosa* infection (Figure 1.10) hypothesises that high titres of IgG2, with specificity towards the LPS O-antigen, forms a physical barrier distal to the surface of the bacteria. This prevents the access of other protective antibodies and complement proteins to their surface related targets and leads to the failure of MAC insertion into the cell membrane and the inhibition of cell lysis (Maclennan *et al.*, 2010; Wells *et al.*, 2014). Wells *et al*. (2014) were able to demonstrate that inhibitory IgG2 specifically targets the O-antigen produced by the colonising *P. aeruginosa* strain. Serum containing inhibitory antibody could still elicit a bactericidal effect against strains isolated from other patients, which is indicative of serotype specificity of the antibodies. The density of O-antigen, the production of branched chain O-antigen and O-antigen modification have been implicated in serum resistance (Onsere *et al*., 2015). However, the association with inhibitory antibody is yet to be elucidated.

1.4.7 *P. aeruginosa* vaccine development and the implication of inhibitory antibodies

Although the screening of potential *P. aeruginosa* vaccine candidates began in the early 1970's and had promising outcomes, a licenced vaccine is still unavailable for the prevention of *P. aeruginosa* infections (Alexander and Fisher, 1970; Baker *et al*., 2019). Vaccine development has been particularly problematic due to: the highly adaptive nature of *P. aeruginosa*, which itself is impacted by the variable host conditions at different sites of infection; complications arising from the immunocompromised status of the patients who are most likely to be infected by the bacterium; and the lack of suitable animal models of disease (Worgall, 2012). It has been

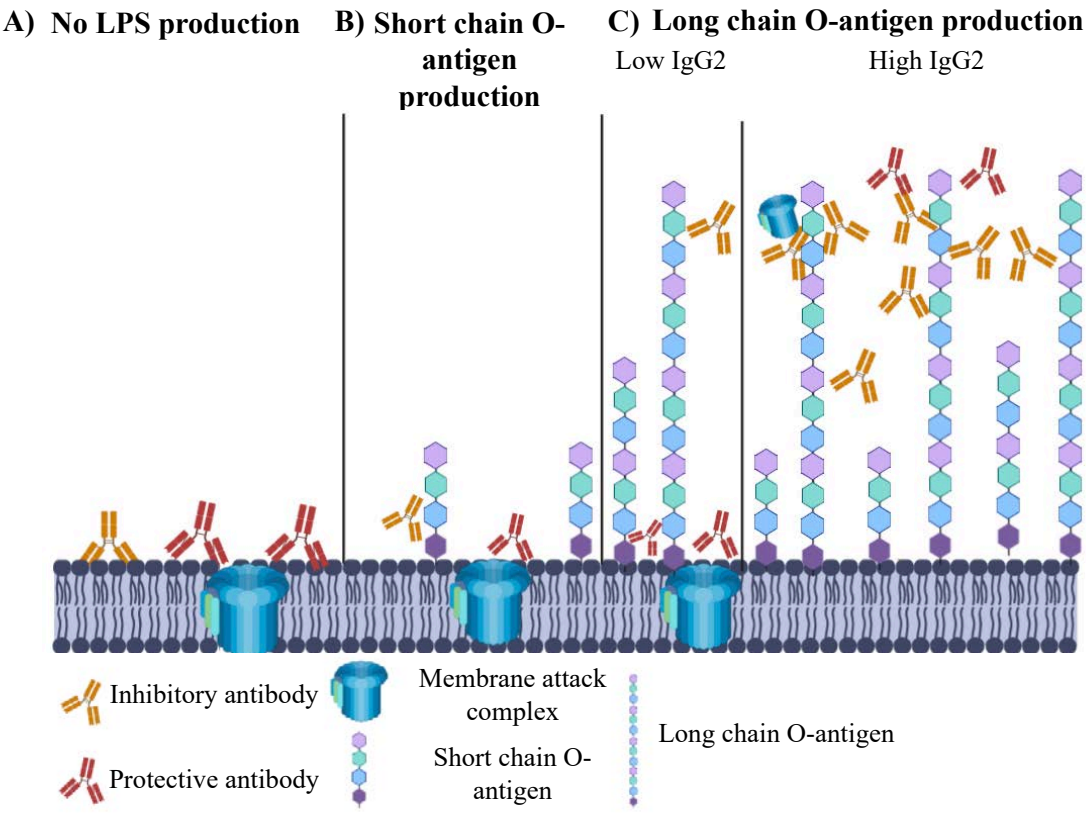


Figure 1.10 Proposed mechanism of inhibitory antibodies

A) In the absence of LPS production, protective antibodies are not hindered from accessing bacterial cell surface antigens and successfully recruit the complement system to the cell membrane. This ultimately results in pore formation through the insertion of the membrane attack complex and cell lysis. **B**) The production of short chain O-antigen does not block protective antibody access to cell surface antigens and complement recruitment by inhibitory antibodies might still occur at a distance that still permits membrane attack complex insertion into the membrane. **C**) In the presence of long chain O-antigen and low inhibitory antibody titres, protective antibody might still be able to make contact with cell surface antigens. However, in the presence of long chain O-antigen and high titres of inhibitory antibody, protective antibodies are blocked from reaching the cell surface. Complement recruitment by inhibitory antibodies occurs too far away from the surface and the membrane attack complex is unable to insert into the cell membrane.

highlighted that vaccination strategies might need to vary depending on the type of infection caused by *P. aeruginosa* and the immune status of the patient (Worgall, 2012).

1.4.7.1 Passive vaccination strategies

Passive vaccination strategies are used to provide immediate immunity and involve the administration of preformed antibodies directed against *P. aeruginosa* antigens including; LPS, pili, flagella, PcrV and exotoxin A (Casadevall, 2002; Sharma *et al*., 2011). It is thought that this strategy would be more suitable for patients with underlying immune defects that hinder the activation of a humoral response or for patients at high-risk of infection including; burns, cancer and intensive care patients, where colonisation occurs too rapidly to allow protection to develop through active immunisation strategies (Hemachandra *et al*., 2001; Sharma *et al*., 2011). However, passive immunisation does not confer long-term immunity and protection might only last for weeks to months (Casadevall, 2002).

1.4.7.2 Active vaccination strategies

Active vaccination strategies involve the administration of bacterial derived antigens in order to stimulate a long-lasting humoral or cellular immune response (Baxter, 2007). Potential candidates for vaccine development have included: O-antigens, flagellar proteins, alginate, outer membrane proteins and live-attenuated *P. aeruginosa* strains (Priebe and Goldberg, 2014). It is believed that an active immunisation strategy would be more suitable to prevent *P. aeruginosa* colonisation in patients who are more susceptible to developing chronic infections such as CF and bronchiectasis patients. However, there is still insufficient clinical evidence to support the use of vaccines for these patients (Johansen and Gøtzsche, 2015).

Animal studies with O-antigen vaccines have found that these vaccines are able to elicit the production of protective antibodies (Pier, 2007). However, the diversity of *P. aeruginosa* O-antigens makes it difficult to produce a vaccine that can confer immunity to all serotypes, with polyvalent vaccines being unsuccessful at providing full immunity (Pier, 2003; Priebe and Goldberg, 2014). It is believed that vaccines based on the O-antigen might not be suitable for CF patients as the *P. aeruginosa* strains infecting these patients stop expressing the O-antigen and exhibit a mucoid phenotype, prompting research into whether alginate can stimulate a protective immune response (Pier *et al*., 2004). Monoclonal antibodies to alginate have been generated that confer protection against mucoid and non-mucoid strains of *P. aeruginosa* and a conjugate vaccine consisting of flagellin and the mannuronic acid epitope of alginate has also been successful in stimulating the production of protective antibodies against mucoid and non-mucoid strains in animal studies (Pier *et al*., 2004; Campodónico *et al*., 2011).

Outer membrane proteins (OMPs) are more conserved between strains of *P. aeruginosa*, which makes them appealing vaccine candidates. Several studies have shown that immunisation with the OMPs OprF and OprI elicits a strong humoral response in mice and humans (Priebe and Goldberg, 2014; Hassan *et al*., 2018; Adlbrecht *et al*., 2020). Genetic approaches have also been used to identify alternative vaccine candidates that are conserved, cell surface exposed and are not homologous to host-antigens. Screening of these antigens also revealed that vaccines containing a combination of antigens involved in various stages of the infection cycle can increase the survival rate in murine models of infection (Biaconi *et al*., 2019).

It has been suggested that an effective *P. aeruginosa* vaccine should activate both the humoral and cellular immune response (Worgall, 2012). Live-attenuated vaccines have been shown to

provide immunity through the stimulation of protective antibodies, IL-17 secretion and neutrophil recruitment (Priebe *et al*., 2008). The outer membrane protein, OprL; the type 3 secretion system translocation protein, PopB; and the pyoverdine outer membrane receptor, FpvA have also been shown to stimulate an antibody-independent IL-17 response (Wu *et al*., 2012). However, vaccines that elicit a neutrophil response might not be suitable for all patients. An IL-17 inducing vaccine could promote inflammation in CF and non-CF bronchiectasis patients who already have a heightened inflammatory response. This could have a negative impact on these patients and exacerbate the disease (Wu *et al*., 2012).

1.4.7.3 Implication of inhibitory antibodies in vaccine design

Many of the studies into passive vaccination strategies have developed antibodies that target the O-antigen of *P. aeruginosa* (Sharma *et al*., 2011). Although active vaccination strategies appear to have moved away from developing vaccines containing O-antigen, vaccines containing live-attenuated strains of *P. aeruginosa* with a smooth-LPS phenotype still expose the immune system to the O-antigen, which can stimulate a humoral response. As inhibitory antibodies target the O-antigen, this must be taken into consideration during vaccine development as the aetiology of inhibitory antibodies has yet to be determined (Wells *et al*., 2014). It could be hypothesised that a vaccine containing O-antigen might stimulate the production of inhibitory antibodies, which could lead the prevention of *P. aeruginosa* clearance in susceptible patients. Other surface exposed proteins have been identified as targets for inhibitory antibodies in other bacterial species and the O-antigen has not been confirmed as the only target of inhibitory antibodies in *P. aeruginosa* infections (Rice *et al*., 1986; Munkley *et al*., 1991; Wells *et al*., 2014). Therefore, it could be hypothesised that other antigens included in the vaccines might also induce the production of inhibitory antibody. As such, the inhibitory

potential of the antibodies produced following vaccination should be also be assessed during the evaluation of animal studies and clinical trials.

1.5 Plasmapheresis

1.5.1 The plasmapheresis procedure

Plasmapheresis (also known as therapeutic plasma exchange) is a medical procedure which is used to remove pathogenic molecules from circulation. Plasma is separated from other blood components, substituted with a replacement fluid and returned to the patient (Figure 1.8) (Reeves and Winters, 2014). Examples of replacement fluid include: fresh frozen plasma; 5% albumin solution; 5% albumin-frozen plasma mix and 4% succinylated gelatin (Shemin *et al*., 2007; Aguirre-Valencia *et al*., 2019). Albumin is the most commonly used replacement fluid due to the reduced risk of complications (Reeves and Winters, 2014). There are two main methods of plasma separation; centrifugation and filtration (Winters, 2012). The filtration method can comprise of one or two filtration steps (Figure 1.11). In procedures employing one filtration step, the plasma is removed and substituted with replacement fluid. If two filtration steps are implemented, a plasma fractioner is used to specifically remove macromolecules and the filtrate can either be returned to the patient or substituted with replacement fluid (Reeves and Winters, 2014). Evidence has shown that 65 to 70% of the target molecule can be removed following the standard 1 to 1.5 plasma volume exchange (Williams and Balogun, 2014).

1.5.2 The use of plasmapheresis in disease management

The use of this procedure to manage disease has been extensively reviewed by Nguyen *et al.* (2012). Such diseases include: thrombotic microangiopathies, autoimmune disorders, Wilson's disease and post-ABO incompatible organ transplantation. Patients with acquired thrombotic

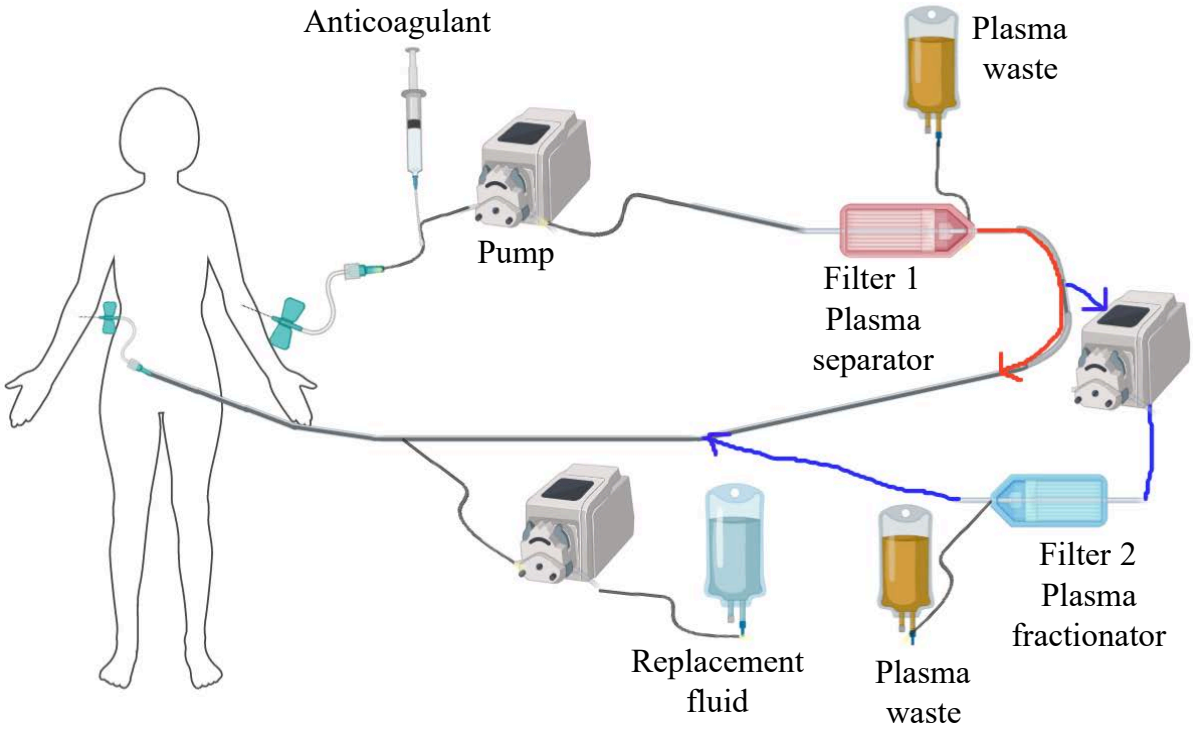


Figure 1.11 Plasmapheresis using the filtration procedure

The filtration procedure for plasma exchange can encompass the use of one or two filtration systems. Procedures with one filter (red arrow) uses a plasma separator to extract the plasma, which contains the pathogenic molecules, from the blood. The plasma is discarded and the remaining blood constituents are returned to the patient along with replacement fluid. Procedures with two filters uses the plasma separator to extract the plasma, which is then passed through the plasma fractionator (blue arrow) to selectively remove larger molecules. The filtered plasma can then be returned to the patient, with the addition of replacement fluid if required. (Adapted from Williams and Balogun, 2014; created with Biorender.com).

thrombocytopenic purpura often undergo plasma exchange to remove autoantibodies, which cause increased blood clotting in patients by inhibiting the cleavage of von Willebrand factor (Nguyen *et al*., 2012).

Plasma exchange for these patients has increased the survival rate from <10% to 80–90% (Kremer Hovinga *et al*., 2017). There is a general consensus that the use of plasma exchange as a treatment for autoimmune disease can improve disease status and reduce morbidity and mortality for the majority of patients (Aguirre-Valencia *et al*., 2019). Minor complications can arise due to the use of plasma exchange, particularly when albumin is not used as the replacement fluid (Shemin *et al*., 2007). Several studies assessing plasma exchange-related complications have found that no deaths have occurred as a result of the treatment (Shemin *et al*., 2007; Ara *et al*., 2017; Knaup *et al*., 2018; Aguirre-Valencia *et al*., 2019; Maheshwari *et al*., 2019).

1.5.3 Plasmapheresis as a novel therapeutic procedure for infection

The use of plasmapheresis for the removal of inhibitory antibody from two patients with non-CF bronchiectasis and chronic *P. aeruginosa* infection has recently been reported (Wells *et al*., 2017). Both patients had previously received multiple courses of alternating broad-range antibiotics, which had failed to improve disease symptoms; experienced respiratory failure; and were deemed unfit to receive lung transplants. Plasmapheresis was used as a novel rescue therapy with the hypothesis that the removal of inhibitory antibodies from the serum would improve the disease status of the patients by enabling the clearance of *P. aeruginosa* by their immune systems.

The patients received the treatment for four hours a day over five days. Plasma was separated from the other blood components, using the filtration method with a single filter, and substituted with an albumin and electrolyte replacement fluid (Figure 1.11). As the removal of plasma removes the majority of immunoglobulins from the blood, healthy donor immunoglobulin was provided for five days post-treatment (Wells *et al*., 2017).

The authors observed a significant decrease in serum inhibitory antibody titre following plasmapheresis and the restoration of serum-mediated killing of the *P.* aeruginosa isolated from the patient. The authors monitored serum inhibitory antibody titres and *P. aeruginosa* colonisation for several months following treatment. They observed an increase in inhibitory titre over time that coincided with the return of *P. aeruginosa* in sputum samples after approximately three months. The authors reported an improvement in the quality of life and a reduction in both hospitalisation frequency and antibiotic use for both patients following plasmapheresis. The return of high titres of inhibitory antibodies indicated that patients might need to receive the treatment periodically in order to maintain these improvements. Further studies are still required to assess the complication risks and to evaluate the efficacy of the use plasmapheresis as a therapeutic treatment for patients with inhibitory antibody.

1.6 *P. aeruginosa* genomics

As highlighted previously, the ability of *P. aeruginosa* to inhabit a variety of environmental and clinical niches is attributed to the large, flexible genome (Klockgether *et al*., 2011). The introduction of next-generation sequencing technologies and advances in bioinformatics have provided a useful tool to obtain whole genome sequences, which have been used to

simultaneously compare the genomes of hundreds of strains of *P. aeruginosa* in order to improve our understanding of this bacterium (Besser *et al*., 2018; Freschi *et al*., 2019).

The first complete reference genome sequence was obtained for strain PAO1 in 2000, followed by PA14 in 2006, LESB53 in 2009, PA7 in 2010 and PAK in 2019 (Stover *et al*., 2000; Lee *et al*., 2006; Winstanley *et al*., 2009; Roy *et al*., 2010; Cain *et al*., 2019). Hundreds of draft and complete genome sequences are also available (https://www.ncbi.nlm.nih.gov/genbank/). Comparative analysis of genomes has enabled the core genome of *P. aeruginosa* to be defined. Early studies with a relatively small number of genomes (n = < 25) identified that the core genome represented ~90% of the total genome (Wolfgang *et al*., 2003; Kung *et al*, 2010; Valot *et al.,* 2015; Subedi *et al*., 2018). However, studies comparing 389 and 1311 genomes have revealed that the core genome represents much less of the genome at 17.5% and 1.2%, respectively (Freschi *et al*., 2015; Freschi *et al.,* 2019).

Phylogenetic analysis of *P. aeruginosa* isolates based on the core genomes initially identified 3 main groups (Stewart *et al*., 2014; Freschi *et al*., 2015). These studies revealed that approximately 80% of isolates belong to group 1, which also contains the reference strains PAO1, LESB53 and PAK; approximately 17% of isolates belong to group 2, which contains the reference strain PA14; and approximately 3% of isolates belong to the phylogenetically distant group 3, which contains the reference strain PA7 (Stewart *et al*., 2014; Freschi *et al*., 2015). Recently, strains belonging to two further groups (groups 4 and 5) have been identified, which are located phylogenetically between groups 1-2 and group 3 (Freschi *et al*., 2019). Strains of *P. aeruginosa* can also be genetically typed using multi-locus sequence typing (MLST), which is based on the allelic profile of the 7 housekeeping genes: *acsA, aroE, guaA,*

mutL, nuoD, ppsA and *trpE* (Curran *et al*, 2004). MLST is also a useful tool for phylogenetic and evolutionary analyses of a bacterial population and can be used to understand the epidemiology of outbreaks (Belén *et al*., 2014).

Analysis of the PAO1 genome revealed that this strain contains an increased number of genes that are associated with colonisation, environmental sensing, metabolism, drug efflux, virulence and regulation, which enables the bacterium to survive in a variety of environments (Stover *et al*., 2000; Kung *et al*., 2010). The accessory genome of *P. aeruginosa* contains genes that are present in some but not all strains and commonly encodes genes that are associated with antibiotic resistance, surface antigen modification, virulence and metabolism, allowing the bacterium to adapt to specific environments (Kung *et al*., 2010). These genes are often acquired through horizontal gene transfer and are inserted into regions of genomic plasticity, which are commonly located near core genome tRNAs (Mathee *et al*., 2008; Kung *et al*., 2010). Genetic elements contributing to the accessory genome include: plasmids, prophage, pathogenicity islands and transposons (Kung *et al*., 2010).

1.7 Transposon-directed insertion site sequencing

1.7.1 Transposons

Transposons (transposable elements) can be defined as genetic elements that are able to move between positions within the genome (Muñoz-Lopéz and García-Pérez, 2010). Transposons were first described and implicated in the control of gene expression in maize by Barbara McClintock in the 1950's (McClintock, 1950). Since then, transposable elements have been identified in almost all prokaryotic and eukaryotic organisms (Pray, 2008). These elements have been found to make up 45% of the human genome (Klein and O'Neil, 2018), between 4 and

60% of the currently available vertebrate genomes (Sotero-Caio *et al*., 2017) and between 14 and 85% of plant genomes (Ma *et al*., 2019). Retrotransposons are class I transposable elements and require the transposon to be transcribed into messenger RNA prior to transposition (Wicker *et al*., 2018). DNA transposons are class II transposable elements that can move around the genome via a transposase enzyme encoded within the transposon. Transposon sequences are flanked by inverted repeat transposase recognition sequences that enable the transposon to be excised from one position and become re-ligated at another position within the genome (Muñoz-Lopéz and García-Pérez, 2010).

1.7.2 Transposons as genetic tools

Several techniques have utilized transposons in combination with high-throughput sequencing in order to assess bacterial fitness under particular conditions and to link genotypes to phenotypes (van Opijnen and Camilli, 2013; Yang *et al*., 2017). Such techniques include: insertion sequencing, transposon sequencing, high-throughput insertion tracking by deep sequencing and transposon-directed insertion-site sequencing (Goodman *et al*., 2009; van Opijnen *et al*., 2009; Gawronski *et al*., 2009; Langridge *et al.,* 2009). The protocols for these techniques follow the same basic workflow. However, the main differences between the protocols can be found in the methods of transposition, DNA fragmentation and the preparation of DNA for sequencing (Barquist *et al*., 2013).

Transposon-directed insertion-site sequencing (TraDIS) is a high-throughput technique that has been used to sequence high-density transposon mutant libraries and allows the whole genome to be screened for genes essential for survival under specific conditions (Langridge *et al.,* 2009). The technique involves the purification of pooled-library genomic DNA, which is then

fragmented and DNA fragments containing both transposon and genomic sequences are amplified using a PCR based method to create a sequencing library. Transposon-containing fragments are then sequenced and aligned to a reference genome to identify the position of the insertion (Figure 1.12). Regions of the genome containing no transposon insertions are considered essential, as insertions within these regions are lethal to the cells and will not be represented during the sequencing process (Langridge *et al.,* 2009).

Relatively small transposon libraries have previously been made in *P. aeruginosa* strains PA01 and PA14 (Jacobs *et al*., 2003; Lewenza *et* al., 2005; Liberati *et al*., 2006). However, in recent years, larger libraries consisting of approximately 1,000,000 mutants have been constructed (Table 1.6) (Lee *et al*., 2015; Poulsen *et al*., 2019). Such libraries have been used to determine the essential genome of *P. aeruginosa* strains and to identify conditionally essential genes, such as those required for the survival in the presence of antibiotics, sputum or serum (Juhas, 2015).

Transposomes™ consisting of a transposon containing an antibiotic resistance marker flanked by 19-base-pair inverted repeat Tn5 transposase recognition sequences and the Tn5 transposase are commercially available (Lucigen, UK). These systems are based on the Tn5 transposon systems found in Gram-negative bacteria, which has been modified to create a hyperactive Tn5 transposase with an increased transposition efficiency up to 1000-fold greater than that of the original transposase (Goryshin and Reznikoff, 1998). The availability of these systems provides a useful tool for the construction of large-scale transposon insertion mutant libraries.

1.8 Thesis theme and aims

Non-CF bronchiectasis patients chronically infected with *P. aeruginosa* often experience poor

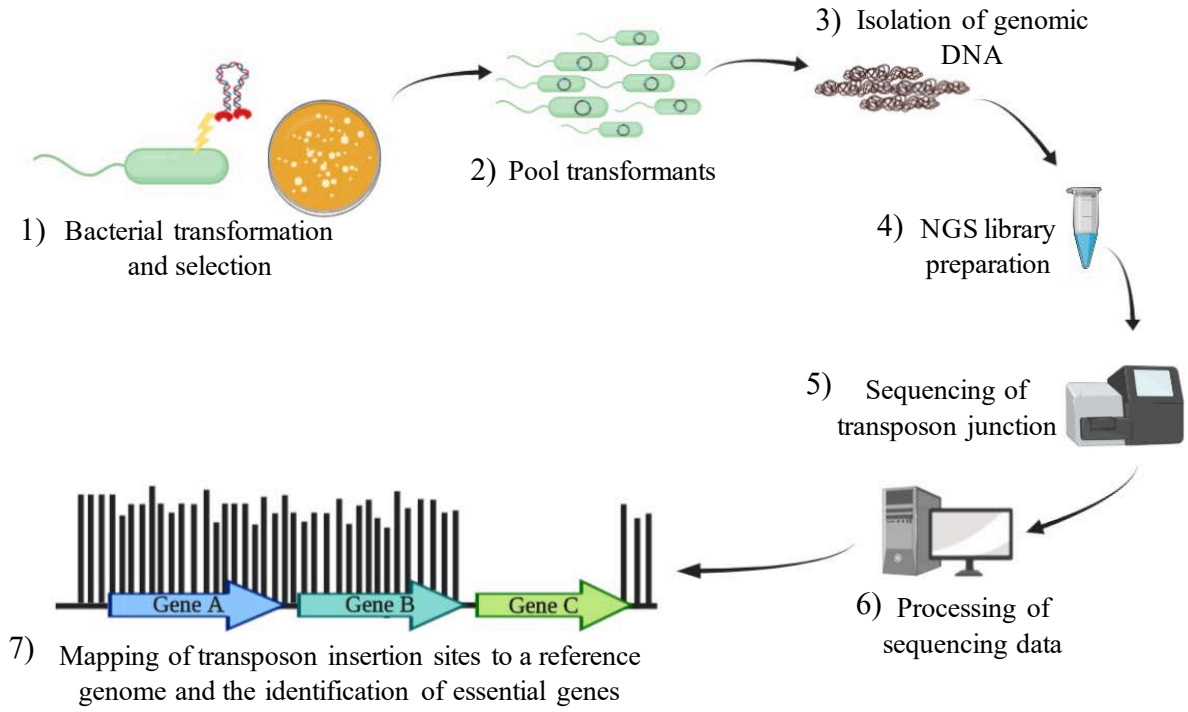


Figure 1.12 An overview of the workflow of a TraDIS experiment

1) The transposome is introduced into the cell by electroporation and upon activation by intracellular Mg^{2+}, the transposase randomly inserts the transposon into the genome. Transformed mutants can be selected for by growing transformants on LB plates containing the corresponding antibiotic. **2)** Transposon mutants are pooled and **3)** genomic DNA is isolated. **4)** Genomic DNA is fragmented by shearing and transposon containing DNA fragments are enriched for using a PCR based method to create a next generation sequencing (NGS) library. **5)** The transposon-genome junctions are sequenced and **6)** the sequencing data is processed to remove: short DNA sequences; those that do not contain transposon sequences; and primer, Illumina barcode and transposon introduced sequences prior to **7)** mapping of the transposon insertion sites to the reference genome and identifying essential genes.

Table 1.6 Existing *P. aeruginosa* transposon insertion libraries

Strain	Approximate number of mutants	method	Reference
PAO1	30100	Conjugation	Jacobs *et al*., 2003
PAO1	18500	Conjugation	Lawenza *et al*., 2005
PA14	34176	Conjugation	Liberati *et al*., 2006
PA14	300000	Conjugation	Skirnik *et al*., 2013
PAO1	1000000	Conjugation	Lee *et al*., 2015
BL23 (eye isolate) **BWH005 (urine isolate)** **BWH013 (lung isolate)** **BWH015 (urine isolate)** **CF77 (lung isolate)** **PS75 (environmental isolate)** **PA14 (wound isolate)** **X13273 (blood isolate)** **19660 (eye isolate)**	5000000	Conjugation	Poulsen *et al*., 2019

lung function and increased exacerbation frequencies resulting in increased hospital admissions and a poor quality of life (Chalmers *et al*., 2012; Wells *et al*., 2014). The control of *P. aeruginosa* infection in non-CF bronchiectasis patients relies heavily on antibiotic regimens and the high level of antibiotic resistance exhibited by *P. aeruginosa* poses a severe threat to the management of infection in these patients (Aliberti *et al*., 2016). A subset of these patients also produce inhibitory antibodies, which further hinders the control of infection (Wells *et al*., 2014). Non-CF bronchiectasis patients often require lung transplants. However, receiving a lung transplant is not always possible. Plasmapheresis has been used as a last resort therapy to remove inhibitory antibodies from circulation in order to improve the disease status of two patients who were deemed unfit to received lung transplants. Plasmapheresis resulted in a decrease in the bacterial load in the lungs and lowered the requirement for antibiotic therapy (Wells *et al*., 2017). However, as the treatment has never been used to treat patients with inhibitory antibody, the effect of removing these antibodies on the *P. aeruginosa* population in the lung remain unknown. The mechanism of action of these inhibitory antibodies is not completely understood.

P. aeruginosa rarely causes disease in healthy individuals (Sadikot *et al*., 2005). Most work has focused on cell-mediated *P. aeruginosa* clearance mechanisms in healthy individuals (Lovewell *et al*., 2014). However, previous work has reported *P. aeruginosa* isolates from chronic lung infections that are sensitive to the bactericidal activity of healthy sera suggesting that antibody also has a crucial role in protecting against *P. aeruginosa* infection (Wells *et al*., 2014). A more in-depth understanding of the role of antibodies in controlling this bacterium will hopefully help in the development of improved treatments for patients with chronic *P. aeruginosa* infection. Therefore, the aims of this thesis were as follows:

1. To understand how the removal of inhibitory antibodies using plasmapheresis affects the *P. aeruginosa* population within the lungs of chronic bronchiectasis patients by phenotypically and genotypically characterising longitudinal isolates obtained both prior to and post-treatment. This will help to both determine whether or not strain replacement occurs as a result of the treatment and help to evaluate the effectiveness of plasmapheresis as a viable treatment option for patients with inhibitory antibody and chronic *P. aeruginosa* infection.

2. To further elucidate the mechanism of serum-mediated killing of *P. aeruginosa* in healthy individuals by assessing the importance of the presence of antibody and active complement in human sera and to provide an insight into the development of inhibitory antibodies by assessing the inhibitory potential of antibody purified from the serum of healthy individuals.

3. To develop a tool to provide further insight into the mechanism of inhibitory antibodies by constructing a high-density transposon mutant library in a clinical strain of *P. aeruginosa* isolated from a patient with inhibitory antibodies. This will allow the essential genes to be determined for a clinical respiratory isolate of *P. aeruginosa*. The transposon mutant library can be used for future evaluation of gene essentiality in the presence of serum containing inhibitory antibody, which might help to provide improved treatments for these patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

2.1.1 *P.* aeruginosa isolation from sputum

P. aeruginosa strains B1, B3, B4, B6, B7 and B10 were isolated previously from the sputum of patients by streaking the sputum onto a chocolate blood agar plate to single colonies. Colonies were subsequently checked for growth on *Pseudomonas* isolation agar (Sigma, UK) (Wells *et* al., 2014; Whitters, 2017). *P. aeruginosa* strains BN1- and BN3- were isolated from patient sputum by streaking the sputum on LB agar to single colonies. Unless multiple colony morphologies were detected, a single colony was also checked for growth on *Pseudomonas* isolation agar (PIA). If growth was observed on the PIA plate, the corresponding colony on the LB plate was stocked at -80°C (see section 2.1.4). Strains were also confirmed as *P. aeruginosa* through whole genome sequencing (see section 2.4.3). All bacterial strains used in this study are listed in Table 2.1.

2.1.2 Preparation of growth media

Growth media were prepared and sterilised by autoclaving at 121°C for 15 min prior to use. LB agar was left to cool prior to the addition of antibiotics and mixed thoroughly before pouring. M9 minimal media was autoclaved prior to the addition of filter sterilised supplements. The composition of growth media and antibiotics can be found in Tables 2.2 and 2.3.

2.1.3 Preparation of bacterial overnight cultures

Overnight cultures were prepared in 50 ml conical centrifuge tubes by inoculating 5 ml Luria-Bertani (LB) broth with a single colony picked from a LB agar plate with a 1 μl loop. Cultures were incubated overnight at 37°C with orbital shaking at 180 rpm.

Table 2.1 List of bacterial strains used in this study

Strain Name	Species	Serotype	Origin	Extra details
BW25113	*E. coli*	N/A	Datsenko and Wanner, 2000	Parental strain of Keio
BW25113-Cm	*E. coli*	N/A	Discuva ltd, Cambridge, UK	Chloramphenicol resistant
PAO1	*P. aeruginosa*	O5	Stover *et al*., 2000	Laboratory reference isolate Burn wound
PA14	*P. aeruginosa*	O10	Lee *et al*., 2006	Laboratory reference isolate Burn wound
BN1-1	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1, Freeman Hospital, Newcastle, UK	Isolated 13/12/12
BN1-2	*P. aeruginosa*	O1	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 13/6/13
BN1-3	*P. aeruginosa*	O1	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 31/10/13
BN1-4	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 10/12/14
BN1-5	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 22/1/15
BN1-6	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 20/2/15

BN1-7	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 20/2/15
BN1-8	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 7/5/15
BN1-9	*P. aeruginosa*	O6	Sputum sample Non-CF bronchiectasis patient PN1 Freeman Hospital, Newcastle, UK	Isolated 1/6/15
BN3-1	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 14/3/13
BN3-2	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 14/3/13
BN3-3	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 17/4/13
BN3-4	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 24/8/13
BN3-5	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 14/11/13
BN3-6	*P. aeruginosa*	Probable O3	Sputum sample	Isolated 12/2/14

			Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	
BN3-7	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 13/3/14
BN3-8	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 14/3/14
BN3-9	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 14/3/14
BN3-10	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 10/7/14
BN3-11	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 22/10/14
BN3-12	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 30/10/14
BN3-13	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 15/1/15
BN3-14	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient	Isolated 15/1/15

			PN3 Freeman Hospital, Newcastle, UK	
BN3-15	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 05/3/15
BN3-16	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 16/4/15
BN3-17	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3 Freeman Hospital, Newcastle, UK	Isolated 02/7/15
BN3-18	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient PN3, Freeman Hospital, Newcastle, UK	Isolated 08/9/15
B1	*P. aeruginosa*	Probable O3	Sputum sample Non-CF bronchiectasis patient P1, Queen Elizabeth Hospital, Birmingham, UK	Isolated 31/8/10
B3	*P. aeruginosa*	O3	Sputum sample Non-CF bronchiectasis patient P3, Queen Elizabeth Hospital, Birmingham, UK	Isolated 20/7/10
B4	*P. aeruginosa*	O9	Sputum sample Non-CF bronchiectasis patient P4, Queen Elizabeth Hospital, Birmingham, UK	Isolated 11/5/10
B6	*P. aeruginosa*	010	Sputum sample Non-CF bronchiectasis patient P6, Queen Elizabeth	Isolated 09/6/10

			Hospital, Birmingham, UK	
B7	*P. aeruginosa*	O5	Sputum sample Non-CF bronchiectasis patient P7, Queen Elizabeth Hospital, Birmingham, UK	-
B10	*P. aeruginosa*	Strain not sequenced	Sputum sample Non-CF bronchiectasis patient P8, Queen Elizabeth Hospital, Birmingham, UK	Isolated 14/9/10
KO1	*P. aeruginosa*	O1	Keratitis isolate	Winstanley *et al*., 2005
KO2	*P. aeruginosa*	O2	Keratitis isolate	Winstanley *et al*., 2005
KO3	*P. aeruginosa*	O3	Keratitis isolate	Winstanley *et al*., 2005
KO4	*P. aeruginosa*	O4	Keratitis isolate	Winstanley *et al*., 2005
KO6	*P. aeruginosa*	O6	Keratitis isolate	Winstanley *et al*., 2005
KO8	*P. aeruginosa*	O8	Keratitis isolate	Winstanley *et al*., 2005
KO10	*P. aeruginosa*	O10	Keratitis isolate	Winstanley *et al*., 2005
KO11	*P. aeruginosa*	O11	Keratitis isolate	Winstanley *et al*., 2005

Table 2.2 The composition of media used in this study

Media	Composition
LB broth	1% NaCl, 1% bacto tryptone, 0.5% yeast extract in MilliQ ultrapure H_2O
LB agar	1% NaCl, 1% bacto tryptone, 0.5% yeast extract in MilliQ ultrapure H_2O, 1.5% agar
0.3% LB agar	1% NaCl, 1% bacto tryptone, 0.5% yeast extract in MilliQ ultrapure H_2O, 0.3% agar
M9 minimal medium	20 ml 5 x M9 salts (Sigma-Alderich), 200 µl 1 M $MgSO_4$, 10 µl 1 M $CaCl_2$, 0.02% casamino acids (BD Biosciences), 2 ml 20% glucose
SOC (Sigma)	20 g/L tryptone, 5 g/L yeast extract, 4.8 g/L $MgSO_4$, 3.603 g/L dextrose, 0.5 g/L NaCl, 0.186 g/L KCl
***Pseudomonas* isolation agar (Sigma)**	13.6 g/L agar, 1.4 g/L magnesium chloride, 20 g/L peptic digest of animal tissue, 10 g/L potassium sulfate, 0.025 g/L triclosan (Irgasan), 20 ml/L glycerol

Table 2.3 List of antibiotics, corresponding solvents, concentrations and storage conditions

Antibiotic	Solvent	Storage	Stock concentration	Supplier
Kanamycin	H_2O	-30°C	50 mg/ml	Sigma-Alderich
Chloramphenicol	Ethanol	-30°C	35 mg/ml	Merck
Trimethoprim	DMSO	Freshly made when required	50 mg/ml	Sigma-Alderich

2.1.4 Strain storage

For long term storage, strains were grown overnight as described in section 2.1.2 and 700 µl of culture was transferred into a cryotube. Glycerol (ThermoFisher) was added to a final concentration of 15%. Stocks were mixed by pipetting and stored at -80°C.

2.2 Bacterial analysis

All buffer compositions for the following assays can be found in Table 2.4

2.2.1 Bacterial colony morphology

Overnight cultures were diluted to 10^{-6} in LB broth to allow for single colony formation. Diluted cultures were plated as 5 µl spots, in triplicate, onto square LB plates and incubated overnight at 37°C.

2.2.2 Bacterial growth kinetics

Overnight cultures were diluted to an OD_{600} = 0.025 with LB broth and 150 µl aliquots of each culture were added in triplicate to the corresponding wells in black, clear bottom Costar 96-well assay plates (Corning Incorporated). Plates were sealed with a Breathe-Easy® sealing membrane (Sigma-Aldrich). An automated protocol using a CLARIOstar 96-well plate reader (BMG Labtech) was used to measure the OD_{600} of the cultures every 30 min for 16 h. Plates were incubated at 37°C with constant shaking at 200 rpm.

2.2.3 Swimming motility

Strains were grown overnight and a 1 µl loop was used to inoculate cultures into the centre of

Table 2.4 The composition of buffers used in this study

Buffer	Composition
10 x Phosphate buffered saline (PBS)	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in MilliQ ultrapure H_2O, pH 7.4
10 x Tris buffered saline (TBS)	24g Tris-base, 88g NaCl, to 1 L with MilliQ ultrapure H_2O, pH 7.6
Cell lysis buffer	1 M Tris, 2% SDS, 4% β-mercaptoethanol, pH 6.8
ELISA block buffer	1 x PBS pH 6.8, 1% bovine serum albumin (Sigma-Alderich) in MilliQ ultrapure H_2O
ELISA wash buffer	1x PBS pH 6.8, 0.05% Tween 20 in MilliQ ultrapure H_2O
ELISA dilution buffer	1 x PBS, pH 6.8, 0.05% Tween 20, 1% bovine serum albumin
Western blot block buffer	5% milk powder, 1 x TBS + 0.5% sodium azide in MilliQ ultrapure H_2O
Western blot wash buffer (TBS-T)	1 x TBS, 0.1% Tween 20 (Sigma-Alderich) in MilliQ ultrapure H_2O
Protein binding buffer	0.02 M sodium phosphate, pH 7.0 in MilliQ ultrapure H_2O
Protein elution buffer	0.1 M glycine-HCl, pH 2.7 in MilliQ ultrapure H_2O
Protein neutralising buffer	1 M Tris-HCl, pH 9.0 in MilliQ ultrapure H_2O
1 x TAE	40 mM Tris, 20 mM Acetate and 1 mM EDTA, pH 8.6
Protein G neutralising buffer	1 M Tris-HCl, pH 9.0
Protein G binding buffer	0.02 M sodium phosphate, pH 7.0
Protein G elution buffer	0.1 M glycine-HCl, pH 2.7
Protein G storage buffer	20% ethanol

a 0.3% LB agar plate in triplicate. Plates were incubated for 18 h at 37°C and the diameter of the swim colonies were recorded.

2.2.4 Biofilm formation

Overnight cultures of each strain were diluted to a final OD_{600} = 0.2 in fresh M9 medium. Seven 150 µl replicates of each strain were plated per column in Greiner-Bio-one U-bottom 96-well cell culture plates with one M9 only negative control per column. Plates were sealed with Breathe-Easy® sealing membranes and incubated at 37°C for 18 h in a static incubator.

Biofilm formation was quantified using a crystal violet staining protocol. Briefly, the liquid cultures were discarded and excess liquid was removed by tapping plates onto a paper towel. The plates were incubated at room temperature (RT) for 30 min with 150 µl of 0.1% crystal violet per well. The crystal violet was discarded and the plates were washed 6 times by submerging the plate into distilled H_2O. Excess liquid was removed as above and 150 µl of an 80% ethanol: 20% acetone solution was added to each well and the plates were incubated at room temperature for 30 min with agitation. The absorbance at OD_{595} was recorded using Multiskan™ GO Microplate Spectrophotometer (ThermoFisher). To standardise the absorbance readings, the average for the negative control wells was calculated and removed from each sample absorbance reading.

2.2.5 LPS purification

LPS was extracted from *P. aeruginosa* as previously described with modifications (Wells *et al*., 2014). Strains were grown overnight and standardised to 1 ml of OD_{600} = 1.0. Cell pellets were collected by centrifugation and resuspended in 100 µl of cell lysis buffer. Samples were boiled for 5 min, immediately frozen at -80°C for 5 min followed by boiling for a final 5 min

to ensure sufficient lysis of the cells. Samples with increased viscosity at this stage were boiled for up to 1 h. Samples were spun and the supernatants were transferred to fresh microcentrifuge tubes containing 5 µl of 5mg/ml Proteinase K and incubated at 60°C for 1 h. Samples to be analysed by enzyme-linked immunosorbent assay (ELISA) were heated at 95°C for 5 min. Samples to be analysed by Western immunoblot or silver stain were combined with Laemmli buffer (Sigma, UK) in a 1:1 ratio prior to heating. Samples were stored at -30°C.

2.2.5.1 Visualisation of LPS by silver stain

LPS was separated by SDS-PAGE using 10, 12 or 15 well 1.0 mm NuPAGE™ 4-12% Bis-Tris protein gels (ThermoFisher) and run with 1 x NuPAGE™ MES running buffer (Invitrogen). Gels were loaded with 5 µl BLUeye Pre-Stained Protein Ladder 10-245kDa (Geneflow) and 10 µl of each sample and ran at 180 V for 45 to 60 min. Gels were washed briefly with Milli-Q ultrapure H_2O and stained using a SilverQuest silver staining kit (ThermoFisher) using the fast stain protocol.

2.2.6 Outer membrane protein purification

Overnight cultures were diluted 1:100 into LB broth and cultures were grown to late log-phase (OD_{600} = 1.0). Cells were pelleted at 4°C by centrifugation at 10, 000 x *g* for 10 min followed by resuspension in 10 mM Tris buffer pH 7.4 and pelleted as above. The pellets were resuspended in 10 mM Tris buffer pH 7.4, 2 mM phenylmethylsulfonyl fluoride to prevent protein degradation. Cells were lysed by high pressure homogenisation (Biorupter Emulsiflex-C3) followed by centrifugation at 4°C at 6000 x *g* for 10 min to remove unbroken cells. The supernatant was syringe filtered into a new centrifuge tube using a 0.45 µm filter and the cell envelope was separated from the cytoplasmic fraction by centrifugation at 4°C at 30,000 x *g*

for 45 min. The envelopes were resuspended in 10 mM Tris Buffer pH 7.4, 2% (v/v) Triton X-100 (Sigma) and incubated at RT to allow the inner membranes to solubilise. Samples were centrifuged at 4°C at 30,000 x *g* for 45 min and pellets were resuspended in 1 ml 10 mM Tris buffer pH 7.4 and transferred into a microcentrifuge tube. Inner membranes were removed by a further two centrifugation and resuspension steps at 30,000 x *g* for 30 min with 1 ml 10 mM Tris buffer pH 7.4. Outer membranes were stored in 250 µl aliquots at -30°C.

2.2.7 Statistical analysis

The area under the curve (AUC), normality tests and statistical analyses were completed using GraphPad Prism version 8.

2.3 Analysis of sera

Blood samples were obtained from patients or healthy control donors a using vacutainer® system (Becton Dickson Ltd Oxford UK) and red cap blood collection tubes. Serum was separated by centrifugation at 4000 x *g* for 30 min at RT. Sera were stored in 0.5 ml or 1 ml aliquots at -80°C until required.

All buffer compositions for subsequent assays can be found in Table 2.4, sera can be found in Table 2.5 and antibodies can be found in Table 2.6.

2.3.1 Serum bactericidal assay (SBA)

Serum bactericidal assays were done as previously described (Wells *et al*., 2014). In brief, the equivalent of 1.5 ml OD_{600} = 0.6 of an overnight culture was pelleted by centrifugation at 3381 x *g* for 10 min in 1.5 ml microcentrifuge tubes. Pellets were washed in 1 ml of 1 x PBS, spun

Table 2.5 List of sera used in this study

Sample	Origin	Corresponding strain	Serum sample date
PN1	Patient, UK	BN1-	24/03/17
PN3	Patient, UK	BN3-	02/07/15
LATS	Patient, UK	N/A	03/11/16
LAWC	Patient, UK	N/A	25/05/17
LAPS	Patient, UK	N/A	14/08/17
LANC	Patient, UK	N/A	17/01/18
PCCF82	Patient, Australia	N/A	-
HCS1	Healthy donor, UK	N/A	31/7/17
HCS2	Healthy donor, UK	N/A	31/7/17
HCS3	Healthy donor, UK	N/A	31/7/17
HCS4	Healthy donor, UK	N/A	31/7/17
HCS5	Healthy donor, UK	N/A	31/7/17
HCS6	Healthy donor, UK	N/A	31/7/17
HCS7	Healthy donor, UK	N/A	31/7/17
Pooled-HCS	UK/Australia	N/A	Variable
Pooled-Patient	Equal volume combination of the above UK patient sera	N/A	-

Table 2.6 List of antibodies used in this study

Primary antibody	Dilution (ELISA/Western blot)	Product details
HCS / pooled-HCS	1:100/1:2000	N/A
Patient sera	1:100/1:5000	N/A
Baby rabbit complement	1:2000	S7764 Complement sera from rabbit
Secondary antibody		
Anti-human IgG -AP	1:2000/1:10000	Sigma A9544 Anti-human IgG (Fc specific)-AP produced in goat.
Anti-human IgG2	1:2000/1:10000	Sigma I9513 Monoclonal anti-human IgG2 (Fc specific) antibody produced in mouse
Anti-human IgA-AP	1:2000/1:10000	Sigma F5259 Anti-human IgA (α-chain specific)-AP produced in goat
Anti-human IgM-AP	1:2000/1:10000	Sigma A3437 Anti-human IgM (μ-chain specific)-AP produced in goat
Anti-human IgGAM-AP	1:10000	Sigma A3313 Anti-human Polyvalent immunoglobulins (α, γ and μ-chain specific)-AP produced in goat
Anti-rabbit IgG-AP	1:10000	Sigma A3687 Anti-rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat
Tertiary antibody		Anti-human polyvalent immunoglobulin
Anti-mouse IgG-AP	1:2000/1:10000	SouthernBiotech; Cat: 1030-04 Goat anti-mouse IgG human adsorbed-AP

and resuspended in 1 ml of 1 x PBS to form the stock culture. Stock cultures were diluted 1:10 and 5 µl was inoculated into 45 µl of neat or 50% patient serum, healthy control serum (HCS); or 45 µl of a 50:50 mix of patient and HCS (Figure 2.1A). The stock culture was serially diluted in 1 x PBS and plated as 10 µl spots in triplicate on to LB agar plates to determine the starting inoculum. Serum samples were incubated at 37°C shaking for 3 h and 10 µl of each sample was taken at 45 min and 3 h intervals, serially diluted to 10^{-6} in 1 x PBS, plated in triplicate and incubated overnight at 37°C to determine viable cell number after incubation in serum Figure 2.1B and C).

2.3.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Nunc-Immuno™ 96-MicroWell™, ThermoScientific) were coated with 1 µg/ml of LPS and incubated overnight at 4°C. Plates were washed 3 times with wash buffer, blocked with blocking buffer for 1.5 h at RT followed by 3 more washes. The primary antibody was prepared by diluting test sera 1:100 in dilution buffer and 100 µl of primary antibody was added to each well. The plates were incubated with primary antibody for 1 h at 37°C in a humid chamber, subsequently washed 3 times as above and then incubated with a 1:2000 dilution of anti-human IgG/IgA/IgM-AP or anti-human IgG2 secondary antibody for 1 h at 37°C in a humid chamber. Following incubation with secondary antibody, the plates with AP-conjugated antibody were washed a further 4 times and developed with SIGMA*FAST*™ *p*-Nitrophenyl phosphate substrate (Sigma-Aldrich). Antibody binding was quantified by recording the OD_{405} every 30 min. Plates with the IgG2 secondary antibody were washed 3 times and incubated for 1 h at 37°C with a 1:2000 dilution of anti-mouse IgG-AP. Plates were then washed and developed as above.

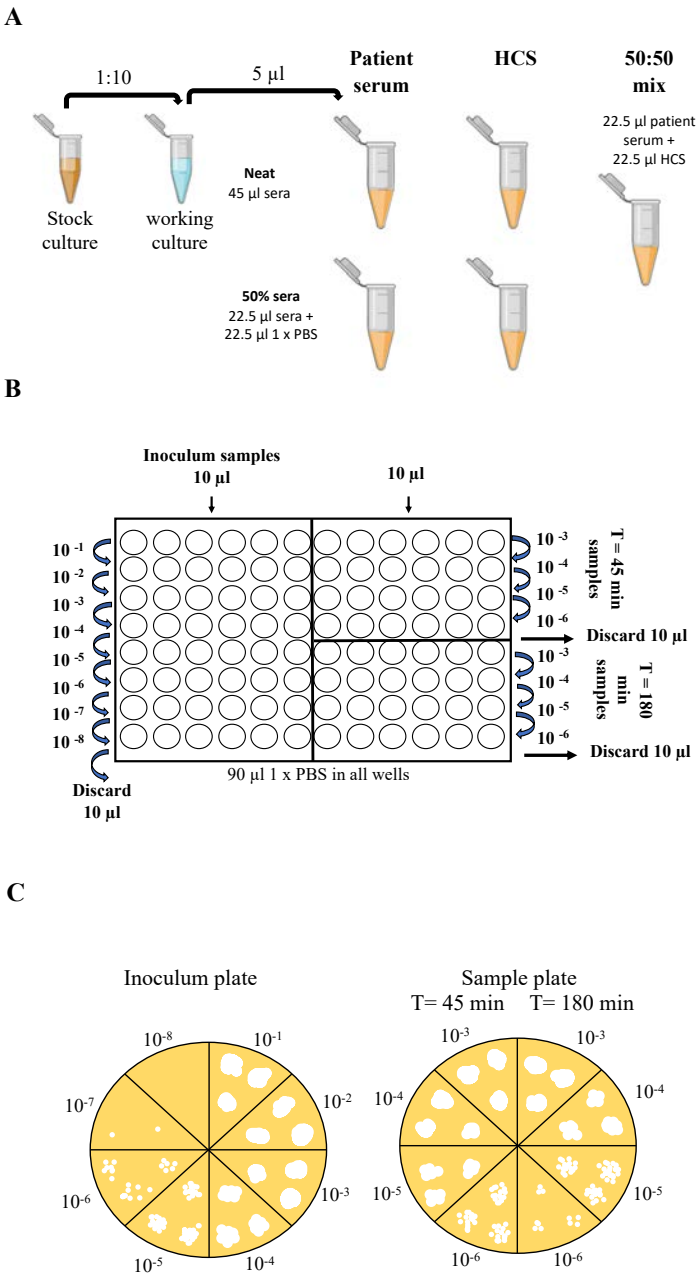


Figure 2.1 Schematic of a serum bactericidal assay

Preparation of sera and bacteria. The stock culture was diluted 1:10 in 1 x PBS to make the working culture. 45 µl of neat sera, sera diluted 50% in 1 x PBS or a 50:50 mix of patient and healthy sera (HCS) were added to 1.5 ml microcentrifuge tubes and inoculated with 5 µl of working culture. **B) Example dilution series of stock culture and time point samples.** The stock cultures were serially diluted from 10^{-1} to 10^{-8} in 1 x PBS. Time point samples at 45 min and 3 h had already been 1:100 diluted from the stock culture so samples were serially diluted from 10^{-3} to 10^{-6}. **C) Plating of serial dilutions using the Miles and Misra plating method.** 10 µl of each dilution was spotted into the corresponding section of a LB plate in triplicate. Following incubation overnight at 37°C, the number of colonies and the dilution of the sample were recorded and used to determine the change in viable cell number after incubation in sera.

2.3.3 Antibody purification

IgG antibodies were purified from sera using an automated protocol with an ÄKTA pure system and a 1 ml HiTrap™ Protein G HP column (GE Life Sciences) following the manufacturer's instructions (protocol 71-7001-00 AR). Sera was diluted 1:10 in binding buffer, the column was washed with 5 ml of MilliQ ultrapure H_2O and then equilibrated with 10 ml of binding buffer. Serum was passed through the column and then the column was washed with 10 ml of binding buffer. IgG antibodies were eluted with 5 ml of elution buffer. Eluted samples were collected in tubes containing 200 µl of neutralising buffer. The column was regenerated by passing through 10 ml of binding buffer and stored in 20 % ethanol. Buffer composition can be found in Table 2.4.

The eluted IgG samples were combined (total 5 ml) and buffer exchanged with 40 ml of 0.5 x PBS using a 5 kDa viva spin column. Samples were centrifuged at 3220 x *g* at 4°C until 1 ml of purified IgG remained. Collected samples were stored at 4°C.

2.3.4 Western Immunoblot

Following gel electrophoresis (see section 2.2.3.1**)**, LPS was transferred to a nitrocellulose or PVDF membrane using the Invitrogen™ iBlot™ 2 system (ThermoFisher) and membranes were blocked overnight at 4°C in blocking buffer. Membranes were incubated for 1.5 h at RT in primary antibody (1:2000 and 1:5000 dilution of HCS and patient serum in blocking buffer, respectively) with agitation, followed by three 5-min washes in 1 x TBS-T. Membranes were then incubated in the required secondary antibody for 1.5 h at RT (Table 2.6). Membranes incubated in an alkaline phosphatase (AP) conjugated secondary antibody were washed 4 times in 1 x TBS-T followed by a brief wash in MilliQ ultrapure H_2O prior to developing with 1 ml BCIP®/NBT-Purple Liquid Substrate System (Sigma). Membranes requiring a tertiary

antibody were washed 3 times in 1 x TBS-T and incubated in tertiary antibody for 1 h at RT and then washed and developed as described above.

2.4 Genetic analysis

2.4.1 Genomic DNA and plasmid DNA extraction

Genomic DNA was extracted using the RTP Bacteria DNA Mini kit (Stratec) following Protocol 2: Isolation of DNA from bacteria pellets (1 x 10^9 bacterial cells). Samples were eluted in 100 µl pre-warmed elution buffer (EB). The final incubation time was increased to 5 min and eluted samples were passed back through the column for a second time to increase the DNA yield. Plasmid DNA was isolated from overnight cultures using the QIAprep® Spin Miniprep Kit (Qiagen) following the quick start protocol. Plasmid DNA was eluted in 30 µl of pre-warmed EB as above.

2.4.2 Quantification of DNA

The DNA concentration in samples was determined using the Qubit dsDNA HS Assay kit as per the manufacturer's instructions using 1 µl of undiluted DNA. Samples were diluted 1:10 or 1:100 if the input concentration was outside the upper detection limit of the fluorometer.

2.4.3 Whole-genome sequencing

Whole genome sequencing of the BN1 and BN3 isolates was provided by MicrobesNG (http://www.microbesng.uk), using the Illumina next-generation sequencing technology offered by the standard whole genome service. A single colony for each strain was mixed in 100 µl of sterile 1 x PBS and transferred to a LB plate. The culture was spread over half of the plate to produce a lawn and streaked to single colonies over the second half of the plate to

check for contamination. Following growth overnight at 37°C, the lawn was collected with a sterile loop and transferred into cryogenic vials containing beads provided by MicrobesNG and sent to the facility at RT.

The B3 reference genome was provided by MicrobesNG using a combination of the Illumina short read and the Oxford Nanopore long read sequencing technologies offered by the enhanced genome service. A single colony of B3 was mixed into 200 µl of sterile 1 x PBS and 100 µl was then transferred into a 250 ml flask containing 50 ml of LB and grown to OD_{600} = 0.9. The remaining 100 µl was streaked onto a LB plate to check for contamination. Once the culture had reached the late exponential phase, the cells were pelleted by centrifugation at 5000 x *g* for 10 min, the supernatant was discarded and the pellet was weighed to confirm that there was between 300 to 600 mg of cells. The pellet was resuspended in 500 µl of the cryopreservant from the cryogenic vials containing beads provided by MicrobesNG and transferred back into the vial. The tube was kept at room temperature and sent to the facility to be sequenced.

2.4.4 Sanger sequencing

The sequence of the transposon encoding chloramphenicol resistance was determined using the Sanger sequencing service provided by the Functional Genomics, Proteomics and Metabolomics Facility (University of Birmingham, UK). The samples contained 10 ng of purified transposon DNA, 1 µM of primer and were made up to a final reaction volume of 10 µl with nuclease free H_2O. Four samples containing four individual TnCm primers were used to check the transposon sequence (Table 2.7).

2.4.5 PCR, gel electrophoresis and PCR purification

The transposon DNA was amplified from chromosomal DNA extracted from BW25113-Cm using the Phusion DNA polymerase protocol (New England Biolabs). The TnCm forward and reverse primers can be found in Table 2.7 and the reaction composition and PCR thermal profile can be round in Table 2.8.

PCR products were mixed in a 5:1 ratio with 5 x DNA loading buffer blue (Bioline) and separated by gel electrophoresis in 50 ml 1% agarose gels containing 1.5 µl Midori green (Nippon Genetics) in 1 x Tris-acetate-EDTA buffer (Table 2.4). Gels were run at 100 V for 1 h and visualised using the Syngene G:Box gel imaging system with the Genesys image capture software using the DNA agarose gel green pre-loaded protocol.

PCR products were purified using the QIAquick PCR purification kit using the microcentrifuge protocol (Qiagen). DNA was eluted in 20 µl of EB after a 5 min incubation at room temperature and passed through the column one more to increase DNA yield.

2.4.6 Serotype and multi-locus sequence type (MLST) analysis

Serotype and MLST analyses were done using PAst 1.0 (Thrane *et al*., 2016) available at (https://cge.cbs.dtu.dk/services/PAst/) and MLST 1.8 (Larsen *et al*, 2012) available at (https://cge.cbs.dtu.dk/services/MLST/). The whole genome sequence for each isolate in fasta format was used as the input file.

2.4.7 Pangenome analysis

Core gene alignments, gene presence-absence and core/accessory genome data for longitudinal isolates were generated using Roary (Page *et al*., 2015). Whole genome sequences in the general feature format were used as the input files and the programme was run using the

Table 2.7 List of primers used in this study

Primer	Sequence 5′ to 3′
TnCm forward	CTG-TCT-CTT-ATA-CAC-ATC-TAC-CGG-GTC-GAA-TTT-GC
TnCm reverse	CTG-TCT-CTT-ATA-CAC-ATC-TAC-CG
TnCm location forward	TTG-GAA-CCT-CTT-ACG-TGC-C
TnCm location reverse	ATA-AGC-GGA-TGA-ATG-GC
TnTm PCR-1 forward	GAG-TCG-ACC-TGC-AGG-CAT-GCA-AGC-TTC-AGG-GT
TKK. PCR-1 reverse	GAC-TGG-AGT-TCA-GAC-GTG-TGC-TCT-TCC-GAT-C
TnTm 6.1	AAT-GAT-ACG-GCG-ACC-ACC-GAG-ATC-TAC-ACT-CTT-TCC-CTA-CAC-GAC-GCT-CTT-CCG-ATC-TCG-TAC-GGC-AAG-CTT-CAG-GGT-TGA-GAT-GTG-TA
TnTm 7.4	AAT-GAT-ACG-GCG-ACC-ACC-GAG-ATC-TAC-ACT-CTT-TCC-CTA-CAC-GAC-GCT-CTT-CCG-ATC-TTA-GCT-AGG-CAA-GCT-TCA-GGG-TTG-AGA-TGT-GTA
TnTm 8.2	AAT-GAT-ACG-GCG-ACC-ACC-GAG-ATC-TAC-ACT-CTT-TCC-CTA-CAC-GAC-GCT-CTT-CCG-ATC-TGC-ATG-CAT-GCA-AGC-TTC-AGG-GTT-GAG-ATG-TGT-A
TnTm 9.2	AAT-GAT-ACG-GCG-ACC-ACC-GAG-ATC-TAC-ACT-CTT-TCC-CTA-CAC-GAC-GCT-CTT-CCG-ATC-TAT-CGA-TCG-AGC-AAG-CTT-CAG-GGT-TGA-GAT-GTG-TA

Table 2.8 PCR reaction components and thermocycler conditions for the amplification of the TnCm transposon DNA

PCR	50 µl reaction components	Thermocycler conditions
TnCm transposon	For 50 µl reaction: 10 µl – GC buffer 1 µl – 10 mM dNTPs 2.5 µl – 10 mM F primer 2.5 µl – 10 mM R primer 100 ng – BW25113-Cm chromosomal DNA 0.5 µl – Phusion DNA polymerase to 50 µl – nuclease free-H20	**Initial denaturation**: 98°C -1 min **30 cycles**: 98°C – 15 secs 64°C – 20 secs 72°C- 2 mins **Final extension:** 72°C- 7 mins 4°C – hold

following command: roary -e –mafft -p 6 *.gff. The option '-e –mafft' creates a core gene alignment using the tool MAFFT, the option -p 6 runs the command using 6 threads and *.gff runs the command using all files with the file extension '.gff'. Gene presence/absence data was visualised using Phandango (Hadfield *et al.*, 2018; available at http://jameshadfield.github.io/phandango/#/). Core gene SNP distance matrices were produced from the core gene alignment output from Roary using the programme snp-dists (https://github.com/tseemann/snp-dists).

2.4.8 Phylogenetic analysis

Phylogenetic trees were generated from the core gene alignment output from Roary using RaxML 8.2.10 (Stamatakis, 2014) using the following command: raxmlHPC-PTHREADS-AVX -T 6 -f a -p 12345 -s core_gene_alignment.aln -x 12345 -# 100 -m GTRGAMMA. Trees were visualised and edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5 *P. aeruginosa* transposon library construction

2.5.1 Antibiotic susceptibility testing

Antibiotic susceptibility testing was done using an agar dilution method. Briefly, overnight cultures of test strains were adjusted with LB broth to an OD_{600} = 2.0 and 10 µl of culture was streaked onto LB plates containing 0 to 640 µg/ml of test antibiotic. Plates were incubated at 37°C for approximately 20 h and checked for visible growth.

2.5.2 TnCm transposome construction

A total of 100 ng of purified transposon DNA (described in section 2.4.5) was combined with 2 µl EZ-Tn5™ transposase (Lucigen, USA) and 2 µl glycerol, briefly vortexed and incubated at 25°C for 30 min in a thermocycler and stored at -30°C until required (Figure 2.2A).

2.5.3 TnTm transposome construction

A total of 100 ng of EZ-Tn5™ <DHFR-1> transposon DNA obtained from the EZ-Tn5™ insertion kit (Lucigen) was combined with 2µl EZ-Tn5™ transposase (Lucigen) and 2 µl glycerol, briefly vortexed and incubated at 25°C for 30 min in a thermocycler and stored at -30°C until required.

2.5.4 Electrocompetent cells (old method)

Overnight cultures were diluted 1:100 into 200 ml LB broth and incubated at 37°C with orbital shaking at 180 rpm. When cultures had reached an OD_{600} = 0.5, 100 ml of culture was harvested by centrifugation at 5000 x *g* for 10 min at 4°C. Pellets were washed 4 times by resuspending in cold 300 mM sucrose buffer (*P. aeruginosa*) or cold 10% glycerol (*E. coli*) and centrifuged as above. The pellets were combined after the 4th centrifugation step. After the final wash, cells were resuspended in a final volume of 100 µl buffer and kept on ice until required.

2.5.5 Electrocompetent cells (new method, large scale)

P. aeruginosa isolates were made electrocompetent as previously described with modifications (Choi *et al*., 2006). Briefly, 100 ml of overnight culture was split into two 50 ml conical centrifuge tubes and cells were pelleted by centrifugation at 16,000 x *g* at RT for 2 min and the supernatant was discarded. The pellets were washed twice by resuspension in 25 ml of room temperature 300 mM sucrose buffer and pelleted by centrifugation at 10,000 x *g* for 30 min. The resulting pellets were combined and resuspended in final volume of 1 ml 300 mM sucrose buffer and split into 100 µl aliquots in 1.5 ml microcentrifuge tubes (Figure 2.2B steps 1 to 3).

2.5.6 Transformation of electrocompetent cells and library construction

Electrocompetent cells were incubated on ice for 30 to 60 min prior to the addition of 0.2 µl

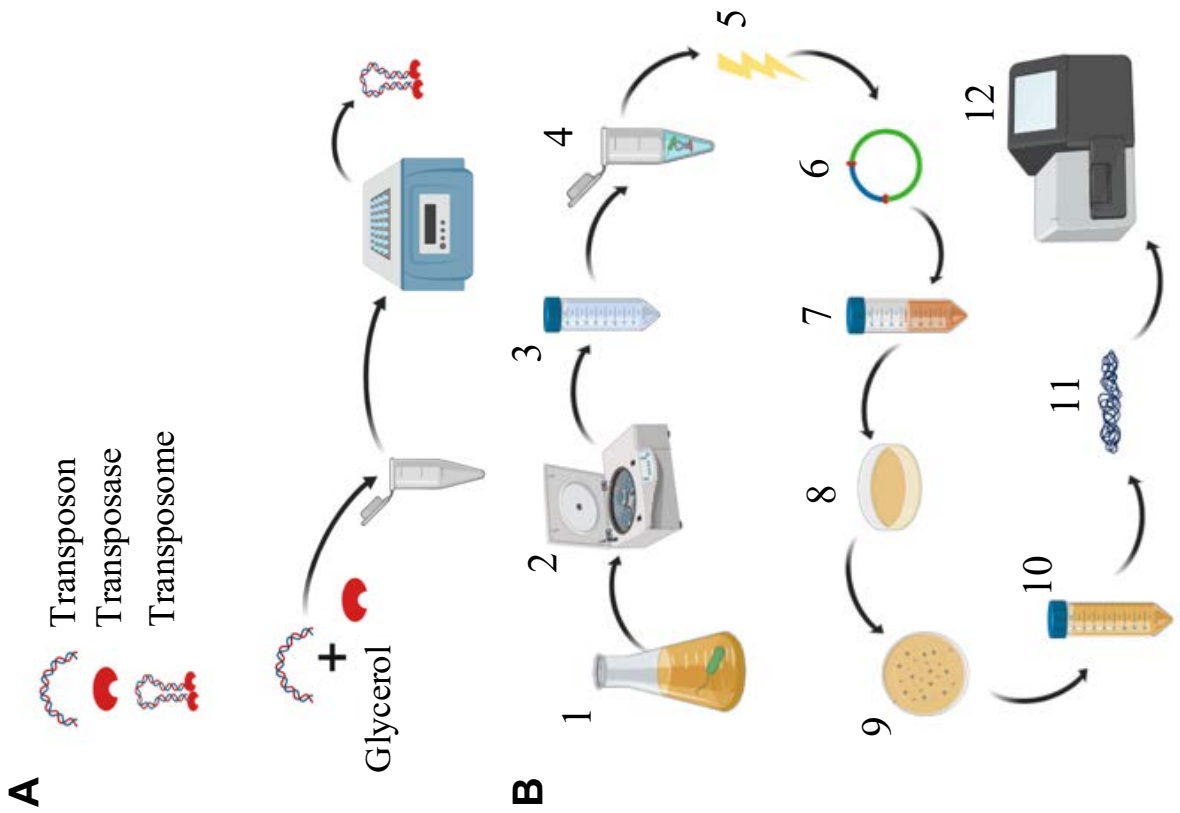


Figure 2.2 Schematic of the transposon library construction protocol

A) Construction of a transposome. EZ-Tn5 transposon DNA was combined with EZ-Tn5 transposase and glycerol in a 0.2 ml PCR tube and mixed by pipetting. The mixture was incubated at 25°C for 30 min in a thermocycler and stored at -30°C. **B) Basic workflow for creating a *P. aeruginosa* transposon mutant library. 1)** 100 ml of parental strain was cultured overnight. **2 and 3)** Bacterial cells were pelleted by centrifugation and washed with twice with 25 ml room RT 300 mM sucrose. Pellets were resuspended in a final volume of 1 ml and divided into 100 µl aliquots and chilled on ice. **4)** 0.2 µl of transposome was mixed with 100 µl of competent cell and incubated on ice. **5 and 6)** The transposon was introduced into the cell by electroporation and incorporated into the chromosome by the transposase. **7 and 8)** Cells were recovered in 1 ml of pre-warmed SOC medium for 2 h at 37°C with orbital shaking at 180 rpm. 50 µl aliquots of undiluted culture were spread onto LB plates containing the appropriate antibiotic and incubated for up to 24 h at 37°C. **9 and 10)** Plates were checked for colonies and colonies were pooled with 1 ml LB broth per plate and transferred into 50 ml conical centrifuge tubes. **11 and 12)** Genomic DNA was extracted from the pooled library, fragmented and transposon containing fragments were amplified using transposon specific primers. An Illumina MiSeq was used to sequence the prepared sequencing library.

transposome. Cells were incubated with the transposome on ice for 30 to 60 min prior to electroporation with an Eppendorf Eporator in a 1 mm gap cold electroporation cuvette (*E.* coli 1.8 kV, *P. aeruginosa* 2.5 kV). 1 ml of pre-warmed SOC medium (Sigma, UK) was added to samples directly after electroporation and cells were transferred into 50ml conical centrifuge tubes and incubated at 37°C shaking for 2 h. 100 µl of 1:10 diluted (*E. coli*) or 50 µl undiluted (*P. aeruginosa*) culture was spread onto LB plates and LB plates containing the appropriate antibiotic using L-shaped spreaders. Plates were incubated at 37°C for 17 to 24 h until the colonies were sufficiently large but not touching. Colonies on each plate were pooled by resuspending in 1 ml of LB broth with a L-shaped spreader and transferred into a 50 ml conical centrifuge tube. Glycerol was added to a final concentration of 20% to the pooled library before freezing at -80°C. Prior to sequencing, all library pools were thawed on ice, combined and mixed thoroughly before dividing into 45 ml and 1 ml aliquots (Figure 2.2 steps 4-10).

2.6 Sequencing of the *P. aeruginosa* transposon insertion library

2.6.1 Sequencing library preparation

Genomic DNA was extracted from the pooled library and quantified as outlined in sections 2.4.1 and 2.4.2. The DNA was diluted to 1 µg in a total of 500 µl of nuclease-free H_2O and sheared into fragments consisting of approximately 200 bp with a Biorupter (Diagenode) using the settings: 13 cycles of 30 s on, 90 s off at low intensity. Samples were transferred into 1.5 ml microcentrifuge tubes, condensed to 50 µl using an Eppendorf vacuum concentrator 5301, made up to 55.5 µl with nuclease-free H_2O and stored overnight at 4°C.

DNA was prepared for sequencing using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's instructions. The volume of

AMPure XP beads (Beckman Coulter) used for the initial size selection of adapter-ligated DNA was based on the fragment size of 200 bp. An additional PCR amplification step was used after size selection to amplify DNA fragments that contained the transposon-genomic DNA junction (Table 2.9, PCR1). The PCR1 reaction was cleaned up following the 'cleanup of PCR reaction' protocol with an elution volume of 17 µl in EB. A second PCR was used amplify the DNA and to incorporate an Illumina barcode and an inline barcode to introduce flow cell and sequencing primer binding sites and to stagger the starting position of the transposon during sequencing (Figure 2.3; Table 2.9, PCR2). The PCR2 reaction was cleaned up following the 'clean-up of PCR reaction' protocol.

2.6.2 Sequencing library quantification and sequencing

The concentration of DNA in the sequencing library was determined by qPCR using the Mx3005P qPCR System and the KAPA Library Quantification Kit (Illumina) following the kit instructions for the universal qPCR master mix with 1:50,000 and 1:500,000 diluted samples. Reaction compositions and the thermocycler profile can be found in Table 2.9.

The sequencing cartridge (150 cycle v3 cartridge, Illumina) and buffer were defrosted at RT and stored at 4°C until required. The sequencing library DNA was diluted to 8 nM in nuclease-free H_2O and 1.5 µl of each library was combined in a 1.5 ml microcentrifuge tube to make the pooled amplified library (PAL). 5 µl of 0.2 M NaOH, 3 µl of TE, pH 8.0 and 2 µl of PAL were combined in a separate 1.5 ml microcentrifuge tube and denatured following the Illumina protocol. Denatured 20 pM PhiX was added to the sample to a final concentration of 5% for quality control. The sample was loaded into the cartridge and sequenced using the Illumina MiSeq system.

Table 2.9 PCR reaction compositions and thermal profiles for sequencing library preparation

PCR reaction	Reaction composition	Thermocycler profile
PCR1	15 µl adapter ligated DNA 25 µl NEBNext Q5 Hot Start HiFi PCR Master Mix 5 µl nuclease-free H_2O 2.5 µl TnTm PCR1 forward (Table 2.6) 2.5 µl TKK PCR1 reverse (Table 2.6)	**Initial denaturation**: 98°C -3 min **10 cycles**: 98°C – 15 s 65°C – 30 s 72°C- 30 s **Final extension:** 72°C- 1 min 4°C – hold
PCR2	15 µl adapter ligated DNA 25 µl NEBNext Q5 Hot Start HiFi PCR Master Mix 5 µl nuclease-free H_2O 2.5 µl TnTm 6.3, 7.4, 8.2 or 9.2 forward (Table 2.6) 2.5 µl Illumina index (NEBNext multiplex oligos for Illumina index primers set 1)	**Initial denaturation**: 98°C -3 min **20 cycles**: 98°C – 15 s 65°C – 30 s 72°C- 30 s **Final extension:** 72°C- 1 min 4°C – hold 4°C – hold
qPCR	2 µl sample DNA (or DNA standard/ nuclease-free H_2O for negative control) 2 µl nuclease-free H_2O 6 µl SYBR + primer master mix	95°C – 5 min **35 cycles:** 95°C – 30 s 60°C – 30 s **Melt curve** 65-95°C– 30 s each

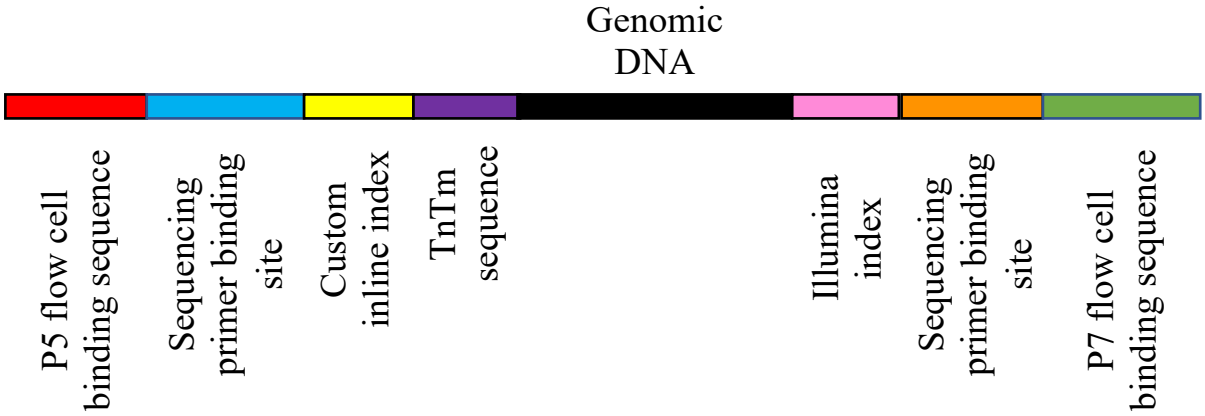


Figure 2.3 Composition of DNA fragments following sequencing library preparation

Amplification during PCR2 incorporates several sequences that allow the fragments to bind to the sequencing flow cell, provide sequencing primer binding sites and barcodes to allow sampled to be identified. The P5 flow cell binding sequence and sequencing primer binding site form part of the custom inline index primer, which adds these sequences to the DNA fragment. The custom inline index also staggers the transposon sequence start site to increase the diversity of the clusters during sequencing. The P7 flow cell binding sequence and another sequencing primer binding site are incorporated by the Illumina index, which also acts as a sample identifier (adapted from Goodall, 2018).

2.7 Sequencing data analysis

2.7.1 The location and frequency of the transposon insertion sites

The sequencing data was processed using scripts provided by Emily Goodall, which were modified from the scripts initially written by Ashley Robinson. The data processing pipeline of these scripts are described in detail in her thesis (Robinson, 2017; Goodall, 2018; Goodall *et al*., 2018). The scripts were modified to contain the corresponding *P. aeruginosa* reference genome size. Briefly, sequencing reads without the custom inline index were removed from the dataset and reads that contained the inline index had the sequence removed. These reads were then checked for the transposon sequence. Reads passing these checks had the transposon sequence removed and reads that were less than 20 bp were removed from the data set. Reads that passed all of the control checks were aligned to the reference genome and custom scripts were used to calculate the number of reads that aligned to each position in the genome and to calculate insertion index scores for each gene by normalising the number and frequency of insertions per gene for gene length. The insertion frequency and position were visualised using the position depth count output file and the corresponding *P. aeruginosa* reference genome: B3 (unpublished), PAO1 (GCF_000006765.1), PA14 (GCF_000014625.1), PA7 (GCA_000017205.1), LESB53 (GCA_000026645.1) using The Artemis genome browser (Carver *et al*., 2012). The total number of unique insertion points and the average length between insertions were calculated using a custom R script provided by Mathew Milner.

2.7.2 Essential gene lists

Essential genes were predicted using custom R scripts kindly provided by Emily Goodall, which were modified from the initial scripts provided by the authors of Langridge *et al*. (Langridge *et al*., 2009; Goodall, 2018; Goodall *et al*., 2018).

CHAPTER 3

THE EFFECT OF PLASMAPHERESIS ON THE *PSEUDOMONAS AERUGINOSA* POPULATION IN THE NON-CYSTIC FIBROSIS BRONCHIECTASIS LUNG

3.1 Introduction

Previous research on patients with non-CF bronchiectasis found that approximately 20% of patients chronically colonised with *P. aeruginosa* produce inhibitory antibodies (Wells, *et al*., 2014). The presence of inhibitory antibody in sera prevented serum mediated killing of the corresponding *P. aeruginosa* isolates. The inhibitory effect was confirmed by mixing patient sera with healthy donor sera that was able to completely kill the *P. aeruginosa* isolates, which were then screened for bactericidal activity. Inhibitory sera were able to block the bactericidal activity of healthy donor sera (Wells, *et al*., 2014).

Following from this work, two patients (PN1 and PN3) were selected as candidates to receive plasmapheresis as a novel treatment to remove the inhibitory antibodies from circulation. Sera from these patients were able to block the bactericidal activity of healthy donor serum when serum mixes contained 30% and 10% patient sera, respectively (Wells *et al*., 2017). These patients were chosen due to the severity of the lung disease and plasmapheresis was used as a treatment of last resort. It was hypothesised that the removal of inhibitory antibodies would enable the immune systems of the patients to clear the *P. aeruginosa* and improve disease severity. Patient sera and sputum were analysed periodically following treatment and for up to 3 months following treatment, *P. aeruginosa* was not isolated from sputum. However, during this period inhibitory antibody titres increased and after 3 months high titres again correlated with the detection of *P. aeruginosa* in their sputum. The *P. aeruginosa* isolates that were detected in the sputum in the subsequent 6-12 months after plasmapheresis were stocked. However, the serum samples collected over the same period were only screened for the presence of inhibitory antibody against the *P.* aeruginosa isolate that the inhibitory antibodies were first detected against (Wells *et al*., 2017). For patient PN1, the original isolate was BN1-1; for patient PN3, the original isolate was B1.

Plasmapheresis has never been used for the management of infections in patients with inhibitory antibodies and it is unclear how this treatment would ultimately impact on the *P. aeruginosa* population in the lung. Previous research characterising the progression of *P. aeruginosa* lung infections in non-CF bronchiectasis patients revealed that the majority of patients are colonised by a single strain, which genetically adapts to the lung environment through a process known as within-host evolution (Woo *et al*., 2012; Hilliam *et al*., 2017). This frequently involves loss-of-function mutations affecting iron acquisition, motility, antibiotic resistance and biofilm formation (Hilliam *et al*., 2017). Similarly, *P. aeruginosa* isolated from the sputum of a single CF patient can belong to the same clonal lineage but exhibit variable phenotypes due to the selective pressures of the CF lung environment, including: nutrient availability, mucus viscosity, antibiotic therapy and host immune defences (Sousa and Pereira, 2014). Bronchiectasis patients can be co-infected with multiple strains of *P. aeruginosa*; although this occurs less frequently than single lineage infections with 10-30% of patients having a multi-lineage infection (Hilliam *et al*., 2017).

As the removal of inhibitory antibodies by plasmapheresis initially resulted in the inability to culture *P. aeruginosa* from sputum samples, it was hypothesised that if the patients were originally colonised by a single strain of *P. aeruginosa,* the decrease in bacterial load could facilitate strain replacement by creating a selective pressure on the resident organism. Alternatively, the same strain may persist in the host, in an immune privileged niche, and give rise to a recrudescent infection.

The aims of this chapter were to determine whether the removal of inhibitory antibodies by plasmapheresis promotes *P. aeruginosa* strain replacement in the lung and to identify any phenotypic changes that might have arisen due to this treatment that could have a negative

impact on the disease status of the patients. As such, a total of 9 isolates from patient PN1 and 18 isolates from patient PN3 that were obtained at various stages both before and after treatment were assessed (Table 3.1). The phenotypes and genotypes of the isolates obtained prior to plasmapheresis were compared to those of the isolates obtained after plasmapheresis. Together, these investigations will contribute to our assessment of the suitability of plasmapheresis as a routine treatment option for patients with inhibitory antibodies and to our understanding of the very nature of *P. aeruginosa* infections in patients with bronchiectasis.

3.2 Results

3.2.1 BN1 and BN3 colony morphology comparisons

It was hypothesised that if the strains isolated from patients following treatment belonged to the same clonal lineage as those present prior to treatment, the strains would have similar phenotypes. A simple indicator of phenotypic variation between strains is the observation of colony morphology differences. In order to determine whether there were any phenotypic differences between colonies, isolates were incubated overnight at 37°C on LB agar plates. Following incubation, the colony size, shape, colour and opacity for each isolate obtained from the same patient were compared.

Pre-plasmapheresis isolate BN1-1 formed large, irregular, non-mucoid colonies whereas pre-plasmapheresis isolates BN1-2 and BN1-3 formed small, circular, non-mucoid colonies. Post-plasmapheresis isolates BN1-4 to BN1-9 all formed small, circular, non-mucoid colonies. However, these colonies appeared larger than those produced by isolates BN1-2 and BN1-3 when grown on the same medium over the same period of time. Another colony difference was observed when the bacteria were subsequently prepared for serum bactericidal assays (See section 3.2.4). Isolates BN1-1, 4, 6, 8 and 9 produced a greenish yellow pigment, characteristic

Table 3.1 *P. aeruginosa* isolates obtained from each patient

Patient	Sputum isolate *	Isolation date	Pre- or post-plasmapheresis isolate	Plasmapheresis dates
PN1	BN1-1	13/12/2012	Pre	7/1/14
	BN1-2	13/06/2013	Pre	
	BN1-3	31/10/2013	Pre	26/8/14
	BN1-4	10/12/2014	Post	
	BN1-5	22/01/2015	Post	11/6/15
	BN1-6	20/02/2015	Post	
	BN1-7	20/02/2015	Post	
	BN1-8	07/05/2015	Post	
	BN1-9	01/06/2015	Post	
PN3	BN3-1	14/03/2013	Pre	3/10/14
	BN3-2	14/03/2013	Pre	
	BN3-3	17/04/2013	Pre	
	BN3-4	24/08/2013	Pre	
	BN3-5	14/11/2013	Pre	
	BN3-6	12/02/2014	Pre	
	BN3-7	13/03/2014	Pre	
	BN3-8	14/03/2014	Pre	
	BN3-9	14/03/2014	Pre	
	BN3-10	10/07/2014	Pre	
	BN3-11	22/10/2014	Post	
	BN3-12	30/10/2014	Post	
	BN3-13	15/01/2015	Post	
	BN3-14	15/01/2015	Post	
	BN3-15	05/03/2015	Post	
	BN3-16	16/04/2015	Post	
	BN3-17	02/07/2015	Post	
	BN3-18	08/09/2015	Post	

*Isolates were obtained from patient sputum periodically from 12-19 months prior to treatment and 1-12 months post treatment. Colonies were checked on *Pseudomonas* isolation agar and stocked. Strains with the same isolation date originate from the same sputum sample but exhibited a different colony morphology. All isolates were confirmed as *P. aeruginosa* by whole genome sequencing.

of pyoverdine production (Figure 3.1).

When comparing the colony morphologies of the isolates obtained from patient PN3, a mixture of colony morphologies were observed for both pre-plasmapheresis isolates BN3-1 to BN3-10, and for post-plasmapheresis isolates BN3-11 to BN3-18. Isolates formed small or very small colonies that were either round or slightly irregular and slight variations in colony colour and opacity could be seen (Figure 3.2). Unlike BN1 isolates, no pyoverdine production by BN3 isolates was observed.

Isolates obtained from PN1 formed relatively similar colonies and only slight variations were observed. The variation in colony morphology observed for the BN3 isolates does not definitively indicate that isolates are different strains of *P. aeruginosa* as these changes could be the result of differences in gene expression. The colony morphologies of isolates obtained from the same patient suggested that the patients could have been co-infected with two strains of *P. aeruginosa*. However, the minor variations in morphology could also be the result of within-strain mutations and differences in gene expression.

3.2.2 Assessment of the acute to chronic phenotypic switch

During the course of lung infections, *P. aeruginosa* is known to switch phenotypically from an acute infecting strain to its chronic form (Moradeli *et al*., 2017). This involves becoming less immunogenic through the down-regulation of virulence factors and becoming more adapted to survival within the lung by increasing alginate production and biofilm formation (McCaslin *et al*., 2015). Both patients had a chronic *P. aeruginosa* infection. Therefore, in order to determine whether the observed morphological differences could be the result of these adaptive changes or the result of a new *P. aeruginosa* infection following plasmapheresis, the growth kinetics

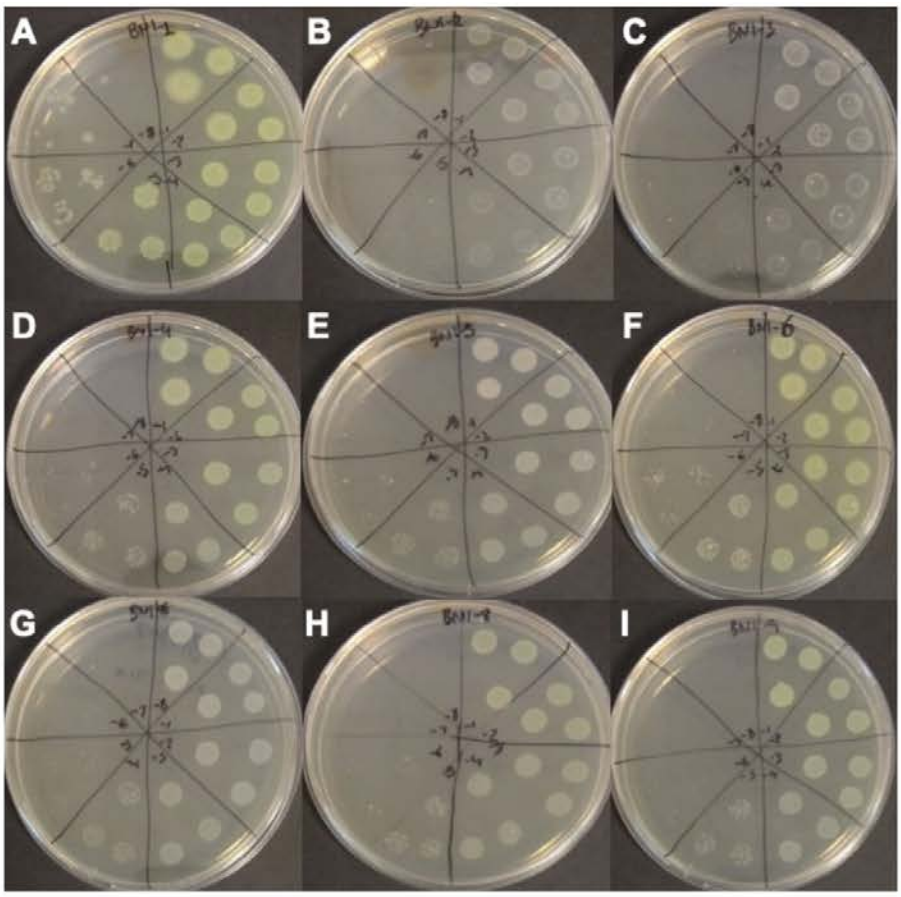


Figure 3.1 Colony morphology comparison of BN1 *P. aeruginosa* isolates

In preparation for serum bactericidal assays, overnight cultures of BN1 isolates were washed twice with 1x PBS. Serial dilutions ranging from 10^{-1} to 10^{-8} were prepared, plated in triplicate onto LB plates and incubated overnight at 37°C. The size, shape, colour and mucoidicity of the colonies were compared. The green-yellow fluorescent pigment observed in A, D, F, H and I indicates the production of pyoverdine by these isolates. **A)** BN1-1 **B)** BN1-2 **C)** BN1-3 **D)** BN1-4 **E)** BN1-5 **F)** BN1-6 **G)** .BN1-7 **H)** BN1-8 **I)** BN1-9.

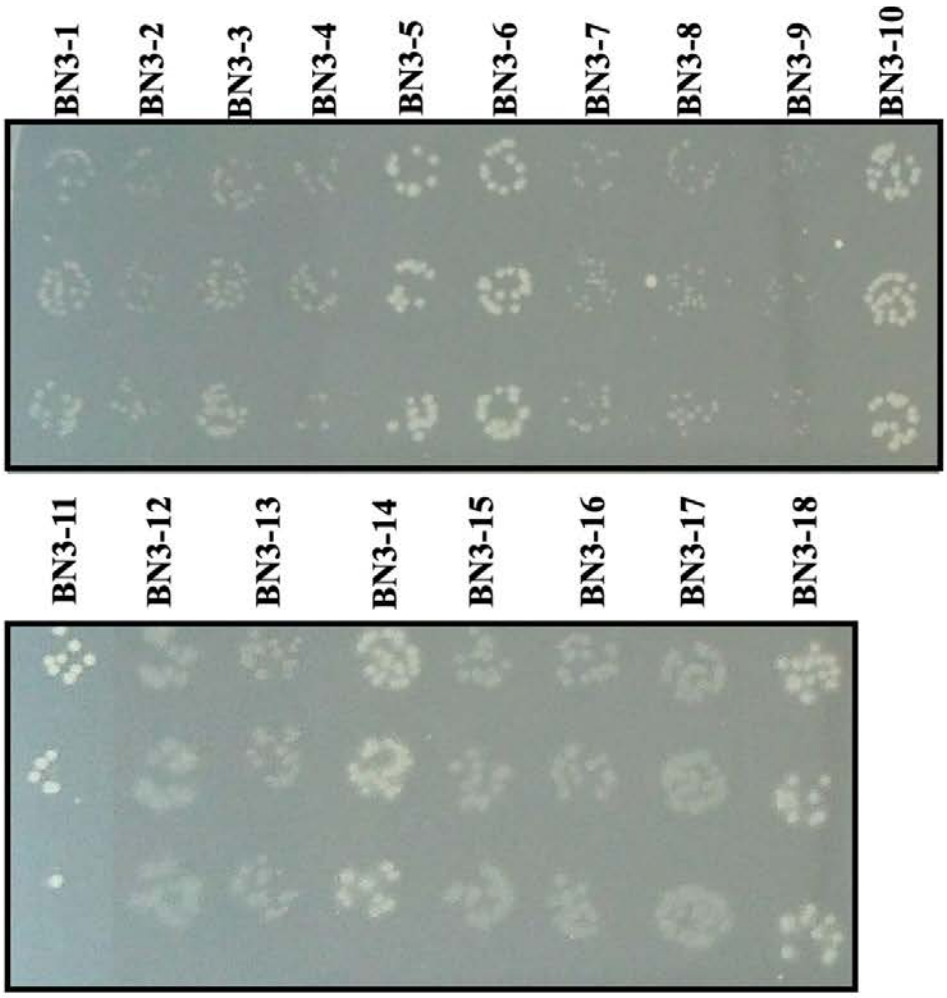


Figure 3.2 Colony morphology comparison of BN3 *P. aeruginosa* isolates

Overnight cultures of BN3 isolates were diluted 10^{-6} -fold into fresh LB broth and 5 µl spots were plated in triplicate onto LB agar plates. Plates were incubated overnight at 37°C and the size, shape, colour and mucoidicity of the colonies were compared. BN3 pre-plasmapheresis isolates are represented in the top panel and BN3 post-plasmapheresis isolates are represented in the bottom panel. Four distinctive colony morphologies are represented by isolates BN3-1, BN3-5, BN3-7 and BN3-12.

and motility phenotypes were assessed.

3.2.2.1 Comparison of the growth kinetics of *P. aeruginosa* isolates

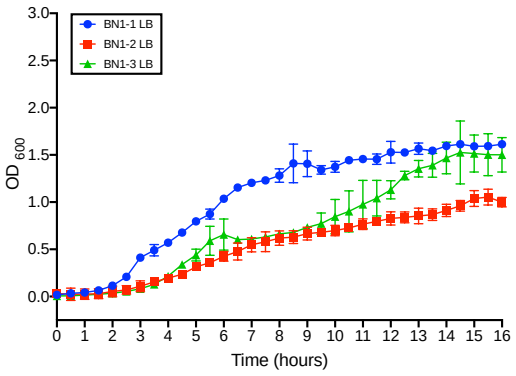
Strains isolated from chronic infections grow more slowly than acute infection isolates (Yang *et al*., 2008). To determine whether the isolates obtained from the patients after plasmapheresis exhibited the acute or chronic growth phenotype, overnight cultures of the isolates were equilibrated to an OD_{600} = 0.025 in fresh LB broth and subsequently grown with aeration in triplicate in 96-well plates.

The OD_{600} of each culture was` recorded every 30 min for 16 h. The area under the curve (AUC) was determined to quantitatively compare the growth of pre- and post- plasmapheresis isolates. An unpaired 2-tailed Welch's t-test was used to test for significant differences between the growth of pre- and post- plasmapheresis isolates and to correct for the unequal sample sizes.

The mean AUC of BN1 pre-plasmapheresis isolates, 15.01, (Figure 3.3A) did not significantly differ from the mean AUC, 17.40, of BN1 post-plasmapheresis isolates (Figure 3.3B). The mean AUC of BN3 pre-plasmapheresis isolates, 13.30, (Figure 3.4A) did not differ significantly from the mean AUC of BN1 post-plasmapheresis isolates (n = 8), 13.94 (Figure 3.4B). Growth curves are presented in log scale in Appendix I.

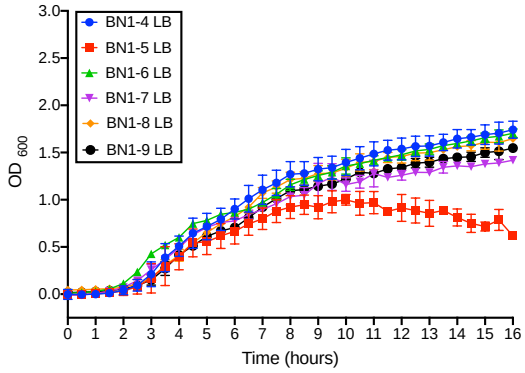
No significant difference in growth was observed between pre- and post-plasmapheresis isolates. However, minor differences for individual isolates could be seen when visually comparing the growth curves. Isolate BN1-1 grew more quickly than isolates BN1-2 and BN1-3 (Figure 3.3A). Isolate BN1-5 reached stationary phase after approximately 8 hours and death phase after approximately 11.5 hours (Figure 3.3B). This was not observed for any other BN1

A



Isolate	BN1-1	BN1-2	BN1-3
AUC	19.98	10.67	14.38

B

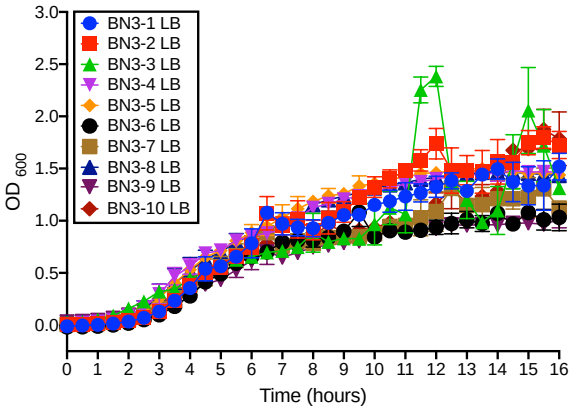


Isolate	BN1-4	BN1-5	BN1-6	BN1-7	BN1-8	BN1-9
AUC	19.77	11.24	19.58	16.72	18.79	17.28

Figure 3.3 Growth kinetics of BN1 isolates

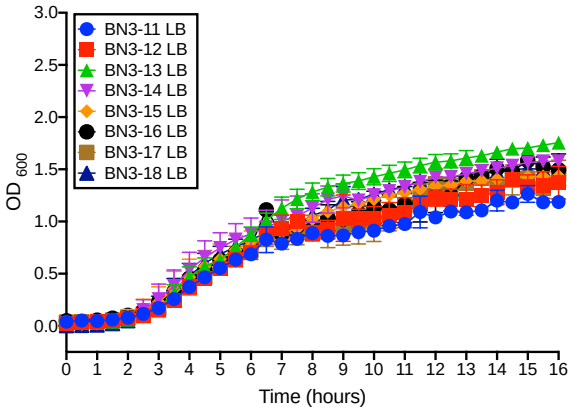
Isolates were grown in triplicate in 96-well plates in LB broth. The OD_{600} was recorded every 30 min for 16 h. **A)** Growth curves of BN1 pre-plasmapheresis isolates (n=3). **B)** Growth curves of BN1 post-plasmapheresis isolates (n=6). The mean OD_{600} was plotted with error bars representing the standard deviation. The area under the curve (AUC) was calculated for each isolate and is shown below the graph. The mean AUC for pre-plasmapheresis isolates, 15.01, was not significantly different from the mean AUC for post-plasmapheresis isolates, 17.40, as determined by an unpaired t-test with Welch's correction (P = 0.4684).

A



Isolate	BN3-1	BN3-2	BN3-3	BN3-4	BN3-5	BN3-6	BN3-7	BN3-8	BN3-9	BN3-10
AUC	13.48	15.08	13.98	14.98	15.35	10.43	11.63	15.01	10.04	12.97

B



Isolate	BN3-11	BN3-12	BN3-13	BN3-14	BN3-15	BN3-16	BN3-17	BN3-18
AUC	11.58	12.69	16.38	15.20	14.08	13.95	13.05	14.62

Figure 3.4 Growth kinetics of BN3 isolates

Isolates were grown in triplicate in 96-well plate format in LB media. The OD_{600} was recorded every 30 min for 16 h. **A)** Growth curves of BN3 pre-plasmapheresis isolates (n=10). **B)** Growth curves of BN3 post-plasmapheresis isolates (n=8). The mean OD_{600} was plotted with error bars representing the standard deviation. The area under the curve (AUC) was calculated for each isolate and is shown below the graph. The mean AUC for pre-plasmapheresis isolates, 13.30, was not significantly different from the mean AUC for post-plasmapheresis isolates, 13.94, as determined by an unpaired t-test with Welch's correction (P = 0.4422).

isolate. Isolate BN3-3 grew irregularly after incubation for 11 hours (Figure 3.4A). Upon inspection of the corresponding wells in the 96-well plate, this isolate had formed aggregates, which had caused the irregular OD_{600} values. The final OD_{600} of BN3 isolates ranged between approximately 1.0 and 1.9. Growth curves are presented in log scale in Appendix II.

3.2.2.2 Comparison of the motility of isolates

Flagella and type IV pili are virulence factors that are known to be associated with the initial attachment and colonisation processes. Downregulation of the production of these virulence factors is common during chronic lung colonisation (Gellaty and Hancock, 2013). Previous work has demonstrated that both type IV pili and flagella allow *P. aeruginosa* to move by twitching, swimming and swarming (Köhler *et al*, 2000; Anyan *et al*., 2014) and that these appendages also mediate biofilm formation (Gellaty and Hancock, 2013; Anyan *et al*., 2014). Increased biofilm formation has been associated with the chronic phenotype (Høiby, 2011).

To determine whether the isolates exhibit the chronic or acute phenotype, the swimming motility and biofilm formation of each isolate was assessed. A 1 µl loop was used to inoculate 0.3% agar plates with overnight cultures of the isolates by stabbing the loop into the centre of agar plate. Plates were then incubated for 18 h at 37°C. The swim diameter was subsequently measured and isolates were allocated into arbitrary groups based on the swim diameter. The swim groups were as follows: 1 = 0-15 mm; 2 = 16-30 mm; 3 = 31-45 mm; 4 = 46-60 mm; 5 = 61-75 mm; and 6 = 76 ≤ 90 mm. To assess biofilm formation, overnight cultures of the isolates were equilibrated to an OD_{600} = 0.2 with fresh M9 minimal medium and 7 technical replicates for each isolate were plated into 96 well plates. Following static growth at 37°C for 18 h, the crystal violet staining method was used to quantify biofilm formation. Isolates were allocated into arbitrary groups for biofilm formation based on the OD_{545} readings. The biofilm

groups were as follows: 1 = 0 ≤ OD_{545} < 1; 2 = 1 ≤ OD_{545} < 2; 3 = 2 ≤ OD_{545} < 3; 4 = 3 ≤ OD_{545} < 4; 5 = 4 ≤ OD_{545} < 5; and 6 = 5 ≤ OD_{545}. The number of isolates belonging to each group prior to plasmapheresis and post-plasmapheresis were compared (BN1 isolates, Figure 3.5. BN3 isolates, Figure 3.6). The group allocations for individual isolates can be found in Appendix IV.

For swimming motility, BN1 pre-plasmapheresis isolates (n=3, black bars) were allocated into groups 1, 2 and 3 (Figure 3.5A, n=3, black bars; Appendix II). All BN1 isolates post-plasmapheresis belonged to group 2 (Figure 3.5A, n=6, grey bar; Appendix II). The relatively small swim diameter by all post-plasmapheresis isolates implied a more chronic phenotype. The grouping of post- plasmapheresis isolates indicated that these isolates might be more closely related to pre-plasmapheresis isolate BN1-1 as this isolate belonged to the same group. For biofilm formation, 33.3% of BN1 pre-plasmapheresis isolates were allocated to group 2 and 66.7% were allocated to group 3 (black bars, Figure 3.5B, Appendix III). However, 66.7% of the BN1 post-plasmapheresis isolates were allocated to group 3 and 16.7% were allocated to groups 4 and 5 (grey bars, Figure 3.5B; Appendix III). The increase in biofilm formation by BN1 post-plasmapheresis isolates compared with the pre-plasmapheresis isolates was consistent with the phenotype of isolates that are associated with chronic infections.

For swimming motility, BN3 pre-plasmapheresis isolates were allocated into groups 1, 2, 3 and 6 (Figure 3.6A, n=10, black bars; Appendix ii). BN3 post-plasmapheresis isolates belonged to group 2, 3, 4 and 6 (Figure 3.6A, n=8, grey bars; Appendix II). Seventy percent of pre-plasmapheresis isolates belonged in groups 1 to 3 and the remaining thirty percent belonged to the highest swimming category, group 6. In contrast, post-plasmapheresis isolates split 50:50 into groups 1 to 3 and groups 4 to 6. These data could indicate that patient PN3 might have

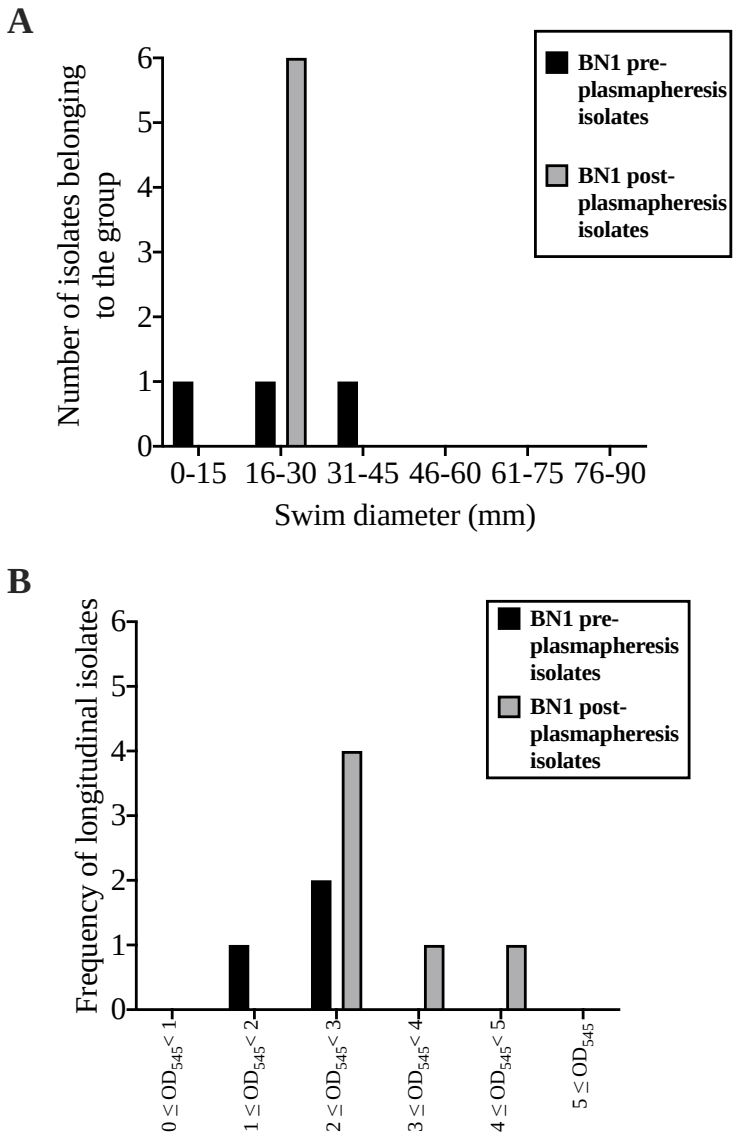


Figure 3.5 The motility of pre-plasmapheresis and post-plasmapheresis BN1 isolates

A) Swimming motility. Overnight cultures of the isolates were spotted into the centre of 0.3% agar plates and incubated at 37°C for 18 h. The diameter of the swim colony was measured and isolates were placed into one of six measurement groups. The number of isolates belonging to each group was plotted based on pre-plasmapheresis (BN1-1 to BN1-3, black bars) and post-plasmapheresis (BN1-4 to BN1-9, grey bars). **B) Biofilm formation in M9 minimal media**. Overnight cultures of the isolates were diluted to an OD_{600} = 0.2 into fresh M9 medium and 7 technical replicates per isolate were plated into 96-well plates, which were then incubated for 18 h at 37°C without aeration. Biofilms were stained using 0.1% crystal violet and the OD_{545} for each well was recorded. Isolates were allocated into one of six measurement groups. The number of isolates belonging to each group was plotted based on pre-plasmapheresis (BN1-1 to BN1-3, black bars) and post-plasmapheresis (BN1-4 to BN1-9, grey bars). Each assay was repeated 3 times.

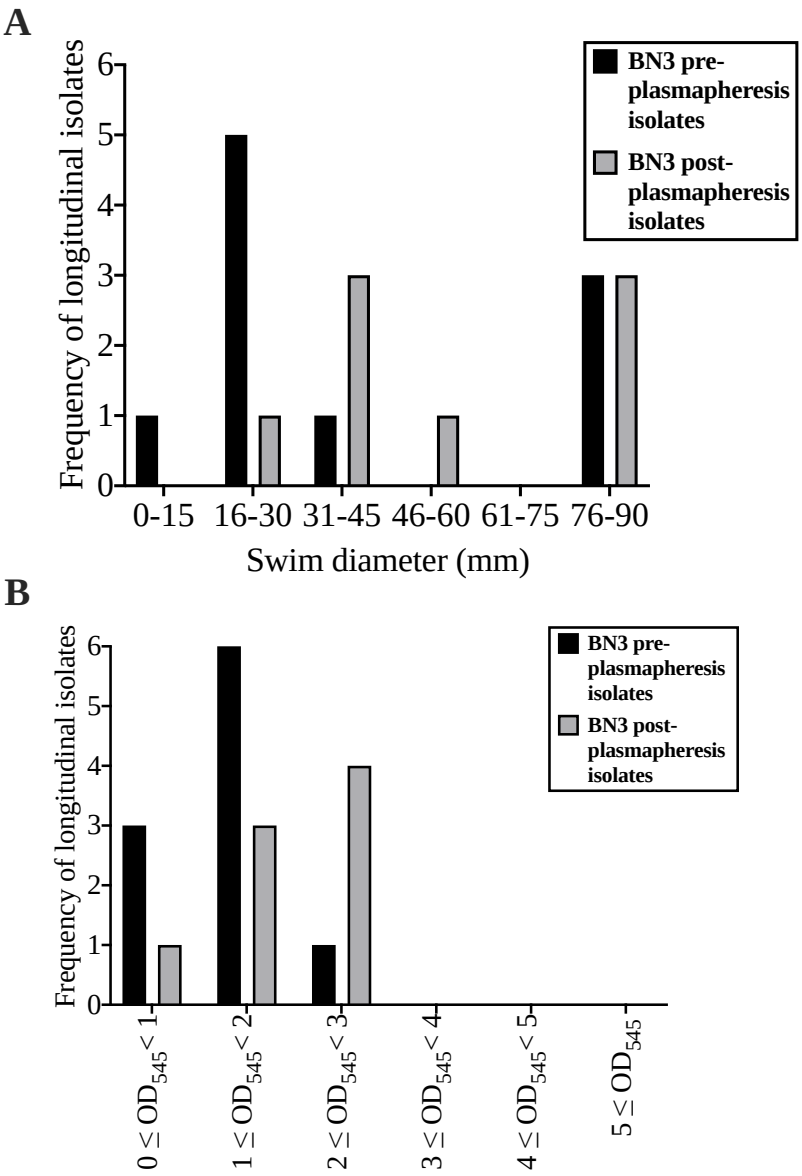


Figure 3.6 The motility of pre-plasmapheresis and post-plasmapheresis BN3 isolates

A) Swimming motility. Overnight cultures of the isolates were spotted into the centre of 0.3% agar plates and incubated at 37°C for 18 h. The diameter of the swim colony was measured and isolates were placed into one of six measurement groups. The number of isolates belonging to each group was plotted based on pre-plasmapheresis (BN3-1 to BN3-10, black bars) and post-plasmapheresis (BN3-11 to BN3-18, grey bars). **B) Biofilm formation in M9 minimal media**. Overnight cultures of the isolates were diluted to an OD_{600} = 0.2 into fresh M9 medium and 7 technical replicates per isolate were plated into 96-well plates, which were then incubated for 18 h at 37°C without aeration. Biofilms were stained using 0.1% crystal violet and the OD_{545} for each well was recorded. Isolates were allocated into one of six measurement groups. The number of isolates belonging to each group was plotted based on pre-plasmapheresis (BN3-1 to BN3-10, black bars) and post-plasmapheresis (BN3-11 to BN3-18, grey bars). Each assay was repeated 3 times.

been co-infected with an acute strain and a chronic strain of *P. aeruginosa* prior to plasmapheresis and that these strains have both remained post treatment. All BN3 pre- and post- plasmapheresis isolates belonged in biofilm groups 1 to 3 (Figure 3.6B; Appendix III). However, similar to the BN1 isolates, an increase in biofilm formation was observed for BN3 post-plasmapheresis isolates (grey bars). This was once again consistent with the chronic infection phenotype.

3.2.3 Comparison of the serum resistance profiles of isolates

As described in the introduction to this chapter, the removal of inhibitory IgG2 antibodies by plasmapheresis resulted in the serum-killing of the original isolate cultured from the patient by patient sera obtained following treatment. The return of circulating inhibitory IgG2 saw the reappearance of *P. aeruginosa* in the sputum of the patients. As phenotypic variations between longitudinal isolates had been observed, the serum-resistance profiles of the isolates were investigated further. Due to the specificity of inhibitory antibodies, it was hypothesised that if the isolates from the same patient were all the same strain of *P. aeruginosa*, patient serum with high titres of inhibitory antibody would be able to block the killing of each isolate by pooled-HCS.

To determine whether the IgG2 inhibitory antibodies were able to block the killing of each isolate from the corresponding patient, the sera were screened for inhibitory properties by serum bactericidal assays. Isolates were incubated in the presence of 50% pooled-HCS, 50% patient serum and a 50:50 mix of patient serum and pooled-HCS for 180 mins. Following incubation in serum, isolates were serially diluted and plated in triplicate onto LB agar. After overnight incubation at 37°C, colonies were enumerated and the $\log_{10}$ change in viable cell number was calculated.

The addition of patient PN1 serum to pooled-HCS (green triangles) reduced the killing of isolates BN1-4, 6, 8 and 9 when compared to pooled-HCS alone (blue circles) (Figure 3.7A). The addition of patient serum PN3 to pooled-HCS (green triangles) reduced killing of isolates BN3-5, 6, 10, 11, 13, 16, 17 and 18 when compared to pooled-HCS alone (blue circles) (Figure 3.7B). It should be noted that, with the exception of isolate BN3-10, patient sera were able to cause at least a 0.5 log decrease in viable cell number of their corresponding isolates (Figure 3.7A and B, red squares). This indicated that the titre of inhibitory antibody in patient sera might not have been sufficient to totally abolish serum killing.

Isolates with increased resistance to killing by patient serum compared to pooled-HCS were present both prior to plasmapheresis and post-treatment. Two thirds of BN1 pre-plasmapheresis isolates and BN1 post-plasmapheresis isolates were unable to be killed completely by patient serum. However, the post-plasmapheresis isolates were also more resistant to pooled-HCS (Figure 3.7A, blue circles). In contrast, an increase in the percentage of isolates with increased resistance to patient sera was observed for BN3 post-plasmapheresis isolates compared to BN3 pre-plasmapheresis isolates (62.5% and 30%, respectively). BN3 pre-plasmapheresis isolates, BN3-5, 6 and 10, which had increased resistance to patient serum also had increased resistance to pooled-HCS, whereas all of the BN3 post-plasmapheresis isolates were completely killed by pooled-HCS (Figure 3.7B, blue circles). The individual serum resistance and inhibitory antibody profiles for both sets of isolates can be found in Appendix IV.

As the data presented in section 3.2.3 implied that the isolates originated from the underlying chronic infection and the killing of each isolate by their respective patient sera was variable, it was hypothesised that during the course of infection, the isolates might have produced target antigens with altered structures that could have affected the binding potential of antibody to

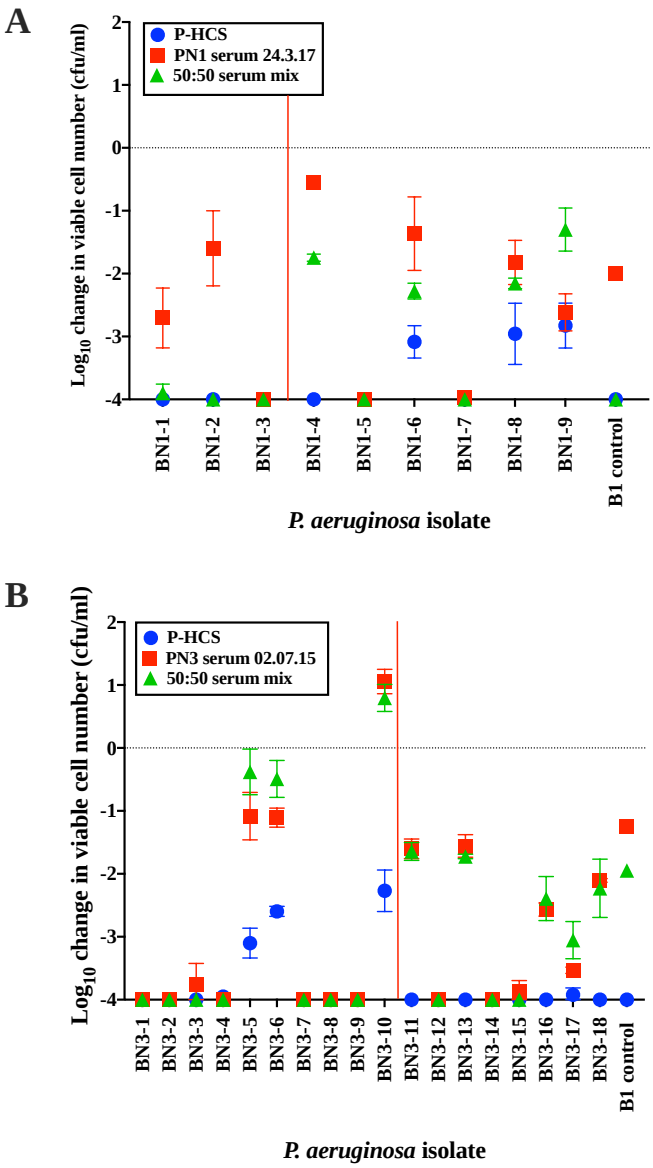


Figure 3.7 Serum bactericidal activity of patient sera against corresponding isolates

The presence of inhibitory antibody in patient sera was determined by serum bactericidal assay. Isolates were incubated in 50% pooled-HCS (blue circles), 50% corresponding patient sera (Patient, red squares) or 50:50 mix of patient and pooled-HCS (50:50 mix, green triangles) at 37°C for 180 min and the $\log_{10}$ change in viable cell number was calculated. Pre-plasmapheresis isolates (obtained periodically between 3-18 months prior to treatment) are to the left of the red line, post-plasmapheresis isolates (obtained periodically between 1-11 months after treatment) are to the right of the red line. Negative y-axis values correspond with a decrease in viable cell number. Sera are considered inhibitory if killing by pooled-HCS is reduced in the presence of patient serum. Data points and error bars represent the mean and standard deviation of at least two biological replicates. B1 is a negative control strain for PN1 and a positive control for PN3 **A)** Inhibitory antibody profiles of BN1 isolates. **B)** Inhibitory antibody profiles of BN3 isolates.

the target antigens.

3.2.4 Antibody recognition of lipopolysaccharide purified from *Pseudomonas aeruginosa* isolates

Analysis of the serum resistance profiles of the isolates revealed that patient sera contained inhibitory antibodies against some but not all strains. IgG, specifically IgG2, has been identified as the inhibitory antibody isotype and subclass and LPS has been suggested to be the target of inhibitory antibody. To determine whether the isolates that could be killed in the presence of inhibitory antibody had produced LPS with an altered structure that might have prevented the binding of inhibitory antibody, LPS was purified from each isolate and separated by SDS-PAGE. These were then transferred to nitrocellulose membranes and the anti-LPS IgG, IgG2 and IgA binding profiles for the serum of each patient were determined by Western blot analysis. The titres of anti-LPS IgG and IgA antibodies in patient sera were also compared by ELISA for LPS isolated from serum-resistant isolates BN1-4 and BN3-6 and serum-sensitive isolates BN1-7 and BN3-15.

Patient PN1 serum and patient PN3 serum contained LPS-specific antibody belonging to both IgG (Figure 3.8A and B), IgG2 (Figure 3.8C and D) and IgA (Figure 3.8E and F). LPS binding by all three antibody types also revealed that patient PN1 had been colonised with *P. aeruginosa* that produced LPS with one of three distinct structures (Figure 3.9). BN1 isolates belonging to group 1 produced LPS with very long and long O-antigen, short chain O-antigen, core antigen and lipid A that was detected by patient antibodies. BN1 isolates belonging to group 2 produced LPS with very long and long O-antigen and lipid A that was detected by patient antibodies. BN1 isolates belonging to group 3 produced LPS with only the lipid A region being detected by patient antibodies (Figure 3.9).

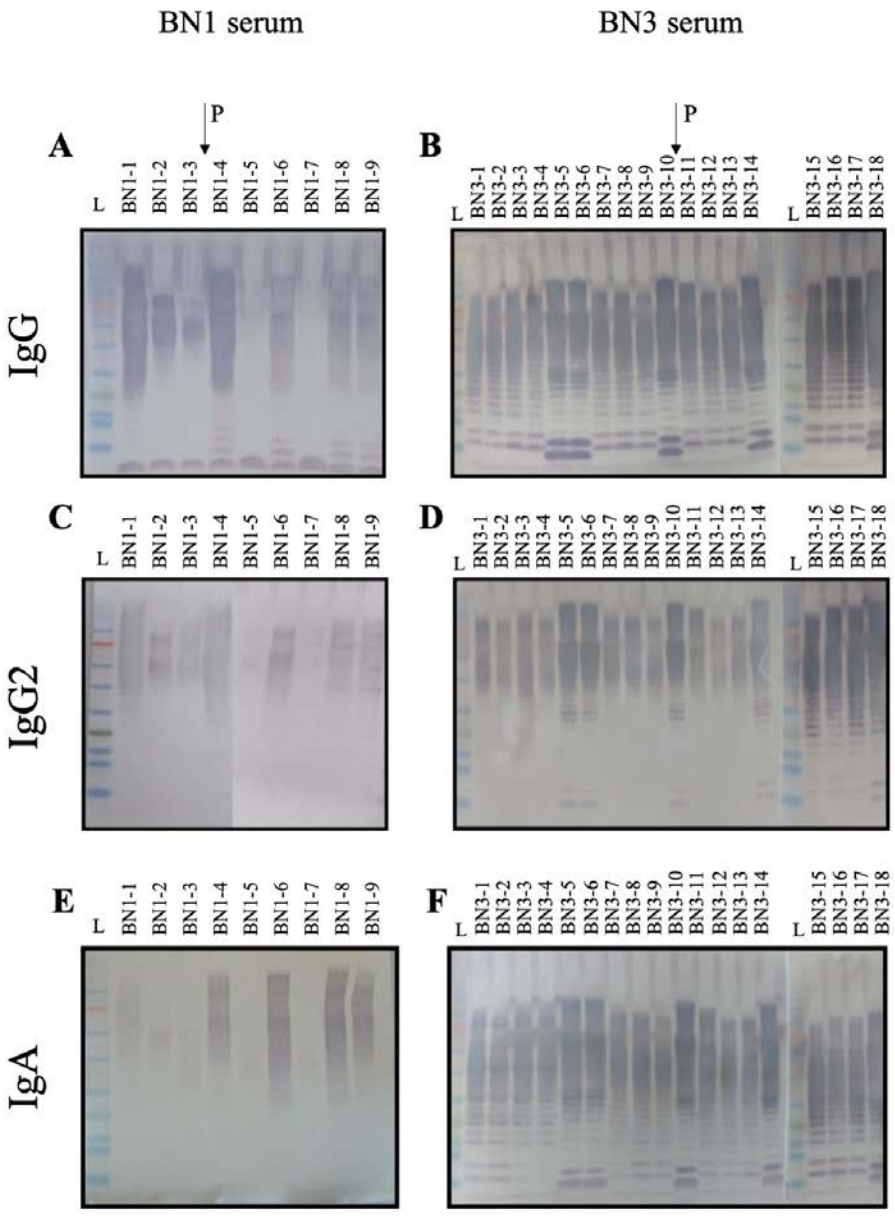


Figure 3.8 Anti-LPS antibody in patient sera directed towards LPS purified from the corresponding *P aeruginosa* isolates

LPS was separated on 10-12% bis-tris polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in blotto followed by 1-hour incubations at room temperature in a primary antibody (1:5000 dilution of patient sera), secondary antibody (1:5000 IgG-AP, 1:5000 IgA-AP or 1:2000 mouse anti-human IgG2) and if required, tertiary antibody (1:2000 anti-mouse IgG-AP). Antibody binding was visualised by the addition of BCIP®/NBT-Purple Liquid Substrate. **A)** PN1 serum probed for anti-LPS IgG to BN1 isolate LPS. **B)** PN3 serum probed for anti-LPS IgG to BN3 isolate LPS. **C)** PN1 serum probed for anti-LPS IgG2 to BN1 isolate LPS. **D)** PN3 serum probed for anti-LPS IgG2 to BN3 isolate LPS. **E)** PN1 serum probed for anti-LPS IgA to BN1 isolate LPS. **F)** PN3 serum probed for anti-LPS IgA to BN3 isolate LPS. The arrow denotes when patients received plasmapheresis (P). L= BLUeye Pre-Stained Protein Ladder, 10-245 kDa.

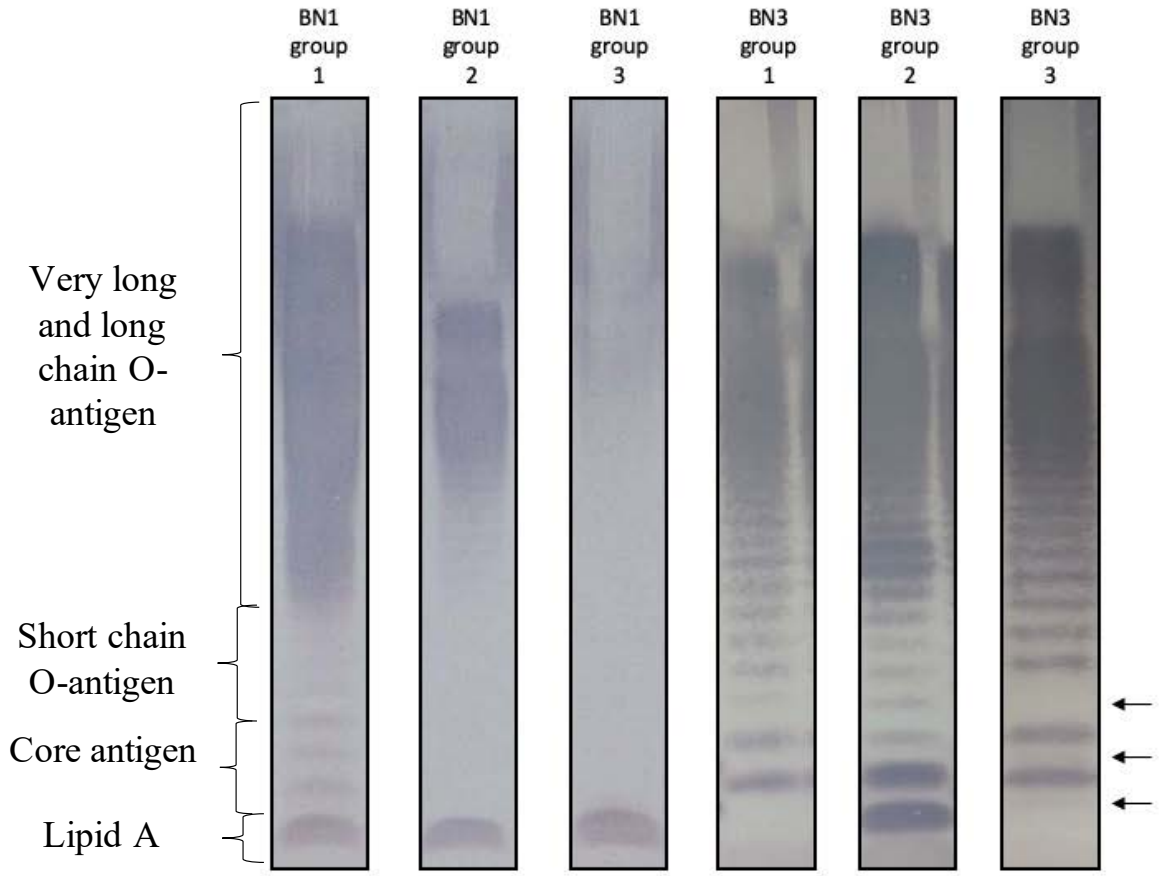


Figure 3.9 Visual representations of the three LPS structures produced by BN1 and BN3 isolates

Patient PN1 serum contained IgG antibodies that recognised all regions of the LPS produced by BN1 isolates belonging to group 1. IgG antibodies were detected in patient serum that recognised the long and very long O-antigen and lipid A of LPS produced by BN1 isolates belonging to group 2. IgG antibodies only recognised the lipid A portion of the LPS produced by BN1 isolates belonging to group 3. Patient PN3 IgG antibodies recognised the lipid A and long and very long O-antigen produced by all three groups of BN3 isolates. A lower molecular weight core antigen was detected in BN3 isolates belonging to group 2 when compared to core antigens produced by group 1 and group 3 isolates. The core antigens of group 1 and group 3 BN3 isolates had similar structures. However, extra bands were detected by patient IgG for group 3 isolates. Differences between group 1 and group 3 LPS structures are indicated by arrows.

Patient PN3 had been colonised with *P. aeruginosa* that also produced LPS with one of three distinct structures. All three LPS groups for BN3 isolates produced LPS with very long and long chain O-antigen and lipid A that was detected by patient antibodies. BN3 isolates belonging to group 2 produced a core antigen with a lower molecular weight than those produced by group 1 and group 3 isolates. BN3 isolates belonging to groups 1 and 3 produced core antigens with similar structures. However, extra bands were detected by patient antibodies for group 3 isolates (Figure 3.9). The different LPS structures were produced by isolates that were obtained both prior to treatment and following treatment (Figure 3.8).

Two thirds of BN1 post-plasmapheresis isolates belonged to the same group as pre-plasmapheresis isolate BN1-1 (group 1), which indicated these isolates are closely related. The remaining post-plasmapheresis isolates did not produce O-antigen (group 3) (Figure 3.8A). Group 1 and group 2 LPS were produced by BN3 isolates obtained before and after treatment. Group 3 LPS was only produced by isolates obtained after plasmapheresis (Figure 3.8B, Appendix IV).

When comparing the level of antibody binding to the LPS purified from a serum sensitive and a serum resistant isolate by ELISA, it was revealed that patient sera contained elevated titres of IgG2 and IgA antibodies towards the LPS produced by the serum-resistant isolates (BN1-4 and BN3-6, blue circles) when compared to the LPS produced by the serum-sensitive isolates (BN1-7 and BN3-15, red squares). However, the titres of IgG1, IgG3 and IgG4 remained the same (Figure 3.10).

Comparison of the anti-LPS antibody binding profiles for BN1 and BN3 isolates confirmed that isolates produced LPS with modified structures. The production of group 1 LPS by BN1

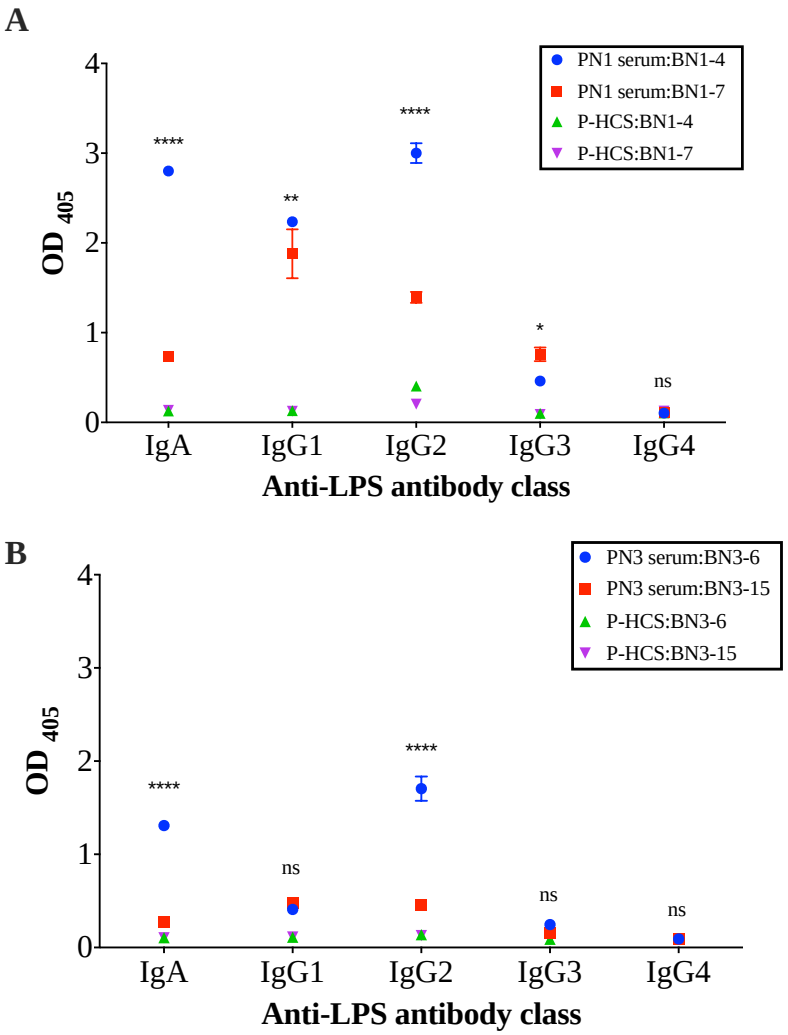


Figure 3.10 Comparison of antibody titre in patient sera directed against the LPS of serum-sensitive and serum-resistant isolates

ELISA plates were coated with LPS extracted from serum-resistant isolates BN1-4 and BN3-6 and serum-sensitive isolates BN1-7 and BN3-15. Plates were incubated with a 1:100 dilution of the corresponding patient sera or pooled-HCS followed by a 1:2000 dilution of anti-IgA-AP, IgG1, IgG2, IgG3 or IgG4 secondary antibody. Plates incubated with an IgG antibody were incubated with a 1:2000 dilution of anti-mouse IgG-AP tertiary antibody. Antibody binding was detected with SIGMA*FAST*™ p-Nitrophenyl phosphate substrate and the OD_{405} was recorded. Data points and error bars represent the mean and standard deviation of 2 biological replicates. A 2-way ANOVA with Sidak's multiple comparison test was used to determine statistical differences between the level of antibody detection in patient serum towards serum-resistant and serum-sensitive isolates. **** $p = < 0.0001$; ** $p = 0.0093$; * $p = 0.0246$; ns $p = > 0.05$. **A)** PN1 serum. **B)** PN3 serum.

isolates correlated with the inhibition of serum-mediated killing of those isolates. The inhibition of serum-mediated killing correlated with the production of group 2 or group 3 LPS for all but BN3-13, which produced group 1 LPS. Elevated titres of IgG2 and IgA to the LPS of the serum-resistant isolates suggested that these antibodies might have an increased affinity for a specific epitope present on the LPS of these isolates. These data suggest that a particular LPS modification produces the target of inhibitory antibodies.

3.2.5 Phylogenetic analyses of BN1 and BN3 *Pseudomonas aeruginosa* isolates

To determine whether the observed phenotypic variances between the isolates were due to *P. aeruginosa* strain differences, whole genome sequences were obtained for each isolate using the Illumina platform provided by MicrobesNG to enable genotypic comparison of the isolates. The pan-genome pipeline identifies core genes, which are present in 95 ≤ to 100% of genomes, and accessory genes, which are present in 0 to < 95%. The pan-genome for both sets of isolates was determined using the Roary pan-genome pipeline (Page *et al*., 2015). Defining the pan-genome is a useful tool as it enables further analysis of the core genome and the genetic relatedness of isolates can be subsequently determined.

The pan-genome of the BN1 isolates consisted of 6936 genes, which corresponds to 5634 core genes and 1302 accessory genes (Appendix V). The core genome was further analysed using the programme snp-dists (https://github.com/tseemann/snp-dists (Torsten Seemann)) to determine and compare the number of SNPs present within the genes of the core-genome. The core gene alignment produced by Roary was used with snp-dists to produce a single nucleotide polymorphism (SNP) distance comparison matrix (Figure 3.11).

The SNP distance matrix produced for BN1 isolates indicated that patient PN1 was colonised

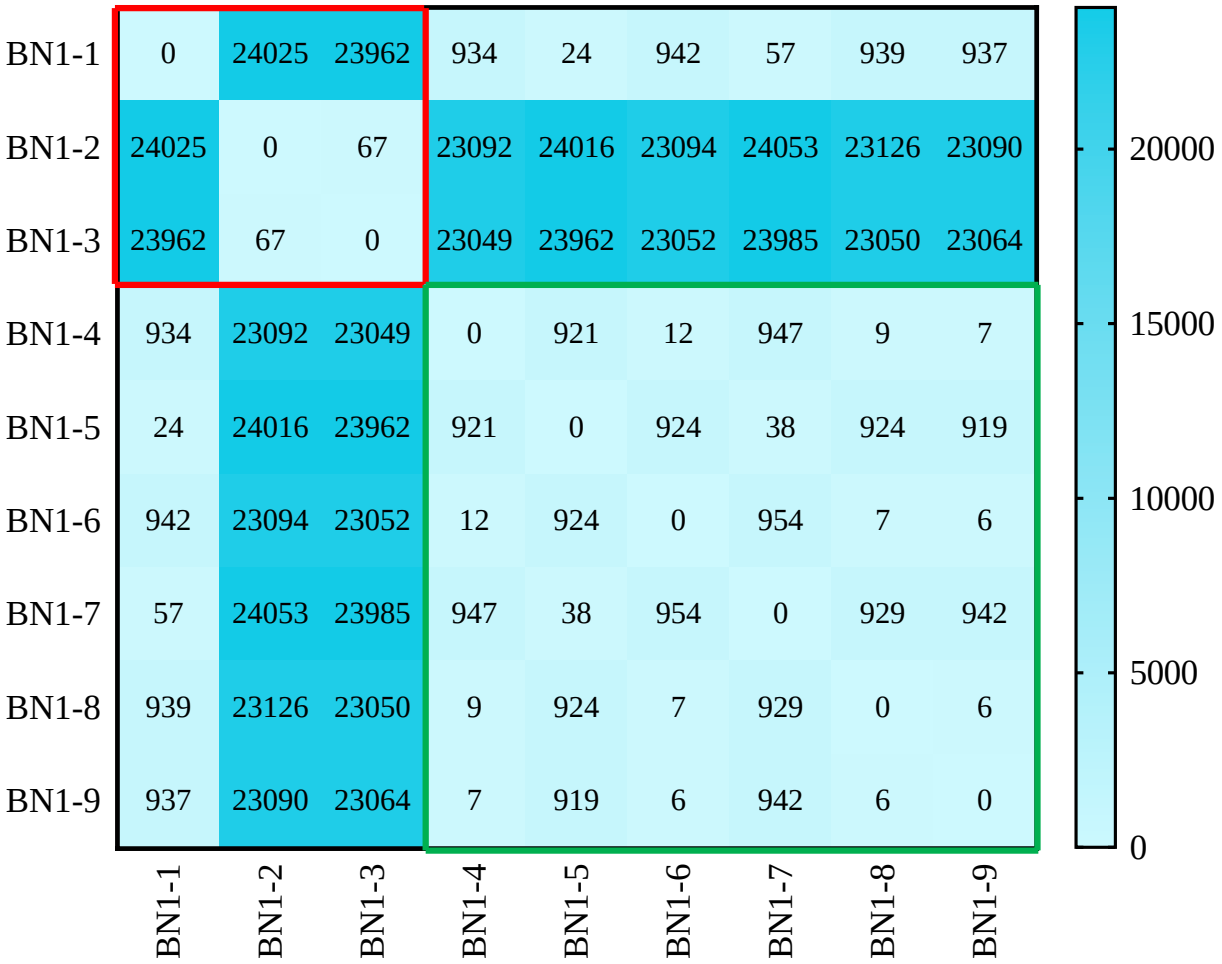


Figure 3.11 Core genome SNP analysis for BN1 *P. aeruginosa* isolates

The SNP distance matrix indicating the number of core-genome SNPs present between isolates. The number of SNPs between the core genomes of the isolates were determined using the core gene alignment produced with Roary with the snp-dists programme (https://github.com/tseemann/snp-dists (Torsten Seemann). The matrix is presented in heat-map format with darker shades representing an increased number of SNPs. The area surrounded by the red box indicates pre-plasmapheresis isolate comparisons. The area surrounded the green box represents post-plasmapheresis isolate comparisons. Areas not surrounded by a box represent comparisons between pre- and post-plasmapheresis isolates.

with two clonal lineages of *P. aeruginosa* prior to plasmapheresis (Figure 3.11, red square). The core-genomes of isolates BN1-2 and BN1-3 differed from isolate BN1-1 by approximately 24000 SNPs. However, only 67 SNPs were identified between isolates BN1-2 and BN1-3. Strains isolated following plasmapheresis (Figure 3.11, green square) belonged to the same clonal lineage as BN1-1, with isolates BN1-5 and BN1-7 being the most closely related to the pre-plasmapheresis isolate (24 and 57 SNPs, respectively). The core genomes of isolates BN1-4, 6, 8 and 9 were nearly identical, with an average of 8 SNPs. However, these isolates differed from isolates BN1-1, 5 and 7 on average by 934 SNPs. These data indicated that isolates BN1-2 and BN1-3 were eradicated following treatment and that the remaining isolates belonged to the same clonal lineage as isolate BN1-1. The low number of SNPs between the remaining isolates indicated the occurrence of clonal expansion.

To investigate this further, the pan-genome was once again determined with the inclusion of the readily available PAO1 genome sequence (AE004091.2) as a reference genome. This genome was annotated using the programme Prokka (Seemann, 2014) prior to the pan-genome analysis. In order to determine the genetic lineage of the isolates, a phylogenetic tree was produced from the new core gene alignment using the programme RaxML v 8.2.10 (Stamatakis, 2014).

In confirmation of the findings of the core genome SNP analysis, isolates BN1-2 and 3 formed a cluster on a distinct branch to the rest of the BN1 isolates in the phylogenetic tree (Figure 3.12A). The inclusion of PAO1 in the core genome SNP analysis also revealed that all of the BN1 isolates differed from PAO1 by 23000-24000 SNPs suggesting that this value is representative of the core genome variation between different strains of *P. aeruginosa*. As suspected, isolates BN1-1, 5, 7 and BN1-4, 6, 8, 9 shared a recent common ancestor. However,

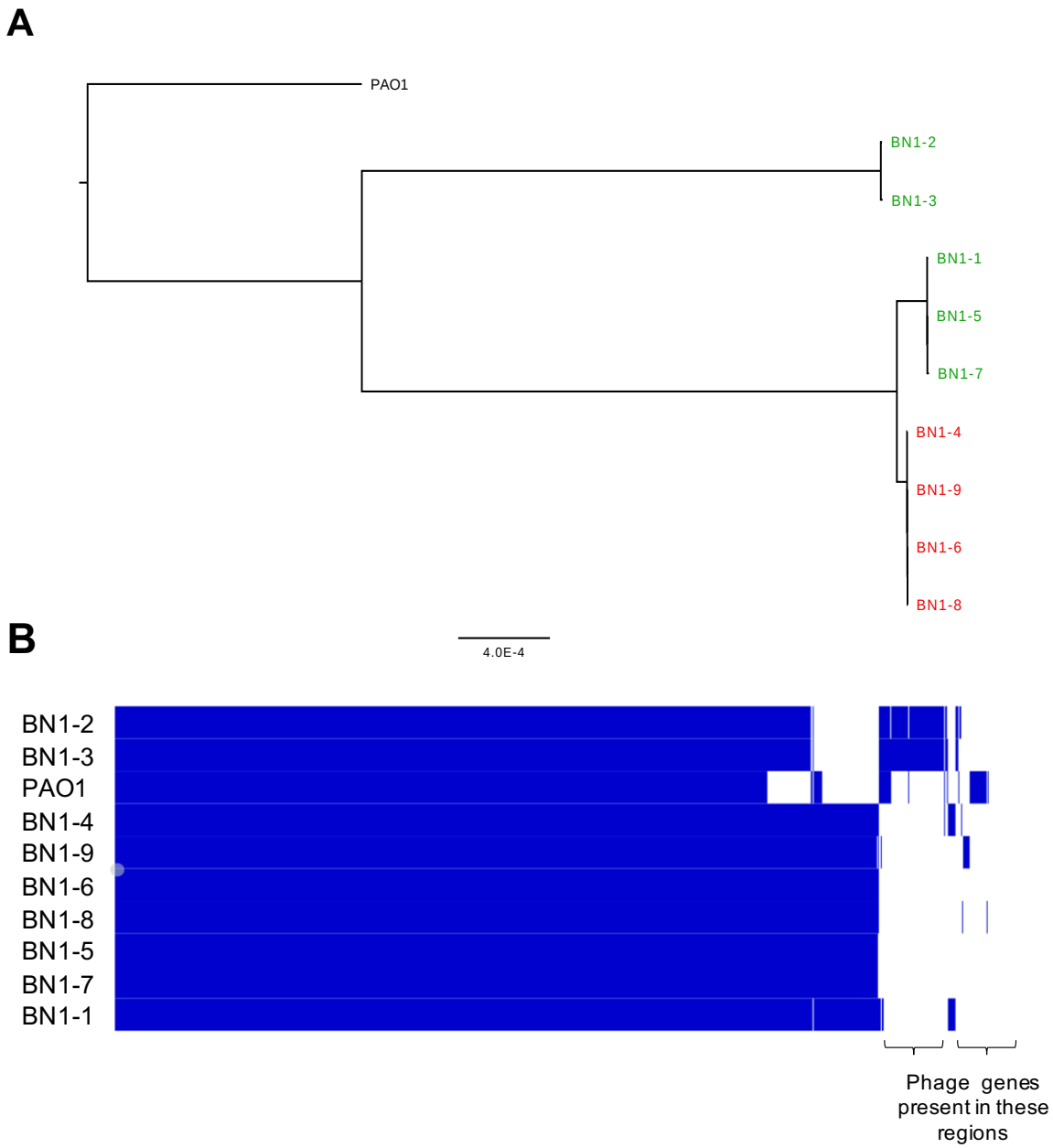


Figure 3.12 Phylogenetic analysis of BN1 *P. aeruginosa* isolates

A Phylogenetic tree of BN1 isolates produced from the Roary core gene alignment using RaxML, which utilizes a maximum likelihood bootstrap model. The tree has been rooted to the reference genome PAO1 (AE004091.2). Green node labels represent non-inhibitory antibody isolates. Red node labels represent inhibitory antibody isolates. **B)** A visual representation of core and accessory gene presence and absence data produced by Roary. Blue lines represent the presence of a gene, white lines represent the absence of a gene. The image was produced using Phandango (Hadfield *et al*., 2017).

the isolates diverged from this common ancestor and formed two further branches on the tree. It was also noted that this divergence correlated with the serum resistance and inhibitory antibody phenotype of the isolates (Figure 3.12A green labels, no inhibitory antibody directed towards isolates; red labels, inhibitory antibody directed towards isolates).

The Roary pan-genome analysis pipeline also provides an output file detailing the presence or absence of the pan-genome genes within each genome. This provides information that can help to explain the observed phenotypic variations between isolates and provide a more in-depth understanding of the isolate's phylogeny. This has been graphically represented using Phandango (Hadfield *et al*., 2017) in Figure 3.12B, which shows a very high level of genetic similarity between the BN1 isolates. Phandango uses blue lines to represent the presence of a gene and white lines to represent the absence of a gene. Upon closer inspection of these data, the presence of bacteriophage genes that were unique to isolates BN1-2 and BN1-3 were identified (Figure 3.12B). Similarly, bacteriophage genes that were unique to isolates BN1-1, 4, 5, 6, 7, 8 and 9 were also identified. The presence of these bacteriophage genes could explain the distinct grouping of isolates BN1-2 and 3 in the phylogenetic tree.

In order to determine whether the observed variations in LPS structure were due to the isolates belonging to different serotypes or the result of chemical modification, the serotypes of the isolates were determined by an *in silico* serotyping technique using the PAst 1.0 programme (Thrane *et al*., 2016). This programme determines the serotype of *P. aeruginosa* strains by isolating the O-specific antigen (OSA) gene cluster and then compares the sequences to a database of known serotype OSA clusters. PAst identified that isolates BN1-2 and BN1-3 produced LPS belonging to the O1 serotype (reference coverage 99.67%). The remaining

isolates produced LPS belonging to the O6 serotype (reference coverage 99.62%) (Appendix IV).

A common technique to understand the evolution of bacterial strains is the application of multi-locus sequence typing (MLST), which is an allele-based technique that compares seven housekeeping genes to determine the sequence type of strains (Larsen *et al*., 2012). In order to confirm the relatedness of the BN1 isolates, the sequence type of each isolate was determined using the *in silico* typing programme MLST 2.0 (Larsen *et al*., 2012). Isolates BN1-2 and BN1-3 were not allocated to an existing sequence type as the allele pattern for these isolates represented a novel sequence type. The closest sequence types were identified as ST1995, 2265, 17 and 2894. The remaining isolates belonged to the sequence type ST-179 (Appendix IV).

Together, the genotypic analyses of the BN1 isolates have confirmed that isolates BN1-1, 4, 5, 6, 7, 8, and 9 are very closely related as they belong to the same sequence type and produce the same serotype of LPS. This suggested that these isolates are the same strain of *P. aeruginosa* and the accumulation of mutations within the core genome over time suggests that clonal expansion might be a contributing factor for the diversion observed on the phylogenetic tree for these isolates. These data also confirmed that patient PN1 was colonised with at least two strains of *P. aeruginosa* prior to plasmapheresis as isolates BN1-2 and BN1-3 produced LPS with a different serotype, belonged to a different sequence type and are found on a distinct branch on the phylogenetic tree when compared to the remaining isolates.

The above genotypic analyses were repeated for the BN3 isolates. The pan-genome of the BN3 isolates consisted of 5975 genes, corresponding to 5736 core genes and 239 accessory genes (Appendix V). A core-gene SNP distance matrix was produced from the core gene alignment

produced by Roary. Analysis of the core gene SNPs between BN3 pre-plasmapheresis isolates (Figure 3.13, red square) indicated that patient PN3 was colonised with one clonal lineage of *P. aeruginosa* as there was a maximum of 1047 SNPs between these isolates. The pre-plasmapheresis BN3 isolates could be split into two groups based on the total number of core-gene SNPs, which is once again indicative of clonal expansion. BN3-1, 3, 4 and 5 formed group 1 and BN3-2, 6, 7, 8, 9 and 10 formed group 2. Comparison of the BN3 post-plasmapheresis isolates also indicated the presence of one clonal lineage, as there was a maximum of 996 SNPs between the isolates, and the clonal lineage could be separated into the two groups as described above (Figure 3.13, green square). Group 1 was formed by isolates BN3-11, 13 and 18 and group 2 was formed by isolates BN3-12, 14, 15, 16 and 17. Isolates belonging to each group were present both pre- and post-treatment and the proportion of isolates belonging to each group remained fairly consistent (pre-treatment: group 1 = 40%, group 2 = 60%; post-treatment: group 1 = 37.5%, group 2 = 62.5%). These results indicated that plasmapheresis did not alter the *P. aeruginosa* population in the lungs of patient PN3.

In order to confirm the above suggestions, a phylogenetic tree was produced from the core gene alignment using RaxML and PAO1 as the reference genome. As suspected, all the BN3 isolates were very closely related, which is represented by the extremely short distances between isolates on the phylogenetic tree (Figure 3.14A). Isolates also diverged into two groups on the tree that corresponded to the groups identified during core gene SNP analysis.

Unlike BN1 isolates, clustering of the BN3 isolates on the phylogenetic tree did not correlate with the serum resistance and inhibitory antibody phenotype of the isolates (Figure 3.14A green labels, no inhibitory antibody directed towards isolates; red labels, inhibitory antibody directed towards isolates). This suggests that the differences between the serum resistance profiles of

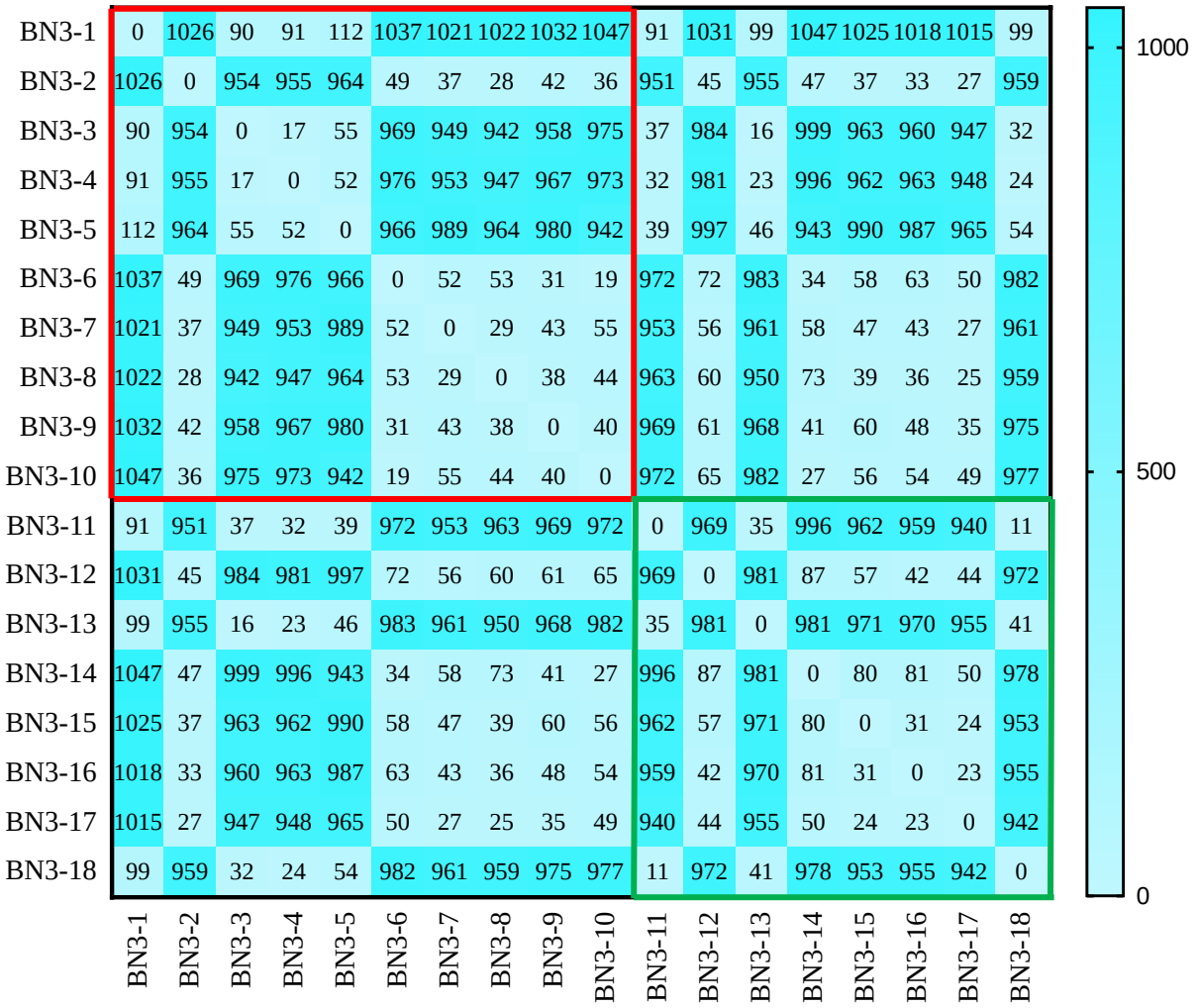


Figure 3.13 Core genome SNP analysis of BN3 *P. aeruginosa* isolates

The SNP matrix with the number of core genome SNPs present between isolates. The number of SNPs was determined using the core gene alignment produced with Roary with by the snp-dists programme (https://github.com/tseemann/snp-dists (Torsten Seemann)). The matrix is presented in heat-map format with darker shades representing an increased number of SNPs. The area surrounded by the red box indicates pre-plasmapheresis isolate comparisons. The area surrounded the green box represents post-plasmapheresis isolate comparisons. Areas not surrounded by a box represent comparisons between pre- and post-plasmapheresis isolates.

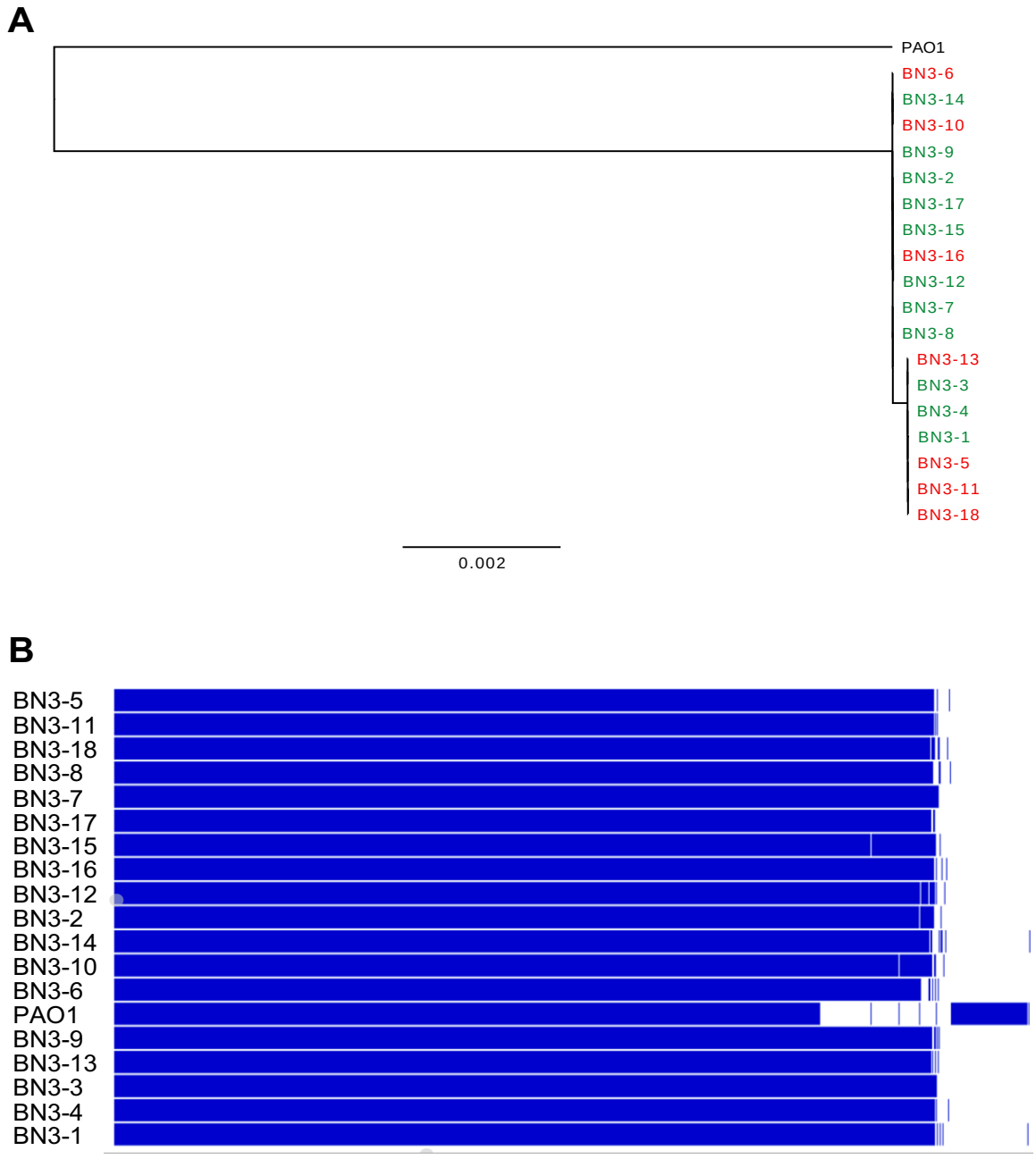


Figure 3.14 Phylogenetics of BN3 isolates

A Phylogenetic tree of BN3 isolates produced from the Roary core gene alignment using RaxML, which utilizes a maximum likelihood bootstrap model. The tree has been rooted to the reference genome PAO1 (AE004091.2). Green node labels represent non-inhibitory antibody isolates. Red node labels represent inhibitory antibody isolates. **B)** A visual representation of core and accessory gene presence and absence data produced by Roary. Blue lines represent the presence of a gene, white lines represent the absence of a gene. The image was produced using Phandango (Hadfield *et al*., 2017).

the BN3 isolates were the result of variable gene expression giving rise to alterations in the inhibitory antibody target.

To further corroborate the relatedness of the isolates, the gene presence and absence data, serotype and the sequence type of each isolate was compared. Only minor differences could be observed following visual representation of the data using Phandango, which confirmed that the genomes of the isolates were highly similar (Figure 3.14B). However, no clear correlation between the gene presence and absence data and the grouping of the isolates could be identified.

Sequence typing of the BN3 isolates using the MLST 2.0 programme revealed that all genomes belonged to the sequence type ST-1328 (Appendix I). Serotyping of each isolate using the PAst programme was unable to confidently determine the serotype of the isolates. All isolates were assigned to the O11 serotype. However, the programme identified the presence of 2 OSA gene clusters within the BN3 isolate genomes and the percentage coverage over the reference OSA clusters was only 15.81%. Positive typing by the programme requires the coverage of the reference OSAs to be > 95% suggesting that the isolates were non-typeable (Thrane *et al*., 2016).

The assignment of non-typeable isolates to the O11 serotype was suggested to be the result of the presence of the O-antigen flanking genes *ihfB* and *wbpM* but the lack of the OSA cluster (Thrane *et al*., 2016). The presence of O-antigen genes was confirmed during manual inspection of the region flanked by *ihfB* and *wbpM*. As such, the DNA sequence for this region was extracted and compared to known *P. aeruginosa* sequences using blastn (Zhang *et* al., 2000). This search identified the BN3 isolate OSA cluster contained 93% sequence identity to

the *P. aeruginosa* O3 putative O-antigen biosynthesis gene cluster (AF498414.1). Patient PN3 sera also contained O3 specific IgG (determined by Western blot, Chapter 4 Figure 4.5B). These data suggested that the BN3 isolates produced LPS with the O3 serotype O-antigen (Appendix iv).

Together, the genotypic analyses of the BN3 isolates have confirmed that all of the isolates are very closely related and produced the same serotype of LPS. These data suggest that the isolates belong to the same clonal lineage of *P. aeruginosa,* sharing a common ancestor, which over time, and due to the accumulation of mutations within the core genome, diverged into the two closely related groups described above.

3.3 Discussion

3.3.1 The acute and chronic phenotypes of BN1 and BN3 *P. aeruginosa* isolates

Both patients PN1 and PN3 had been chronically infected with *P. aeruginosa* prior to receiving plasmapheresis. The work presented in this chapter was designed to determine whether isolates obtained following this treatment originated from the underlying chronic infection or were acquired following treatment.

Studies have shown that the laboratory reference isolate PAO1 forms swim colonies with a diameter ranging between 40-60 mm (Lindhout *et al*., 2009; Pérez-Gallego *et al*., 2016). Phenotypic analyses revealed that all BN1 isolates formed relatively small swim colonies (5-35mm), indicative of the chronic phenotype. Also characteristic of the chronic phenotype, the ability to produce biofilm was detected for all BN1 isolates with the greatest amount of biofilm produced by isolates BN1-5 and BN1-7. The detection of very little IgG towards the O-antigen in patient sera for isolates BN1-5 and BN1-7 suggested that these isolates had downregulated

or lost the ability to produce O-antigen (Figure 3.8A). This might explain the increase in biofilm formation exhibited by these isolates as previous work has implicated the loss of O-antigen with enhanced biofilm formation by *Escherichia coli* (Nakao *et al*., 2012), *Bradyrhizobium japonicum* (Lee *et al*., 2010) and *P. aeruginosa* (Murphy *et al*., 2014). The remaining isolates retained the production of long or very-long chain O-antigen, which is not typically a characteristic of the chronic phenotype. However, this suggests that *P. aeruginosa* does not necessarily need to lose the production of O-antigen during chronic infection.

Isolates obtained from patient PN3 varied greatly in the level of swimming motility, with colony diameters ranging from 5 mm to >90 mm. This suggested that patient PN3 could have been co-infected with two clonal lineages of *P. aeruginosa*, one with the acute phenotype and one with the chronic phenotype. Increased swimming motility appeared to correlate with low levels of biofilm formation implying that isolates BN3-4, BN3-5, BN3-8, BN3-14, BN3-15 and BN3-18 belonged to the lineage with the acute phenotype and the remaining isolates belonged to the lineage with the chronic phenotype. All of the BN3 isolates retained the production of long-chain O-antigen, further supporting the suggestion that *P. aeruginosa* strains isolated from chronic infections do not always lose the expression of long-chain O-antigen (Figure 3.8B).

A-band (common antigen) and B-band (O-specific antigen) production by the isolates was not investigated in this chapter. However, the presence of A-band O-antigen has been implicated in the increase in biofilm formation by *P. aeruginosa* (Murphy *et* al., 2014; Ruhal *et al*., 2015). The variation in the level of biofilm formation by the isolates could therefore be due to differences in A-band and B-band O-antigen expression. Three different LPS structures were identified for strains isolated from both patients. Previous studies have reported that truncation

of the core region of LPS can reduce the swimming and swarming ability of PAO1 (Lindhout *et al*., 2009). Although no distinct correlations between swim group and LPS structure group were made, characterisation and comparison of the chemical composition of the LPS produced by each isolate using mass spectrometry and nuclear magnetic resonance could identify modifications that impact motility. Understanding the exact chemical structure and modifications present within the LPS of each isolate could also identify potential target epitopes for inhibitory antibodies.

With the exception of BN1 isolates targeted by inhibitory antibody, the majority of the observed phenotypic variations did not correspond with the divergence of isolates presented in the phylogenetic trees (Figures 3.11A and 3.13A). This indicated that variations at the genomic level could have impacted other cellular processes and phenotypes not investigated within this chapter.

3.3.2 Pre- vs post- plasmapheresis isolate comparisons

Serotyping and sequence typing of isolates obtained from patient PN1 prior to plasmapheresis identified the presence of two serotypes and sequence types of *P. aeruginosa*. This suggested that patient PN1 was co-infected with at least two strains of *P. aeruginosa*. The work presented in this chapter suggests that one lineage might have been eradicated as a result of plasmapheresis. However, a definitive conclusion cannot be made as only one colony per time point was stocked with the exception of isolates BN1-6 and 7, which were isolated from the same sputum sample. Therefore, isolates belonging to both lineages might have been present at all time points. All *P. aeruginosa* isolates cultured post-plasmapheresis belonged to the same lineage suggesting that this was the dominant strain. Differences in the number of SNPs within the lineage suggest that within-host diversification of this strain had been occurring, which

might have arisen due to other environmental factors such as antibiotic regimes. The percentage of isolates with increased resistance to patient serum did not change for pre- and post-plasmapheresis BN1 isolates suggesting the treatment had no impact on this phenotype. This also indicates that retaining the target antigen in the absence of inhibitory antibody could still have a selective advantage for survival in the lung.

The observed differences in the core gene SNPs for BN3 isolates indicated that one clonal lineage had diverged into two groups. However, the data suggest this divergence was not the result of removal of inhibitory antibodies as isolates belonging to the 2 groups were detected both pre- and post-treatment. Therefore, the divergence of the parental clonal lineage was occurring prior to treatment and both subsequent lineages have persisted following treatment. A rise in the percentage of isolates with increased resistance to patient sera was observed when pre- and post-plasmapheresis isolates were compared. This could imply that the removal of inhibitory antibodies might select for isolates with the resistance phenotype. However, comparison of multiple strains isolated on the same day both before and after treatment would be required to confirm this conclusion.

Analysis of the sputum at each time point surrounding treatment would be useful to provide a further insight into the evolution of the *P. aeruginosa* population within the lung and the impact of plasmapheresis. Unfortunately, this was not possible during this study but sputum analysis should be included for future patients that are being considered for treatment with plasmapheresis. Several aspects of the sputum could be assessed including: viscosity, antibiotic concentration, antimicrobial peptide concentration, immune cell concentration and the microbiome composition. All of these aspects could create a selection pressure that would act on the *P. aeruginosa* population and influence the how the population evolves over time.

Collecting multiple *P. aeruginosa* isolates per time point will reveal the true population dynamics and will enable a more accurate assessment of the impact of plasmapheresis on the population. Analysis of the sputum microbiome will also reveal how the non-*Pseudomonas* population naturally changes over time, if plasmapheresis changes the bacterial composition of the lung microbiome and if these changes correlate with the diversification of the *P. aeruginosa* population.

The persistence of a single strain of *P. aeruginosa* over the course of infection is common in non-CF bronchiectasis patients. One study of 39 patients reported that 67% of patients were infected with a single persisting strain of *P. aeruginosa*, 20% experienced strain replacement and that in 8% the original isolate was temporarily displaced during the course of infection (Woo *et al*., 2018). Another study that analysed two or more *P. aeruginosa* isolates from the same sputum sample for 24 patients reported that multilineage infections were present in 29% of non-CF bronchiectasis patients and that the within-host diversification of a single lineage was the result of loss of function mutations (Hilliam *et* al., 2017).

An average of 1.29 base pair mutations over a 2-month period has been reported for *P. aeruginosa*, which represents a spontaneous mutation rate of 5.18×10^{-4} base pair mutations per genome per generation (Dettman *et al*., 2016). Another study that assessed within-host microevolution of *P. aeruginosa* in CF patients found the average yearly mutation rate within lineages to be 2.7 SNPs/year (Marvig *et al*., 2015). A further CF study found less than 10 SNPs between early infection stage strains isolated 2 years apart. However, a late stage strain (isolated ~15 years later) differed by 959 SNPs, which was attributed to a hypermutator strain (Cramer *et al*., 2011). The hypermutator phenotype of *P. aeruginosa* is extremely prevalent during chronic infection (Maciá *et al*., 2005). Both patients in the present study were infected

with *P. aeruginosa* isolates that had diverged from a single lineage. Comparison of the number of core-gene SNPs between individual isolates from the two divergent groups revealed the presence of approximately 1000 SNPs, which is characteristic of the hypermutator phenotype (Cramer *et al*., 2011). This phenotype has been associated with mutations in the mismatch repair system genes *mutL* and *mutS* (Maciá *et al*., 2005; Cramer *et al*., 2011). Further investigation of the hypermutator phenotype of the isolates could elucidate the difference in the number of core-gene SNPs between divergent groups. Further analysis of SNP location and the effects of corresponding mutation will also provide a deeper insight into the direction of the strain diversification.

In conclusion, this chapter has revealed that BN1 and BN3 isolates demonstrate some variability in their phenotypes. With the exception of BN1-2 and BN1-3, all BN1 isolates belong to the same clonal lineage as the underlying chronic infection. However, divergence of this lineage has occurred, which is characteristic of chronic infection with *P. aeruginosa*. Similar observations were made for BN3 isolates, with all isolates belonging to a single clonal lineage that has diverged. Plasmapheresis resulted in a decrease in the bacterial load following treatment. However, the phenotypic differences and clonal diversification do not appear to be the direct result of the treatment. The re-emergence of the strain that was present prior to plasmapheresis indicated that this strain was able to survive within a specific niche in the lung and remain dormant until the return of high titres of IgG2 provided a selective growth advantage for proliferation. Future in-depth analysis of WGS data and the acquisition of multiple isolates for all collection dates would be beneficial for confirming whether plasmapheresis resulted in the eradication of a strain.

CHAPTER 4

INVESTIGATING THE MECHANISM OF SERUM-MEDIATED KILLING OF *PSEUDOMONAS AERUGINOSA* BY HEALTHY SERA

4.1 Introduction

Previous work has confirmed that *P. aeruginosa* can be inherently resistant to healthy human sera (Young and Armstrong, 1972; Cevahir *et* al., 2006; Mikucionyte *et al*., 2014; Persyn *et al*., 2019). One study demonstrated that 91% of blood culture isolates and 79% of isolates from various clinical sites not associated with disease were resistant to serum from healthy donors (Young and Armstrong, 1972). Despite the high resistance to serum in blood culture isolates, the sensitivity of *P. aeruginosa* to healthy human sera is highly variable and dependent on clinical isolation site. Serum-resistant strains are commonly isolated from wounds, blood, urine and burns whereas those isolated from the sputum of cystic fibrosis (CF) patients are nearly always sensitive to serum (Muschel *et* al., 1969; Schiller and Hatch, 1983). More recently, contradictions to some of these observations were made by Mikucionyte *et al.* who found that a higher percentage of isolates obtained from wounds, urinary tract and respiratory tract infections were serum sensitive (Mikucionyte *et al.,* 2014).

It has long been recognised that the factor that contributes most to the serum resistance of many Gram-negative bacteria is the O-antigen moiety of LPS (Rowley, 1968). Bacteria that produce long chain O-antigen tend to be more resistant to the bactericidal effect of serum compared to strains that produce short or no O-antigen (Grossman *et al.,* 1987). The accepted mechanism for this resistance is based on two observations. First, it is believed that antibody binding to the O-antigen epitopes can activate the complement system. However, the activated complement proteins are bound at the distal end of the O-antigen and cannot access the bacterial cell surface to permit the insertion of the membrane attack complex (MAC) into the cell membrane, resulting in the release of MAC from the cell (Grossman *et al.,* 1987). Second, the long chain O-antigen produced by Gram-negative bacteria sterically hinders the access of bactericidal antibodies to their cell surface targets (Joiner *et al*., 1986).

Surprisingly, *P. aeruginosa* strains that chronically infect CF patients downregulate the production of long-chain O-antigen and exhibit the rough LPS phenotype (Malonado *et* al., 2016; Hancock *et al*., 1983). Such changes are due to selective pressures and occur in unison with other phenotypic changes that allow *P. aeruginosa* to persist within the host (Demirdjian *et* al., 2017). The loss or reduction in the length of O-antigen chains dampens the host immune response against the bacterium. However, this often results in isolates becoming more susceptible to human serum (Hancock *et al*., 1983; Schiller and Joiner, 1986).

Previous work in our laboratory, with *P. aeruginosa* strains isolated from chronically-infected non-CF bronchiectasis patients, revealed that all of the isolates screened in the study were sensitive to serum obtained from 20 healthy individuals. However, some of these isolates retained the production of long chain O-antigen (Wells *et al.,* 2014). As these previous findings contradict the current understanding of the role of long O-antigen in the serum resistance of Gram-negative bacteria, it was hypothesised that O-antigen production does not have a significant role in protecting *P. aeruginosa* against the bactericidal activity of healthy human sera. The aim of this chapter was to understand the mechanism behind serum-mediated killing of O-antigen positive isolates of *P. aeruginosa* by healthy individuals.

4.2 Results

4.2.1 Serum mediated killing of *P. aeruginosa* strains by healthy individuals

Previous work has revealed that O-antigen positive strains of *P. aeruginosa* isolated from patients with chronic infection can be sensitive to the bactericidal activity of healthy sera (Wells *et al*., 2014). Although associated with chronic infection, these isolates retained the production of long and very long-chain O-antigen. This contradicts the current understanding that *P. aeruginosa* no longer expresses O-antigen during chronic infections (Maldonado *et al*.,

2016). As the production of long chain O-antigen has been associated with an increase in resistance to the bactericidal activity of human sera, the serum-susceptibility of these O-antigen positive isolates were investigated.

Sera obtained from healthy individuals were screened for bactericidal activity against the well-described O-antigen positive *P. aeruginosa* strains, PAO1 and PA14 and seven strains isolated from non-CF bronchiectasis patients with chronic *P. aeruginosa* infection. Strains BN1-1, BN3-6 and B1 produced long chain O-antigen whereas strains B4, B6, B7 and B10 produced no detectable O-antigen. Strains were incubated in the presence of 50% individual healthy sera at 37°C for 180 min. Following incubation, the $\log_{10}$ change in viable cell number (CFU/ml) was calculated for each strain.

All strains of *P. aeruginosa* isolated from chronic infections were susceptible to serum-mediated killing by all healthy sera as no viable cells were recovered following incubation (Figure 4.1A). Bactericidal activity occurred irrespective of the level of O-antigen expression by these strains (Figure 4.1B). In contrast, variable resistance to serum killing by individual healthy sera was observed for strains PAO1 and PA14. The $\log_{10}$ change in the number of viable PAO1 cells ranged between a log increase of 1.2 and a log decrease of -3 whereas the $\log_{10}$ change in the number of viable PA14 cells ranged between a log decrease of -1.2 and -4. This suggested that strain PA14 was more susceptible to the bactericidal effect of healthy human sera (Figure 4.1A). Strain PAO1 produced O-antigen with a range of polysaccharide chain lengths whereas strain PA14 mainly produced long-chain O-antigen, which might explain the variation in serum susceptibility between these strains (Figure 4.1B).

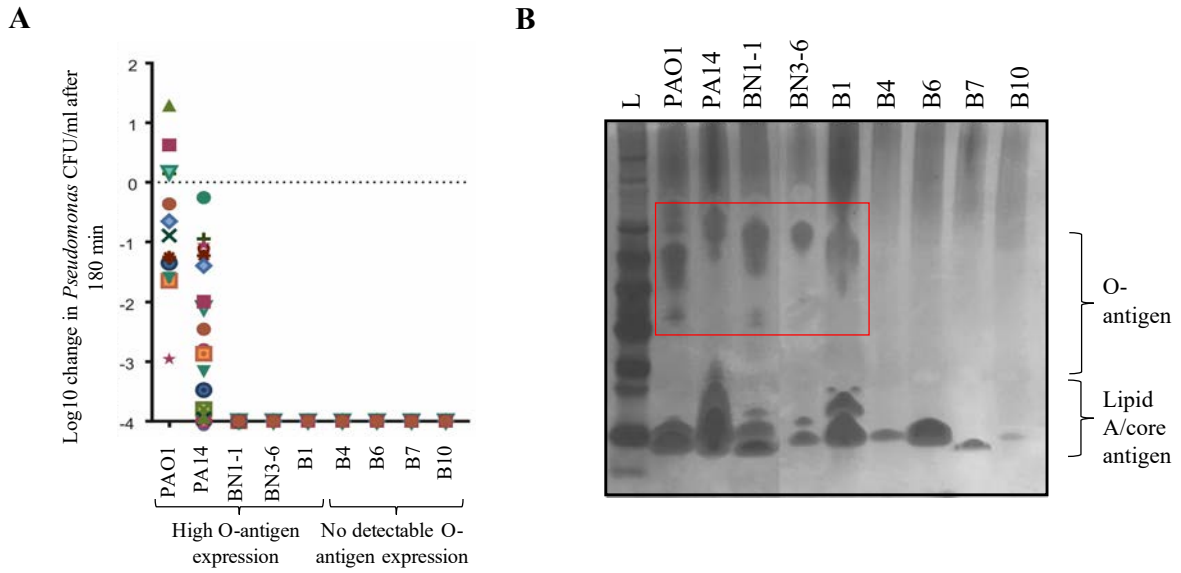


Figure 4.1 Serum-mediated killing of strains of *P. aeruginosa* by healthy individuals

The bactericidal activity of healthy sera against laboratory reference strains (PAO1 and PA14) and strains isolated from non-CF bronchiectasis patients with chronic *P. aeruginosa* infection (BN1-1, BN3-6, B1, B4, B6, B7, B10) were determined by serum bactericidal assays. Bacteria were incubated with sera for 180 min at 37°C and the $\log_{10}$ change in viable cell number (CFU/ml) was calculated for each strain. Negative values represent a decrease in viable cell number compared to the inoculum. Each data point represents an individual healthy serum sample. **B)** Silver stain analysis of LPS from *P. aeruginosa* isolates extracted by the hot-phenol method and separated on 4-12% bis-tris gels. O-antigen is depicted by the red box. L= SeeBlue® Pre-stained protein standard, 4-250 kDa.

In concurrence with the previous findings, the production of long chain O-antigen by isolates BN1-1, BN3-6 and B1 failed to protect these strains against the bactericidal activity of healthy sera. As this contradicts the current understanding of the role of long chain O-antigen in the protection of Gram-negative bacteria from the bactericidal properties of serum, the mechanism of killing by healthy sera was investigated further.

4.2.2 The role of complement in serum killing by HCS

The complement system plays a key role in providing innate immunity against invading pathogens. The activation of the complement system by bacterial antigens must occur close to the bacterial cell surface in order for the MAC to successfully insert into the membrane and elicit bacterial lysis. As the O-antigen positive strains of *P. aeruginosa* isolated from bronchiectasis patients were sensitive to serum-mediated killing by healthy individuals, the role of complement in the serum killing of these isolates was investigated.

To elucidate the importance of functional complement in the killing of the bronchiectasis isolates, sera from healthy individuals were pooled and the complement proteins within the pooled healthy serum (pooled-HCS) were inactivated by heating the serum to 56°C for 30 min. The bronchiectasis isolates were subsequently incubated in 50% heat-inactivated pooled-HCS at 37°C for 180 min and the $\log_{10}$ change in viable cell number was calculated for each strain.

Heat-inactivation of complement proteins within pooled-HCS abolished serum-mediated killing of all 7 of the bronchiectasis isolates (Fig. 4.2, red). An increase in viable cell number was observed for isolates BN3-6, B1, B6 and B10 indicating that these isolates were able to replicate within heat inactivated pooled-HCS whereas no change was identified for isolate

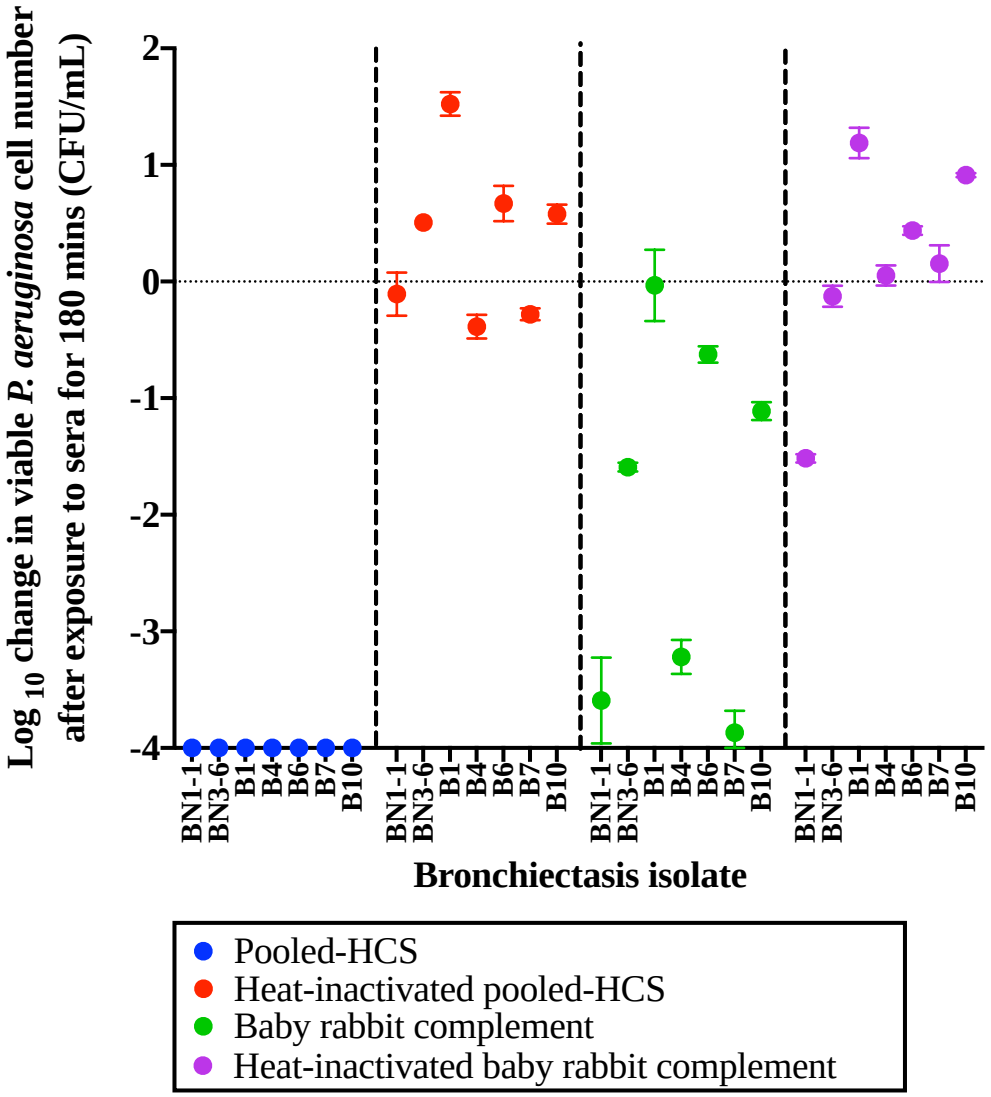


Figure 4.2 The role of complement in the serum-mediated killing of *P. aeruginosa* by healthy individuals

The bactericidal activity of pooled-HCS, heat-inactivated pooled-HCS, baby rabbit complement and heat-inactivated baby rabbit complement against 7 *P. aeruginosa* bronchiectasis isolates was determined by serum bactericidal assays. Following incubation in sera for 180 min, the $\log_{10}$ change in viable cell number was calculated for each isolate. Complement activity was inactivated by heating the sera at 56°C for 30 minutes, which was allowed to cool prior to use. Individual data points and error bars represent the mean and standard deviation of 3 biological replicates for each strain of *P. aeruginosa*. Negative values correspond to a decrease in the viable cell number of *P. aeruginosa*.

BN1-1. A minor decrease in viable cell number was observed for isolates B4 and B7 indicating that some bactericidal activity against these isolates had occurred. However, the lack of bactericidal activity confirmed that the killing of these isolates was complement-dependent.

As killing of all of the *P. aeruginosa* strains was dependent on the presence of functional complement, it was hypothesised that the route of complement activation might be an important factor in the killing of *P. aeruginosa* by healthy individuals. Activation by the classical pathway depends on the presence of antibody. To determine whether the bactericidal activity of complement was dependent on the activation by the classical pathway, each isolate was incubated in 50% commercially available 50% baby rabbit complement, which provided a source of complement without the presence of antibody. Following incubation at 37°C for 180 min, the remaining viable cell number was calculated.

Baby rabbit complement was able to elicit significant bactericidal activity against isolates BN1-1, B4 and B7. This implied that the bactericidal activity observed against these isolates was due to the activation of complement by either the alternative or lectin pathway. No change in viable cell number was detected for strain B1 and low to moderate killing was observed for isolates BN3-6, B6 and B10 (Figure 4.2, green). These data suggest that the bactericidal activity against isolate B1 was dependent on the presence of antibody and complement activation by the classical pathway. However, as baby rabbit complement was also able to cause a reduction in the viable cell number of isolates BN3-6, B6 and B10, complement activation by the alternative or lectin pathway was enough to elicit bactericidal activity.

The increased killing by pooled-HCS compared to baby rabbit complement indicated that the

presence of antibody enhanced this complement-dependent killing. In comparison to both heat-inactivated pooled-HCS and heat-inactivated baby rabbit complement, baby rabbit complement elicited significantly higher killing of all strains (2 way ANOVA, $P < 0.05$) However, heat-inactivated baby rabbit complement was still able to elicit a 1.5 log decrease in viable cell number of isolate BN1-1 (Figure 4.2). No correlation between bactericidal activity of baby rabbit complement and O-antigen production by the isolates was observed.

4.2.3 *P. aeruginosa* specific antibody detection in HCS

The demonstration that baby rabbit complement was unable to kill 4 of the 7 *P. aeruginosa* strains to the same extent as healthy control serum indicated that in order to elicit significant bactericidal activity, complement needed to be activated by the classical pathway. Antibody is known to promote serum-mediated killing through the activation of the complement system via the classical pathway. Thus, the presence of *P. aeruginosa* specific antibodies in healthy serum was investigated.

Pooled-HCS and pooled patient sera were probed for *P. aeruginosa* specific antibodies by enzyme-linked immunosorbent assay (ELISA) and Western immunoblot techniques. To identify the presence of antibody belonging to the isotypes IgG, IgA and IgM in the sera, 96-well ELISA plates were coated with LPS isolated from 8 *P. aeruginosa* O-antigen serotypes, which were subsequently incubated with a 1:100 dilution of sera followed by an anti-human IgGAM-AP secondary antibody. The binding of LPS-specific antibody was detected by measuring the absorbance at 405 nm following a 30 min incubation with a *p*-nitrophenyl phosphate substrate. To determine the binding profiles of anti-*P. aeruginosa* IgG, the LPS from 8 *P. aeruginosa* O-antigen serotypes and OMPs isolated from strains PAO1, PA14, BN1 and

BN3 were separated by SDS-PAGE and transferred to a PVDF or nitrocellulose membrane. Western immunoblotting was then used to visualise antibody binding.

O-antigen specific antibody to all 8 LPS serotypes was detected in pooled-HCS by ELISA. However, antibody titres against all LPS serotypes were lower in pooled-HCS when compared to the titres detected in pooled patient sera. With the exception of serotypes: O1 and O3; O1 and O11; and O6 and O11, no significant differences were observed between the level of detection of each serotype by pooled-HCS (Figure 4.3).

Western blot analysis also confirmed the presence of LPS-specific IgG antibodies in pooled-HCS. The most prominent antibody binding was observed against the O5 LPS serotype (Figure 4.4). IgG antibodies that bound to *P. aeruginosa* OMPs were also detected in pooled-HCS. Fewer bands were detected for membranes probed with pooled-HCS when compared to membranes probed with pooled patient serum (Figure 4.4). This implied that pooled patient sera contained IgG antibodies with increased specificity that were produced by the adaptive immune system whereas the less specific antibodies detected in in pooled-HCS might have been produced as part of the innate immune response.

As serotype-specific variations in the level of antibody detection was observed in pooled-HCS, the presence of *P. aeruginosa* specific antibody in the sera of 7 healthy individuals were compared by ELISA and Western immunoblot. It should be noted that the individual healthy sera originated from a separate cohort to that of the pooled-HCS.

ELISA examination revealed that HCS contained high titres of IgG to various O-antigen serotypes that was comparable to patient sera in some instances. LPS belonging to the serotypes

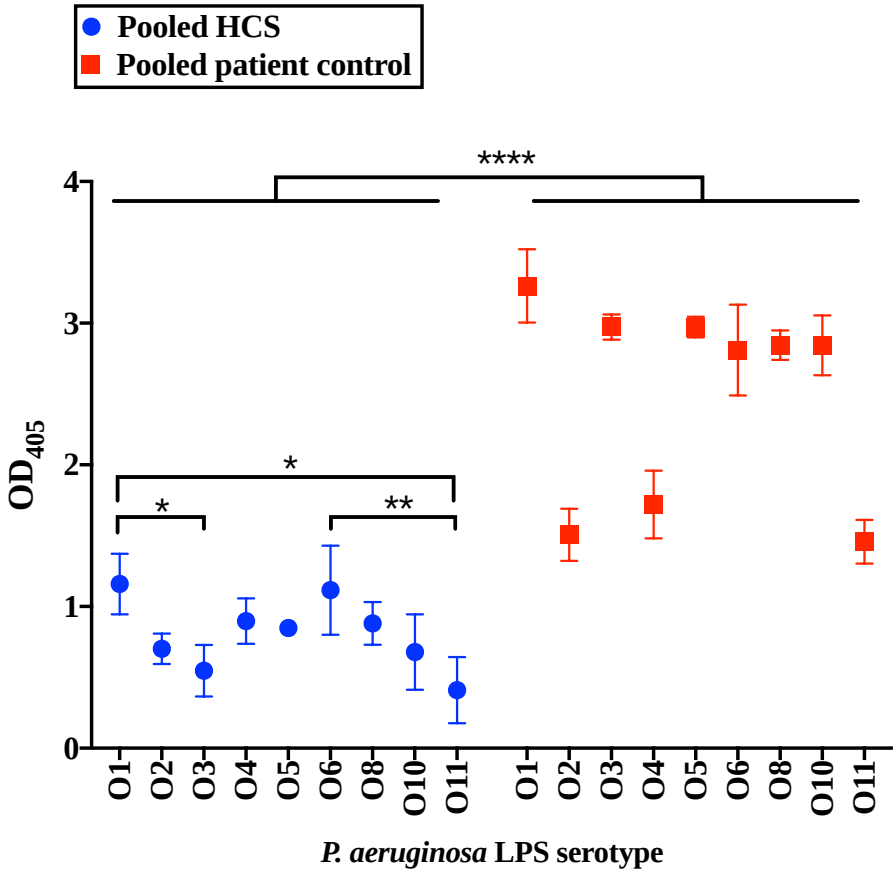


Figure 4.3 Detection of lipopolysaccharide-specific antibody in pooled-HCS by enzyme-linked immunosorbent assay

Pooled-HCS and pooled patient sera were probed for LPS specific IgG/IgA/IgM. Plates coated with 1 µg/ml LPS from 8 *P. aeruginosa* serotypes were incubated with 1:100 dilution of sera, washed and subsequently incubated in 1:2000 dilution of anti-human IgGAM-AP. The absorbance at OD_{405} was measured following washes and a 30 min incubation with SIGMA*FAST*™ p-Nitrophenyl phosphate substrate. Error bars represent the standard deviation of 3 individual replicates. A one-way ANOVA with multiple comparisons was used to determine significant differences in the level of antibody detection between serotypes in pooled-HCS. Significant differences were observed between serotypes O1 and O3; O1 and O11; and O6 and O11 (P = 0.0324, P = 0.0059 and P = 0.0102, respectively). A two-way ANOVA with multiple comparisons was used to determine significant differences in the level of antibody detection for each serotype between pooled-HCS and pooled patient serum.

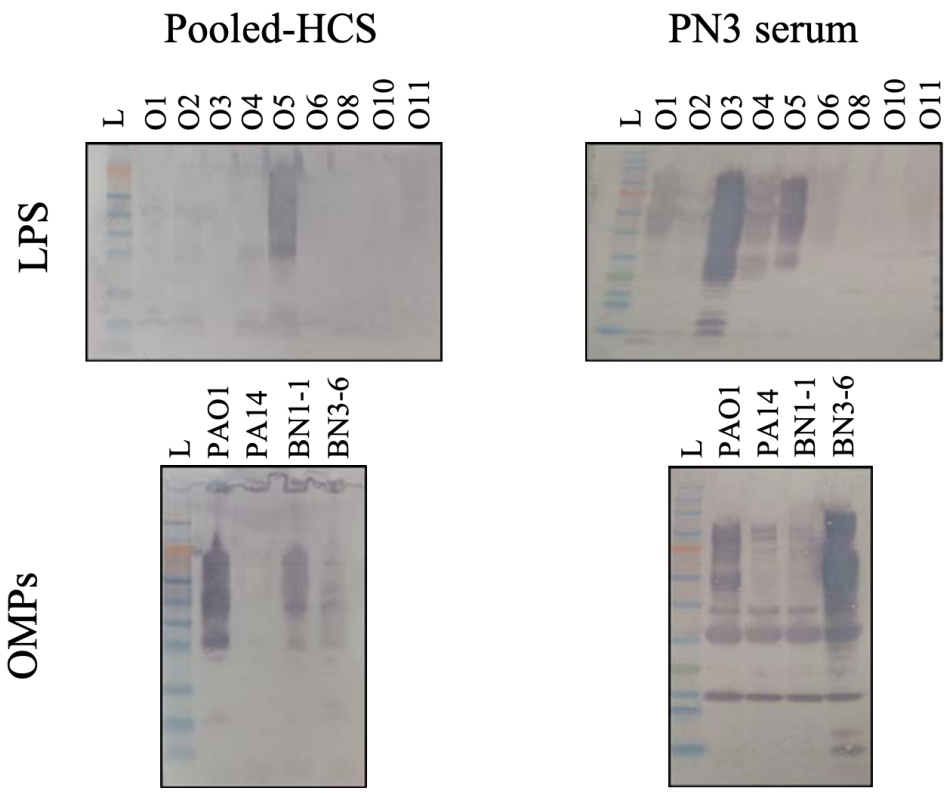


Figure 4.4 Detection of lipopolysaccharide and outer membrane protein specific IgG in pooled-HCS by Western immunoblot

Western immunoblot analysis of LPS prepared from *P. aeruginosa* serotypes O1-O11 and OMPs prepared from *P. aeruginosa* strains PAO1, PA14 and non-CF bronchiectasis isolates BN1-1 and BN3-6. Membranes were probed with a 1:2000 dilution of pooled-HCS and 1:5000 patient PN3 sera followed by a 1:10000 dilution of goat anti-human IgG-AP. Antibody binding was visualised by the addition of BCIP/NBT-purple liquid substrate. L= SeeBlue® Pre-stained protein standard, 4-250 kDa (pooled-HCS) BLUeye Pre-Stained Protein Ladder, 10-245 kDa (PN3 serum). Blots are representative of two independent experiments.

O1, O3, O6 and O11 elicited the highest IgG response in healthy sera (Figure 4.5).

Western blot analysis also revealed that individual healthy sera contained IgG antibodies to at least one LPS serotype. Western blotting provided a visual representation of the variation in serotype and epitope recognition between healthy individuals for both LPS and OMPs (Figure 4.6). The IgG bound mainly to the O-antigen region of the LPS. However, binding to the lipid A and core regions were observed for serum obtained from healthy individuals HCS1, HCS2, HCS3 and HCS7 (Figure 4.6). In contrast to high levels detected in patient serum, very low levels of anti-LPS IgA were detected in healthy serum, (Figure 4.5). Moderate titres of anti-O antigen IgM were found in healthy sera. In general, healthy sera contained increased titres of IgM compared to patient sera (Figure 4.5). The presence of *P. aeruginosa* specific antibodies in all HCS sera tested suggested that antibody contributed to the serum-mediated killing of *P. aeruginosa*.

4.2.4 The role of specific antibody in serum killing by healthy sera

IgG is the most abundant antibody class within sera (Vidarsson *et al*., 2014) and it is produced in response to infection in order to aid in the clearance of invading pathogens (Stavnezer. And Schrader, 2014). As healthy sera contained *P. aeruginosa* specific antibody that predominantly belonged to the IgG isotype, the role of IgG in serum-mediated killing of the 7 chronic bronchiectasis isolates was further investigated.

To determine whether IgG antibodies were able to elicit significant bactericidal activity against *P. aeruginosa*, IgG was purified from pooled-HCS by passing serum through protein G and protein A columns in order to remove other antibody isotypes. IgG antibodies were eluted from

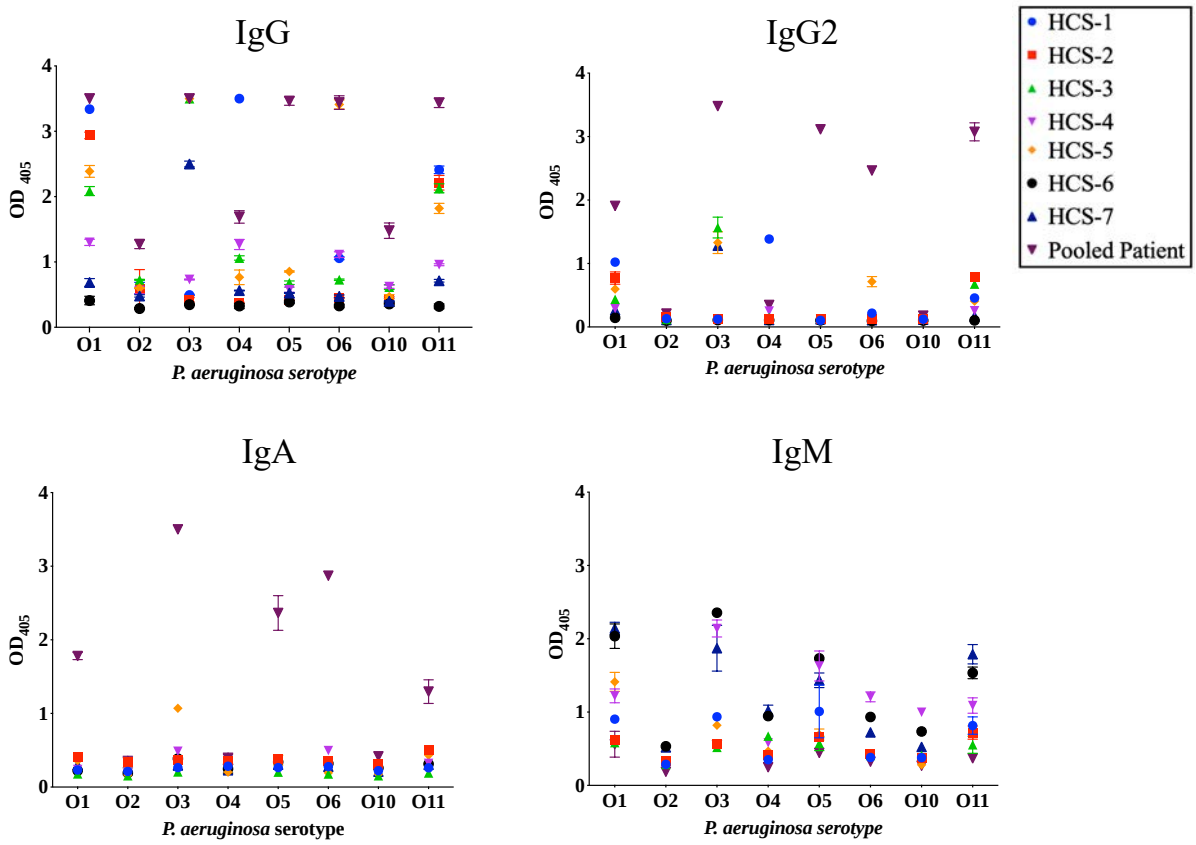


Figure 4.5 Detection of lipopolysaccharide-specific antibody in individual HCS by enzyme-linked immunosorbent assay

Sera from 8 healthy individuals and pooled patient sera were probed for LPS O-antigen specific IgG, IgG2, IgA and IgM. Plates coated with 1 µg/ml LPS from 8 *P. aeruginosa* serotypes were incubated for 1 h with 1:100 dilution of sera, washed and subsequently incubated for 1 h with a 1:2000 dilution of anti-IgG/IgA/IgM-AP or mouse anti-human IgG2. For AP- conjugated secondary antibody plates, the absorbance at OD_{405} was measured following washes and a 2 h incubation with SIGMA*FAST*™ p-Nitrophenyl phosphate substrate. The IgG2 secondary antibody plate was washed and incubated with a 1:2000 dilution of goat anti-mouse IgG-AP for 1 h and washed and developed as above. Error bars represent the standard deviation of 3 technical replicates.

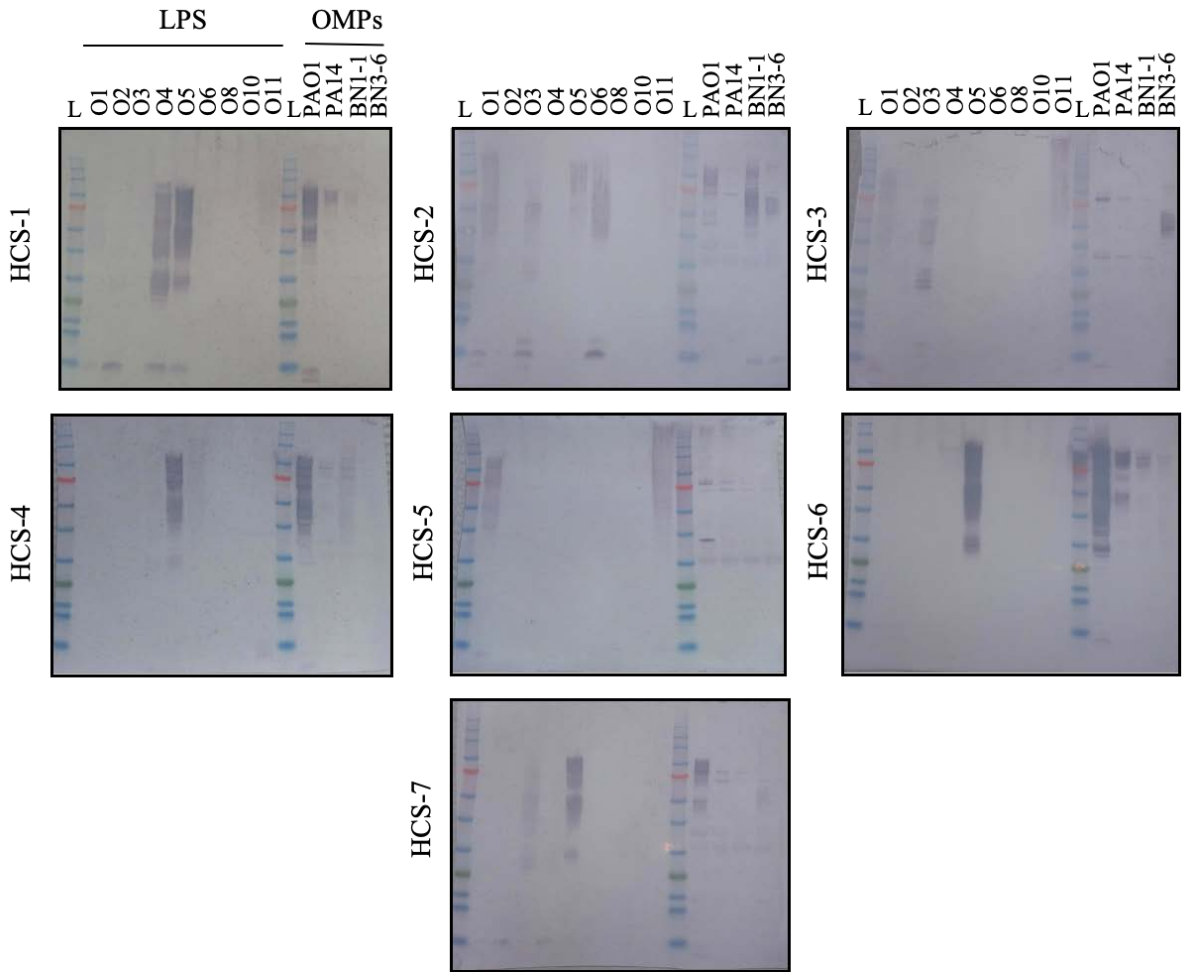


Figure 4.6 Detection of lipopolysaccharide and outer membrane protein specific IgG in individual HCS by Western immunoblot

Western immunoblot analysis of LPS prepared from *P. aeruginosa* serotypes O1-O11 and OMPs prepared from *P. aeruginosa* strains PA01, PA14 and non-CF bronchiectasis isolates BN1 and BN3. Membranes were probed with a 1:2000 dilution of healthy control sera (HCS-1, HCS-2, HCS-3, HCS-4, HCS-5, HCS-6 and HCS-7) followed by a 1:10000 dilution of goat anti-human IgG-AP. Antibody binding was visualised by the addition of BCIP/NBT-purple liquid substrate. L = BLUeye Pre-Stained Protein Ladder, 10-245kDa. Blots are representative of two independent experiments.

the column and concentrated back to *in* vivo concentrations using a 5 kDa viva spin column. The bactericidal activity of the purified IgG alone or in combination with baby rabbit complement was then assessed by serum bactericidal assays.

Purified IgG was unable to elicit bactericidal activity against any of the 7 *P. aeruginosa* isolates. The number of viable BN3-6, B1, B4 and B7 cells remaining after a 3-hour incubation in the presence of purified IgG remained relatively constant and an increase in viable cell number was observed for isolates BN1-1, B6 and B10 (Figure 4.7, green). The addition of purified IgG to baby rabbit complement had no significant effect on the killing of isolates BN1-1 and B7 when compared to baby rabbit complement alone. However, it should be noted that baby rabbit complement was able to kill isolates BN1-1 and B7 in the absence of purified antibody. The bactericidal activity of baby rabbit complement against isolates BN3-6, B1 and B10 was enhanced by the addition of purified IgG whereas an inhibitory effect was observed against isolates B4 and B6. Isolates B4 and B6 exhibited a 1-1.5 log decrease in viable cell number when compared to baby rabbit complement alone (Figure 4.7, purple).

The addition of purified IgG to baby rabbit complement resulted in the enhanced killing of BN3, B1 and B10 suggesting that the mechanism behind sufficient serum-mediated killing of these isolates by HCS was both complement and antibody dependent. Although isolate B6 was sensitive to pooled-HCS, it was not killed significantly by baby rabbit complement or the combination of baby rabbit complement and purified IgG. Killing of this isolate might therefore be dependent on the presence of other serum antibodies including IgA and IgM. Once again, no correlation between the production of O-antigen and enhanced bactericidal activity was observed as the addition of purified IgG to baby rabbit complement enhanced the killing of

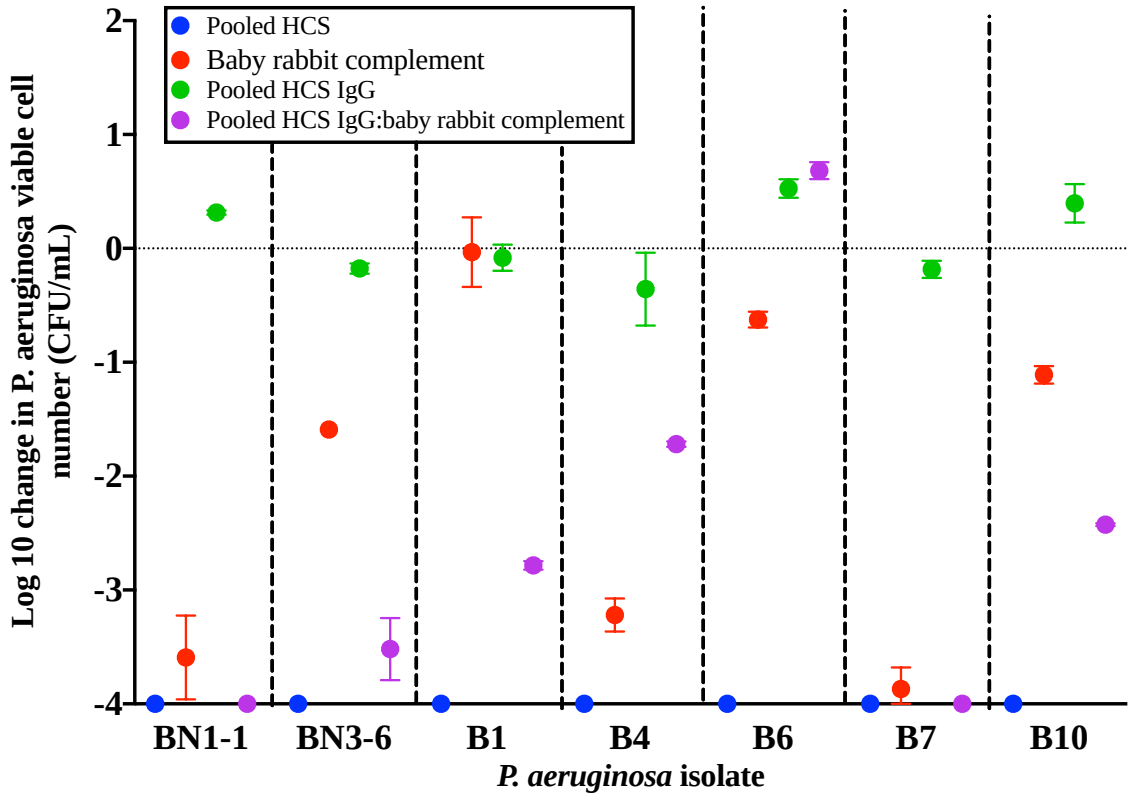


Figure 4.7 The bactericidal activity of purified IgG against *P. aeruginosa*

The bactericidal activity of purified IgG against the 7 non-CF bronchiectasis isolates was determined by serum bactericidal assay. Isolates were incubated in the presence of pooled-HCS, baby rabbit complement, pooled-HCS purified IgG and a 50:50 mix of purified IgG with baby rabbit complement as a source of complement. Following incubation at 37°C for 180 min, the $\log_{10}$ change in viable cell number was calculated for each strain. Individual data points and error bars represent the mean and standard deviation of 3 biological replicates.

isolates that produced long chain O-antigen (BN1-1, BN3-6, B1) and those isolates that lacked O-antigen production (B4, B6, B7, B10).

As the lack of bactericidal activity elicited against isolate B6 by baby rabbit complement and purified IgG implied that other antibody isotypes might have a significant role in the bactericidal activity of human sera, the specific importance of IgG in serum killing was determined. IgG antibodies were depleted from pooled-HCS using protein G and A columns and the IgG-depleted serum was subsequently screened for bactericidal activity against the 7 *P. aeruginosa* bronchiectasis isolates. Following incubation in IgG-depleted pooled-HCS, the $\log_{10}$ change in viable cell number was calculated for isolate.

IgG-depleted pooled-HCS was able to elicit significant bactericidal activity against all 7 isolates (Figure 4.8). IgG-depleted sera still contained complement and other antibody isotypes including IgA and IgM. The combination of antibody and complement in IgG-depleted pooled-HCS also enhanced the killing of strains BN3-6, B1, B4, B6 and B10 when compared to baby rabbit complement alone. This implied that antibodies belonging to other isotypes were also responsible for activating the complement system via the classical pathway resulting in the successful killing of *P. aeruginosa* by healthy sera.

4.2.5 The inhibitory potential of IgG isolated from healthy sera

Previous work has associated high titres of O-antigen specific IgG with the inhibition of serum-mediated killing of several Gram-negative strains of bacteria. Here, the addition of IgG to baby rabbit complement resulted in the reduced killing of two of the isolates. As high levels of IgG, in particular to the O4 and O5 serotypes, were detected in several of the individual HCS, the

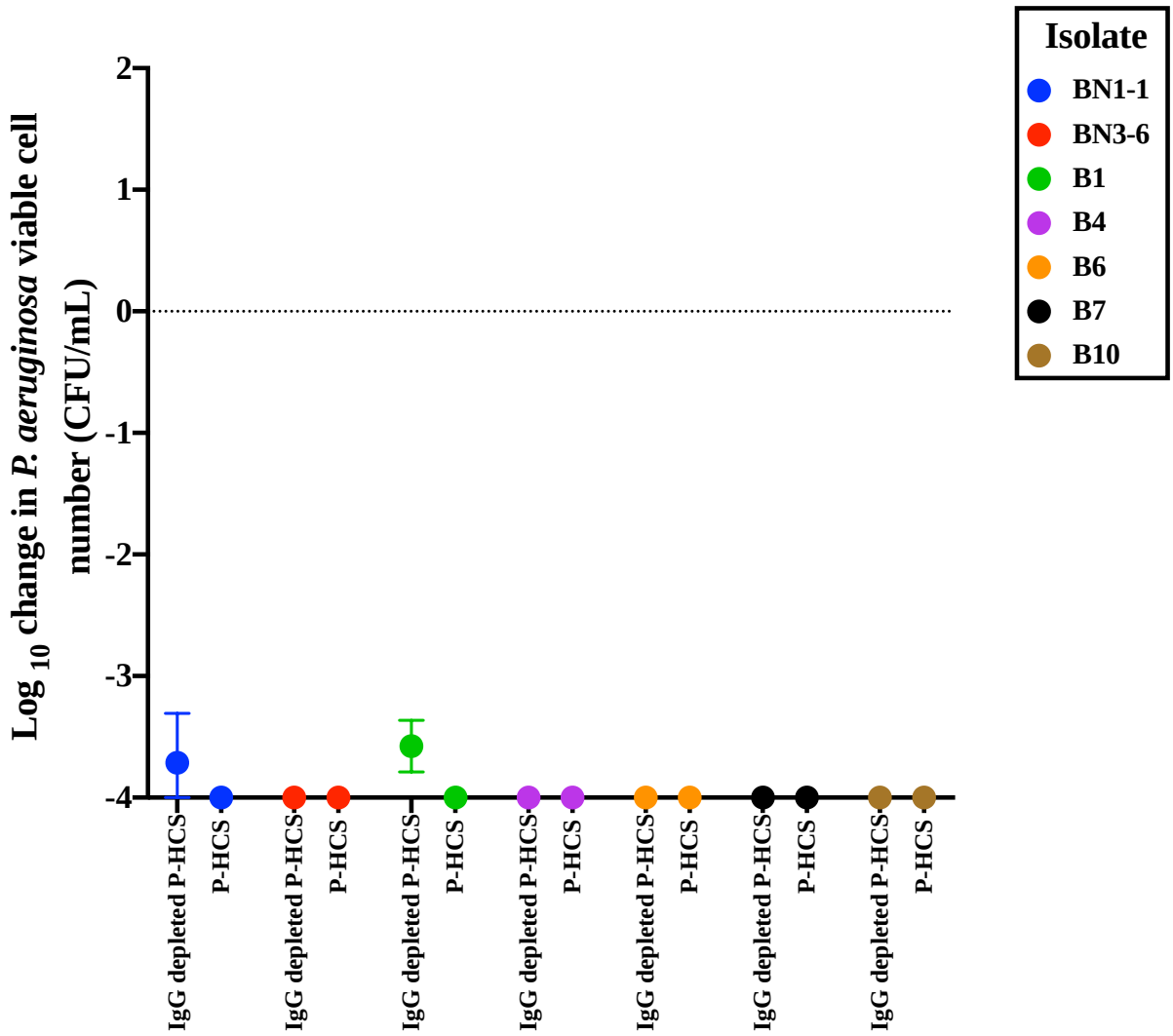


Figure 4.8 The bactericidal activity of IgG-depleted pooled-HCS against *P. aeruginosa*

The bactericidal activity of IgG-depleted pooled-HCS was determined by serum bactericidal assay. The 7 bronchiectasis isolates were incubated in IgG-depleted pooled-HCS at 37°C for 180 min and the $\log_{10}$ change in viable cell number was determined. Individual data points and error bars represent the mean and standard deviation of 3 biological replicates for each strain.

inhibitory potential of IgG in serum from healthy individuals was investigated.

The O5 O-antigen used in this work was isolated from the *P. aeruginosa* strain PAO1, which is generally considered to be resistant to human sera. As previously determined by Western blot analysis, serum obtained from healthy individual 1 (HCS1) contained high levels of serotype O4 specific IgG (Figure 4.6). This serum was unable to elicit significant bactericidal activity against the O4 serotype keratitis strain KO4 when screened by bactericidal assay (Figure 4.9, blue circle). In order to determine the inhibitory potential of IgG antibodies produced by healthy individuals, IgG was purified from HCS1 using protein G and A columns, concentrated back to *in vivo* concentrations and used in subsequent bactericidal assays. Pooled-HCS, HCS1 purified IgG, IgG-depleted HCS1 sera and CF patient PCCF82 sera were screened for bactericidal activity against *P. aeruginosa* isolate KO4.

Serum bactericidal assays revealed that pooled-HCS was unable kill strain KO4 (Figure 4.9, red circle). However, serum obtained from CF patient PCCF82 was able to completely kill KO4 and contained low detectable amounts of anti-O4 serotype specific IgG as determined by Western blot analysis (Figure 4.9A, orange circle; Figure 4.9B). To assess the inhibitory potential of HCS1 purified IgG, patient PCCF82 sera was combined 50:50 with either HCS1 purified IgG or IgG-depleted HCS1 sera, which was subsequently screened for bactericidal activity against *P. aeruginosa* strain KO4.

A 2.7 log decrease in bactericidal activity was observed when patient serum was combined with HCS1 purified IgG compared to patient serum alone (Figure 4.9A, brown circle and orange circle, respectively). No inhibition of serum-mediated killing was observed when patient serum

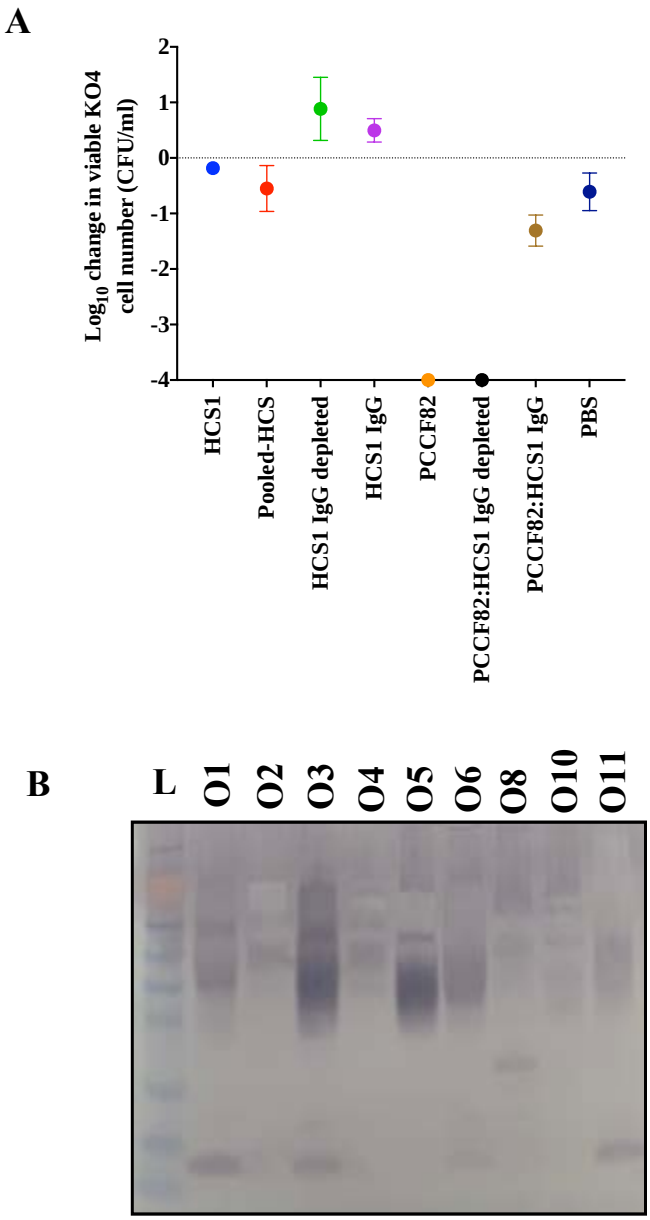


Figure 4.9 Inhibitory effect of IgG purified from healthy serum. A) Pooled-HCS and CF patient (PCCF82) sera were screened for bactericidal activity against keratitis isolate O4 (KO4) by serum bactericidal assays. Pooled-HCS was unable to kill KO4, so IgG purified from HCS1 was tested for its ability to inhibit serum-mediated killing of KO4 by patient serum. Patient sera was mixed 50:50 with HCS1 purified IgG or HCS1 IgG depleted sera. After incubation at 37°C for 180 min, the $\log_{10}$ change in viable cell number (CFU/ml) was calculated for each strain. Data points and error bars represent the mean and standard deviation of 3 biological replicates. **B)** The presence of serotype-specific IgG in PCCF82 sera as determined via Western blot analysis. Antibody binding was visualised with the addition of BCIP/NBT-purple liquid substrate. L= SeeBlue® Pre-stained protein standard, 4-250 kDa.

was combined with IgG-depleted HCS1 (Figure 4.9A, black circle). This confirmed that IgG was the antibody isotype responsible for the inhibitory effect and that IgG antibodies produced by healthy individuals can inhibit the serum-mediated killing of *P. aeruginosa*.

4.3 Discussion

The production of long O-antigen (smooth LPS) by Gram-negative bacteria has long been associated with increased resistance to the bactericidal activity of human serum. This has been demonstrated over a range of organisms including: *Escherichia coli* (Porat *et al*., 1986); *Salmonella spp* (Grossman *et* al., 1987; Powlak *et* al., 2017); *P. aeruginosa* (Hancock *et al*., 1983); *Brucella abortus* (Eisenschenk *et al*., 1999; Mancilla, 2016); and *Klebsiella pneumoniae* (Ciurana and Tomas, 1983; Doordujin *et al*., 2016). The work presented in this chapter confirmed that the O-antigen negative strains of *P. aeruginosa* isolated from chronic infections (strains B4, B6, B7 and B10) were sensitive the bactericidal activity of pooled-HCS. However, other strains that were isolated from chronic infections still retained the production of long-chain O-antigen (strains BN1-1, BN3-6 and B1) and were sensitive to pooled-HCS. These results indicate that the production of long-chain O-antigen does not guarantee that a strain will be resistant to human sera. Conversely, serum resistant strains of *P. aeruginosa* that produce no or very little O-antigen have been identified (Meadow *et al*., 1978; Hancock *et al*., 1983). Although the production of long-chain O-antigen was confirmed by silver stain analysis, the density of the O-antigen production was not determined for each isolate. Sparse distribution of long chain O-antigen across the cell might still permit the access of bactericidal serum components to the cell surface and render the strains susceptible to serum-mediated killing (Browning *et al*., 2013).

The killing of chronic *P. aeruginosa* isolates was complement-dependent, irrespective of the

level of O-antigen production. Baby rabbit complement was able to elicit significant bactericidal activity against isolates BN1-1, B4 and B7 (Figure 4.2, green), indicating that complement activation by either the alternative or lectin pathway can be sufficient to kill *P. aeruginosa*. Previous work has demonstrated that rough LPS strains of *Klebsiella pneumoniae* activate the complement system via the classical pathway, whereas smooth LPS strains activate the alternative pathway (Albertí *et al*., 1993; Doordujin *et al*., 2016). The same conclusion could not be made for the *P. aeruginosa* strains used in this study as, although isolate BN1-1 produced smooth LPS and was killed to some extent in the absence of antibody suggesting the activation of the alternative or lectin pathway, smooth LPS strains BN3-6 and B1 were significantly killed when complement was activated, in the presence of antibody, by the classical pathway. Similarly, rough LPS strains B4 and B7 were killed by complement alone, whereas rough LPS strains B6 and B10 required the presence of antibody. Therefore, a correlation between the mechanism of activation of the complement system and the production of smooth or rough LPS could not be made.

Strain specific variations might explain the differences in the mechanism of complement activation observed for the *P. aeruginosa* isolates. Mannose-binding lectins, which activate the lectin pathway, are able to bind to a variety of different sugars. Several of these sugars are present in *P. aeruginosa* LPS (Lam *et al*., 2011). Sugar composition differences in the O-specific LPS as well as potential variations in the amount of common polysaccharide antigen present may affect the level of complement activation by the lectin pathway.

The outer membrane protein OprF has also been recognised as a *P. aeruginosa* surface located target for complement C3b deposition (Mishra *et al*., 2015). OprF is highly conserved within strains of *P. aeruginosa* (Moghaddam *et al*., 2017) and differences in the level of expression

of OprF or the amount of C3b access to this protein could affect the amount of complement activation by the alternative pathway. Therefore, comparing the genome sequences of the bronchiectasis isolates to identify the presence or absence of known immunogenic surface exposed proteins and comparing the expression of these proteins using RT-PCR could identify key differences in complement activation.

P. aeruginosa can also produce proteases that are able to break down complement proteins (Gellaty and Hancock, 2013). The alkaline protease AprA can block the classical and lectin pathways through the cleavage of complement protein C2 (Laarman *et al*., 2012) suggesting that any variation in the production of proteases by these strains could determine the route of activation of the complement system. Confirming the presence or absence of these proteases in the bronchiectasis and comparing the expression by RT-PCR could again explain the variations in susceptibility to complement in the absence of antibody.

Although it was observed that *P. aeruginosa* can be killed by healthy sera in an antibody-independent manner, the work presented in this chapter confirms that in the presence of IgG, activation of the classical pathway can enhance the serum-mediated killing of *P. aeruginosa* by healthy sera (Figure 4.2, green, isolates BN3-6, B1 and B10). Similarly, IgA and IgM in combination with complement can also elicit significant killing of *P. aeruginosa* (Figure 4.8). Although present in a much lower concentration than IgG in human sera, IgM is normally present in its pentameric form and is a strong activator of C1q and IgA is capable of activating the complement system by the lectin pathway, which could explain the unaffected bactericidal activity of IgG-depleted pooled-HCS (Roos *et al*., 2001; Czajcowsky and Shao, 2009). To further assess the role of antibody subtype in the protection against *P. aeruginosa,* heat-inactivated IgG-depleted serum could be combined with baby rabbit complement and the

bactericidal activity compared with that of baby rabbit complement combined with purified IgG. This would confirm if other antibody subtypes can enhance complement killing of *P. aeruginosa*.

Finally, LPS-specific IgG antibodies purified from healthy sera were able to inhibit serum-mediated killing of *P. aeruginosa*. Most studies have only observed inhibitory antibodies in patients with active bacterial infections (MacLennan *et al*., 2010; Wells *et al*., 2014; Coggon *et al*., 2018). The presence of inhibitory antibody in healthy sera suggests that an active infection is not necessarily required for the production of inhibitory antibody and that environmental exposure to bacterial antigens could be sufficient to stimulate the production of these antibodies. Although patient HCS-1 had an elevated LPS- specific IgG titre, further analysis of donor HCS-1 serum to determine total antibody concentrations would be useful to identify any abnormalities that could affect bactericidal activity.

Inhibitory antibodies in healthy individuals have been reported previously (Trebicka *et al*., 2013). The authors identified one healthy donor serum sample that lacked bactericidal activity against *S.* Typhimurium SL1344 and could block the bactericidal activity of bactericidal sera when mixed in equal volumes. The inhibitory effect was mediated by IgM antibodies that were specific towards the SL1344 LPS. The authors reported that IgG, IgA and IgM titres in the inhibitory sera were consistent with normal concentrations and those found in bactericidal sera, which contradicts the current proposed mechanism of inhibitory antibody (Wells *et al*., 2014). The mechanism of inhibition involved a reduction in complement deposition onto the bacterial cell surface, which was suggested to be the result of abnormal antibody glycosylation. (Trebicka *et al*., 2013). The mechanism of inhibition was not investigated in this study. However, confirmation of antibody binding and complement deposition on the surface of KO4

by HCS-1 and comparison of the inhibitory antibody structure with the bactericidal antibody structure could elucidate the origin of inhibitory antibody and identify antibody modifications that block complement activation.

In conclusion, the work presented in this chapter demonstrates that killing of *P. aeruginosa* by healthy individuals occurs in a complement-dependent manner and the presence of specific antibody enhances serum-mediated killing. Both O-antigen negative and O-antigen positive isolates are able to activate complement in an antibody-dependent manner by the classical pathway and/or in an antibody-independent manner by the alternative/lectin pathways. Therefore, the observed differences in the mechanism of complement activation might be due variations in the expression of surface exposed structures and secreted products rather than the presence or lack of O-antigen. The work presented in this chapter has also revealed that IgG present in healthy serum has the potential to inhibit serum-mediated killing of *P. aeruginosa* and provides an insight into the origin of inhibitory antibodies.

CHAPTER 5

THE CONSTRUCTION OF A TRANSPOSON MUTANT LIBRARY IN A CLINICAL ISOLATE OF *PSEUDOMONAS AERUGINOSA*

5.1 Introduction

Transposon-insertion libraries in combination with high-throughput sequencing technologies have provided a useful tool for defining the essential and conditionally-essential genome of *P. aeruginosa* strains PAO1 and PA14 (Juhas, 2015). Such libraries have been extensively studied and have been used to define the growth condition-specific essential genome of strain PAO1 on a variety of media including: LB, M9 minimal medium, brain heart infusion medium, CF sputum, and human serum (Lee *et al*., 2015). The essential genome of strain PA14 for growth in LB and for mucosal colonisation in a mouse model of neutropenia has also been defined (Skurinik *et al*., 2013). Recently, the conditionally-essential genomes for clinical strains isolated from wound, blood, eye, lung and urinary tract infections were determined for growth in the following media: LB, M9 minimal medium, CF sputum, urine, and serum (Poulsen *et al*., 2019). From this study, the authors concluded that the core essential genome of *P. aeruginosa* species consists of approximately 321 genes.

Several studies have provided evidence to support the current hypothesis that the binding of inhibitory antibodies to the LPS block the access of bactericidal serum components to their targets. Antibodies that block serum-bactericidal activity against *Salmonella* Typhimurium were revealed to target the LPS, block killing when present in high-titres, fail to block killing when the O-antigen chain length was decreased and belong to the IgG isotype (MacLennan *et al*., 2010). Inhibitory antibodies directed against the O-antigen of *P. aeruginosa* and uropathogenic *E. coli* were subsequently identified as the IgG2 subclass (Wells *et al*., 2014; Coggon *et al*., 2018). However, the role of specific epitopes, other cell-surface targets, and other antibody isotypes in the inhibition of complement-mediated killing of *P. aeruginosa* is not completely understood.

We hypothesised that a similar transposon library could be used to provide a detailed understanding of the bacterial targets of inhibitory antibodies. For example, if the hypothesised mechanism of inhibition is correct, loss of the genes encoding the O-antigen should render the bacterium susceptible to complement-dependent killing when the bacterium was incubated with inhibitory antibody. Previously constructed *P. aeruginosa* transposon-mutant libraries could not be used to elucidate the genes that are essential for the survival of *P. aeruginosa* in the presence of inhibitory antibodies because the antibodies are strain-specific and the strains are not respiratory isolates of *P. aeruginosa*. Therefore, the aim of this chapter was to construct a transposon mutant library in a clinically relevant strain of *P. aeruginosa* that was isolated from a non-CF bronchiectasis patient with inhibitory antibodies. Furthermore, screening of the library has the potential to reveal significant insights into the biology of a *P. aeruginosa* respiratory isolate that could in turn lead to improved treatment options for patients.

5.2 Results

5.2.1 Identification of a suitable *P. aeruginosa* isolate

In order to construct a transposon library that could subsequently be used to identify genes that are essential for the survival in human sera containing inhibitory antibody, a suitable clinical strain of *P. aeruginosa* needed to be identified. For reasons outlined previously, it was a requirement that the strain was isolated from a non-CF bronchiectasis patient with inhibitory antibodies. *P. aeruginosa* that were isolated from patients PN1, PN3, P1 and P3 were considered as candidates for the parental strain.

5.2.1.1 Assessing the kanamycin and chloramphenicol resistance phenotypes of *P. aeruginosa* strains BN1-2, BN1-9 and BN3-6

Sera from patients PN1 and PN3 had previously been characterised and were the most readily available (Wells *et al*., 2017). Therefore, isolates obtained from these patients were considered as primary candidates for the parental strain of the transposon insertion library. As transposons containing a kanamycin or chloramphenicol resistance marker were available, the susceptibility to these antibiotics were determined for isolates BN1-2 and BN1-9, which were obtained from patient PN1, and BN3-6, which was obtained from patient PN3.

Overnight cultures were adjusted to an OD_{600} of 2 with LB broth and 10 µl of each isolate was streaked onto LB plates supplemented with 160, 320, and 640 µg/ml of kanamycin or chloramphenicol. The plates were incubated at 37°C for approximately 18 h and subsequently examined for growth. All three isolates were able to grow on the plates supplemented with 640 µg/ml of kanamycin. However, isolates BN1-2 and BN1-9 were susceptible to 160 µg/ml of chloramphenicol and isolate BN3-6 was susceptible to 640 µg/ml of chloramphenicol (Figure 5.1). As the isolates were resistant to kanamycin, the transposon that contained the kanamycin resistance marker could not be used to create the transposon insertion library. Isolates obtained from patient PN1 were more susceptible to chloramphenicol than the isolate obtained from patient PN3. As the strain of *E. coli* containing the chloramphenicol resistance transposon was susceptible to the concentration of chloramphenicol required to kill isolate BN3-6, this isolate was deemed unsuitable to use as the library parental strain. Subsequently, it was revealed that isolate BN1-2 was susceptible to the bactericidal activity of serum obtained from patient PN1 whereas isolate BN1-9 contained inhibitory antibody (Chapter 3, Figure 3.7A); isolate BN1-9 was selected as the library parental strain of *P. aeruginosa*.

5.2.1.2 Construction and assessment of the mini-Tn5 chloramphenicol transposome

A mini-Tn5 chloramphenicol transposome was not commercially available, therefore one

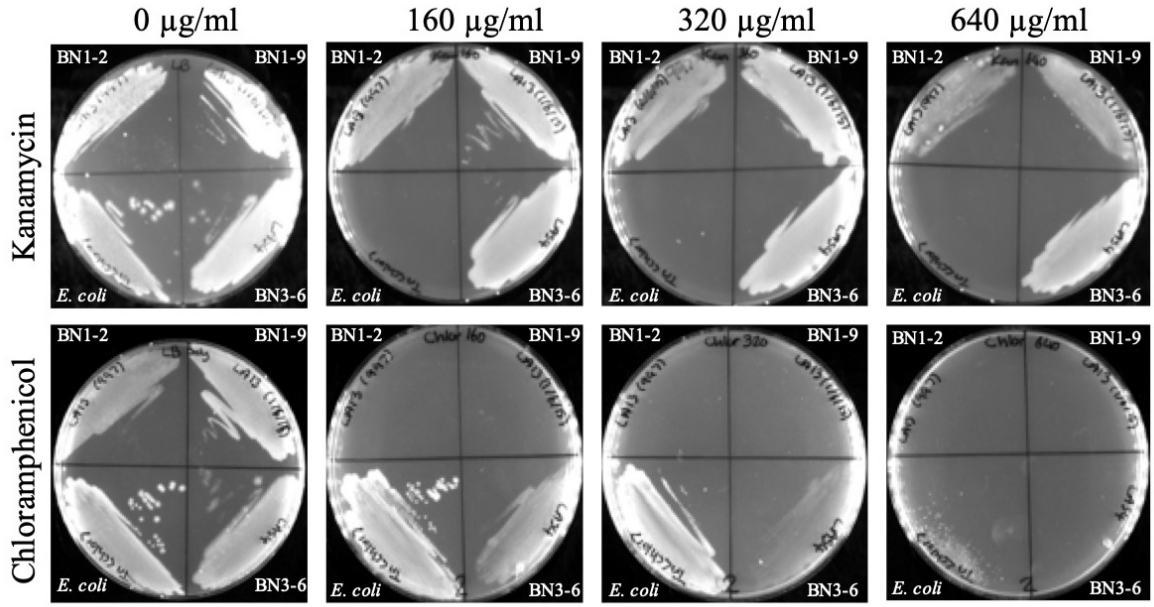


Figure 5.1 The susceptibility of *P. aeruginosa* strains isolated from patients PN1 and PN3 to kanamycin and chloramphenicol

Overnight cultures of each isolate were adjusted to an OD_{600} of 2 with LB broth and 10 µl of each isolate was streaked onto LB plates supplemented with 160, 320, and 640 µg/ml of kanamycin or chloramphenicol. An *E. coli* chloramphenicol transposon mutant was used as a positive control to indicate susceptibility to kanamycin and as a negative control to indicate the resistance to chloramphenicol. The plates were incubated at 37°C for approximately 18 h and subsequently examined for growth. Plate layout clockwise from the top left quadrant: BN1-2; BN1-9; BN3-6; *E. coli* chloramphenicol transposon mutant.

needed to be constructed in house. In order to create the mini-Tn5 chloramphenicol resistance transposome, chromosomal DNA was extracted from an *E. coli* BW25113 chloramphenicol transposon insertion mutant and the chloramphenicol resistance transposon was amplified from the genomic DNA (Figure 5.2). The sequence of the purified transposon DNA was checked for mutations and the transposome was subsequently constructed by incubating the transposon with the EZ-Tn5 transposase at 25°C for 30 min before storage at -30°C.

In order to confirm that the transposome was functional and to determine the insertion efficiency of the transposome, 0 µl, 0.2 µl, 0.5 µl or 1 µl of the transposome was introduced into *E. coli* BW25113 competent cells by electroporation. Following recovery for 2 h at 37°C, 100 µl of neat or 1:10 diluted cells were spread onto LB plates containing 20 µg/ml of chloramphenicol, which were incubated overnight at 37°C to select for cells that had incorporated the transposon into the genome. Plates were examined for growth and the number of colonies were compared between each volume of transposome introduced. To confirm that the transposon had been successfully inserted into the genome, several colonies were patched onto a new chloramphenicol containing LB plate and the genomic DNA for was checked for the presence of the transposon by PCR.

The *E. coli* formed a lawn on LB only plates, confirming that the cells remained viable during the transformation process (Figure 5.3A, column 1). The number of colonies for each volume of transposome introduced and dilution of cells plated were too numerous to count, which implied that the transposome was functional, highly efficient and that the volume of transposome introduced did not significantly affect the transformation efficiency (Figure 5.3A, column 2 and 3). All of the colonies picked for confirmation were able to grow on the second

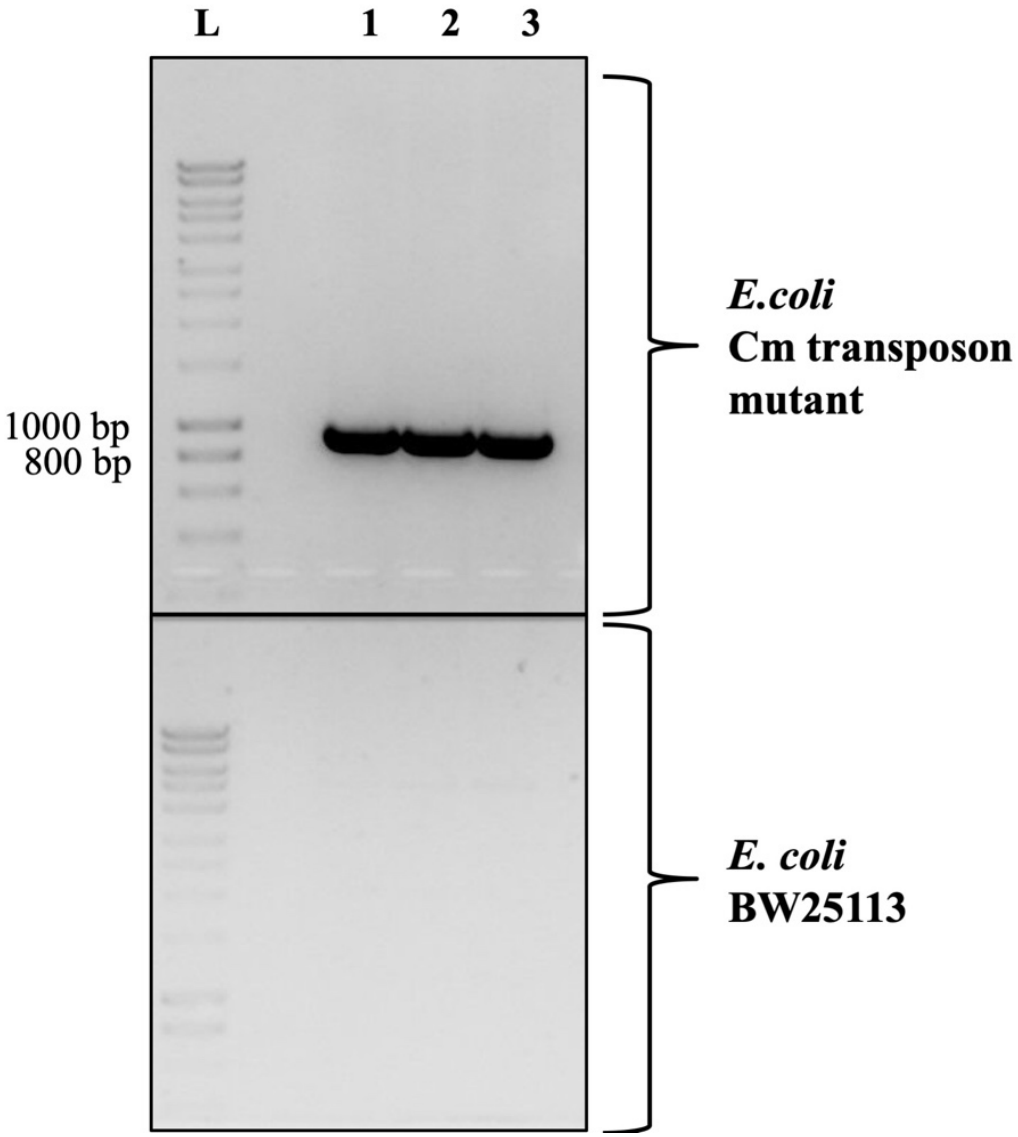


Figure 5.2 Isolation of the mini-Tn5 chloramphenicol resistance transposon DNA

The mini-Tn5 chloramphenicol transposon was amplified out of the *E. coli* chloramphenicol (Cm) transposon mutant by PCR using primers TnCm forward and TnCm reverse, which were designed to target the 19 bp mosaic end sequence and part of the transposon sequence (Chapter 2, Table 2.7). DNA gel electrophoresis with a 1% agarose gel containing 2% midori green was used to determine the size of the PCR product. 20 µl of each PCR product was loaded into the gel and 5 µl of Bioline HyperLadder™ 1 kb was used as the DNA standard (L). DNA bands were visualised using the GeneSys image acquisition software for the Syngene gel doc system. Genomic DNA from the wild type *E. coli* BW25113, which does not contain the chloramphenicol transposon, was used as a negative control.

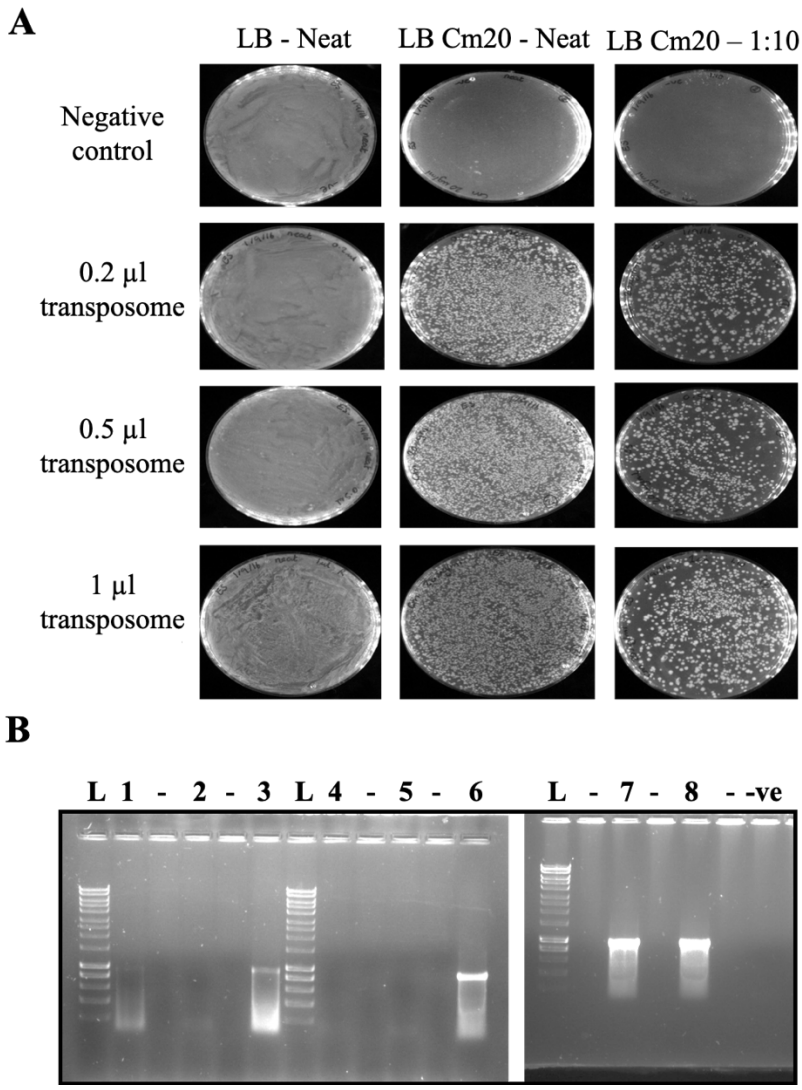


Figure 5.3 Confirmation of mini-Tn5 chloramphenicol resistance transposon insertion into the *E. coli* BW25113 genome

A) *E. coli* BW25113 was made competent by incubating cells to the mid-logarithmic stage of growth followed by a series of washes in decreasing volumes of 4°C 10% glycerol. Competent cells were incubated with 0, 0.2, 0.5 or 1 µl of the EZ-Tn5 chloramphenicol custom transposome for 30 min on ice prior to electroporation. Following recovery for 2 h at 37°C in SOC medium, 100 µl of undiluted or a 1:10 dilution of cells were spread onto LB only plates or LB plates containing 20 µg/ml of chloramphenicol. Plates were incubated for ~18 h at 37°C and inspected for growth. **B)** The incorporation of the mini-Tn5 chloramphenicol transposon in to the *E. coli* BW25113 genome was confirmed by PCR using primers TnCm forward and TnCm reverse (Chapter 2, Table 2.7). A PCR product corresponding to the 924 bp transposon was detected. The PCR confirmation of 4/8 individual transformants are depicted in lanes 3,6 ,7 and 8. BW25113 WT was used as a negative control (-ve). L= Bioline HyperLadder™ 1 kb.

LB plate containing chloramphenicol, which confirmed that the mutants were resistant to chloramphenicol and colony PCR confirmed that the transposon had been successfully inserted into the genome of 4/8 transformants (Figure 5.3B). The DNA sequence of the PCR products were checked using the TnCm primers listed in chapter 2, table 2.7. This confirmed that a functional and efficient transposome had been constructed that could subsequently be used to create the *P. aeruginosa* isolate BN1-9 transposon insertion library.

5.2.1.3 Assessment of the 'quick' method for making electrocompetent *P. aeruginosa* cells

The standard method to make electrocompetent *E. coli* cells typically involves the inoculation of fresh medium with an overnight culture, which is incubated until the culture has reached the mid-logarithmic phase of growth (OD_{600} of 0.4 to 0.6). This is followed by a series of washes with decreasing volumes of ice cold 10% glycerol. Isolate BN1-9 grew much more slowly than *E. coli* and took between 4 and 5 h to reach the mid-logarithmic phase of growth (Chapter 3, Figure 3.3B). The freezing of freshly prepared electrocompetent cells can result in a decrease in the efficiency of transformation reactions. Therefore, the standard method was considered as an impractical method for the preparation of electrocompetent BN1-9 cells and the 'quick' method previously described for the preparation of electrocompetent *P. aeruginosa* was assessed (Choi *et al*., 2006). As the authors had described this method for the *P. aeruginosa* strain PAO1, the method was assessed using strain PAO1.

To make PAO1 electrocompetent, 6 ml of overnight culture was pelleted, washed twice with room temperature 300 mM sucrose and resuspended in a final volume of 100 µl of room temperature 300 mM sucrose. The competent cells were incubated with 100 ng of pBRRMCS-5 plasmid DNA for 30 min and permeabilised by electroporation enabling the access of the plasmid DNA into the cells. Following a 2 h recovery period in SOC medium at 37°C, 100 µl

of a dilution series ranging from 10^{-1} to 10^{-6} were spread onto LB plates containing 100 μg/ml of gentamicin and incubated at 37°C for up to 24 h. Plates were then examined for the presence of transformed cells.

Colonies were observed for all dilutions of the transformation reaction (Figure 5.4) This indicated that PAO1 had successfully been transformed with the pBRRMCS-5 plasmid DNA and that the transformation efficiency was 6 x 10^8 CFU/μg plasmid DNA. This efficiency was 100-fold lower than the transformation efficiencies previously reported (Choi *et al*., 2006). However, the authors also observed a 10-fold difference in the transformation efficiency between plasmids, which suggested that the decrease in transformation efficiency observed could have been caused by the use of the pBRRMCS-5 plasmid. As efficient electrocompetent PAO1 cells were successfully produced, the 'quick' method was subsequently used for the preparation of BN1-9 electrocompetent cells.

5.2.1.4 Transformation of BN1-9 with the mini-Tn5 chloramphenicol resistance transposome

As a functional transposome had been constructed and a suitable method to prepare electrocompetent *P. aeruginosa* cells had been identified, the ability to transform BN1-9 cells with the transposome was assessed by a small-scale transformation reaction. Competent BN1-9 cells were produced, as above, using overnight cultures. However, cells were mixed with 0.2 μl of transposome rather than plasmid DNA. Following a 2 h recovery incubation in SOC medium, 100 μl of 1:10 diluted cells were spread onto LB plates containing 0, 100, 150 or 200 μg/ml of chloramphenicol and incubated at 37°C for up to 24 h. The plates were then examined for the presence of transformed colonies.

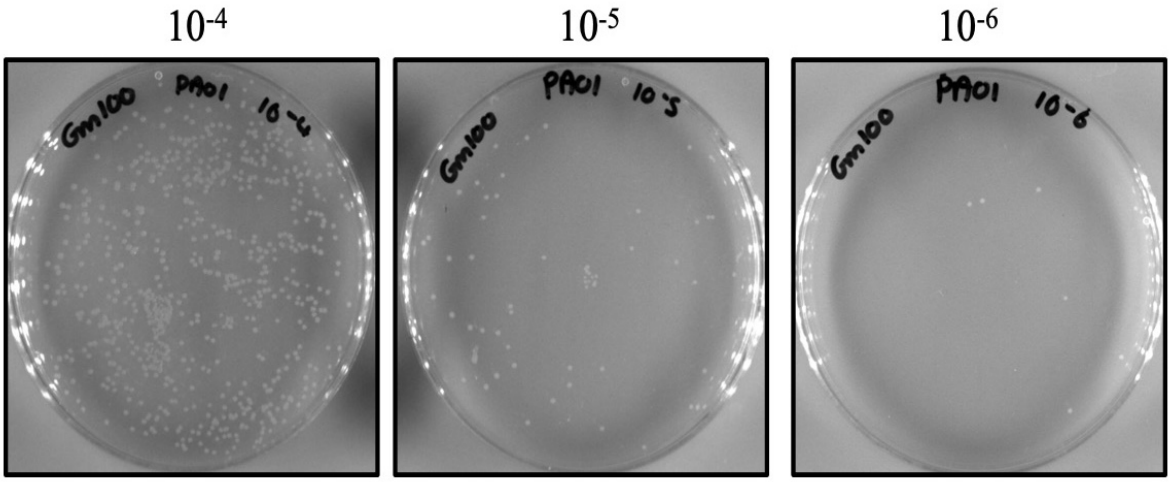


Figure 5.4 Assessment of the transformation efficiency of competent PAO1 cells prepared by the 'quick' method

Overnight cultures of strain PAO1 were made electrocompetent by washing cell pellets twice with room temperature 300 mM sucrose and resuspended to a final volume of 100 µl with 300 mM sucrose. Competent cells were transformed by adding 100 ng of plasmid pBRRMCS-5 DNA to the cells followed by electroporation at 2.5 kV. The cells were recovered for 2 h at 37°C in 1 ml of SOC medium and transformed cells were selected by spreading 100 µl of a dilution series ranging from 10^{-1} to 10^{-6} onto LB plates containing 100 µg/ml of gentamicin and incubated at 37°C for up to 24 h.

The bacteria formed lawns on the LB-only plates, which confirmed that they remained viable after they were made competent and following electroporation (Figure 5.5, top panel). A mixture of lawn and individual colonies were observed on the plates containing 100 and 150 μg/ml of chloramphenicol whereas individual colonies were observed on plates containing 200 μg/ml of chloramphenicol (Figure 5.5, left column). However, similar growth patterns were observed on all of the negative control plates, which indicated that the isolate had become spontaneously resistant to chloramphenicol during the transformation process (Figure 5.5, right column). As isolate BN1-9 became spontaneously resistant to the selection marker, it was deemed unsuitable to be the parental strain for the transposon insertion library.

5.2.1.5 Evaluating the susceptibility of *P. aeruginosa* strains isolated from patients with inhibitory antibodies to trimethoprim

Strains of *P. aeruginosa* isolated from chronic lung infections often exhibit the hypermutator phenotype, which can result in strains becoming spontaneously resistant to antibiotics (Maciá *et al*., 2005). Spontaneous resistance to chloramphenicol has also been described for other bacterial species including *E. coli, Myxococcus xanthus* and *Proteus mirabilis* indicating that chloramphenicol was not a suitable antibiotic resistance marker for the transposon (Carone *et al*., 2015; Yang *et al*., 2019; Charles *et al*., 1985). As the BN1-9 strain became spontaneously resistant to chloramphenicol, *P. aeruginosa* strains B1 and B3, which were isolated from patients P1 and P3 respectively, were subsequently considered as potential parental strains for the library. As a mini-Tn5 trimethoprim transposon was available, the susceptibility of isolates B1 and B3 to trimethoprim was determined.

Isolates were streaked onto plates containing 0, 25, 50, 100 or 200 μg/ml of trimethoprim. The plates were incubated at 37°C for approximately 18 h and subsequently examined for growth.

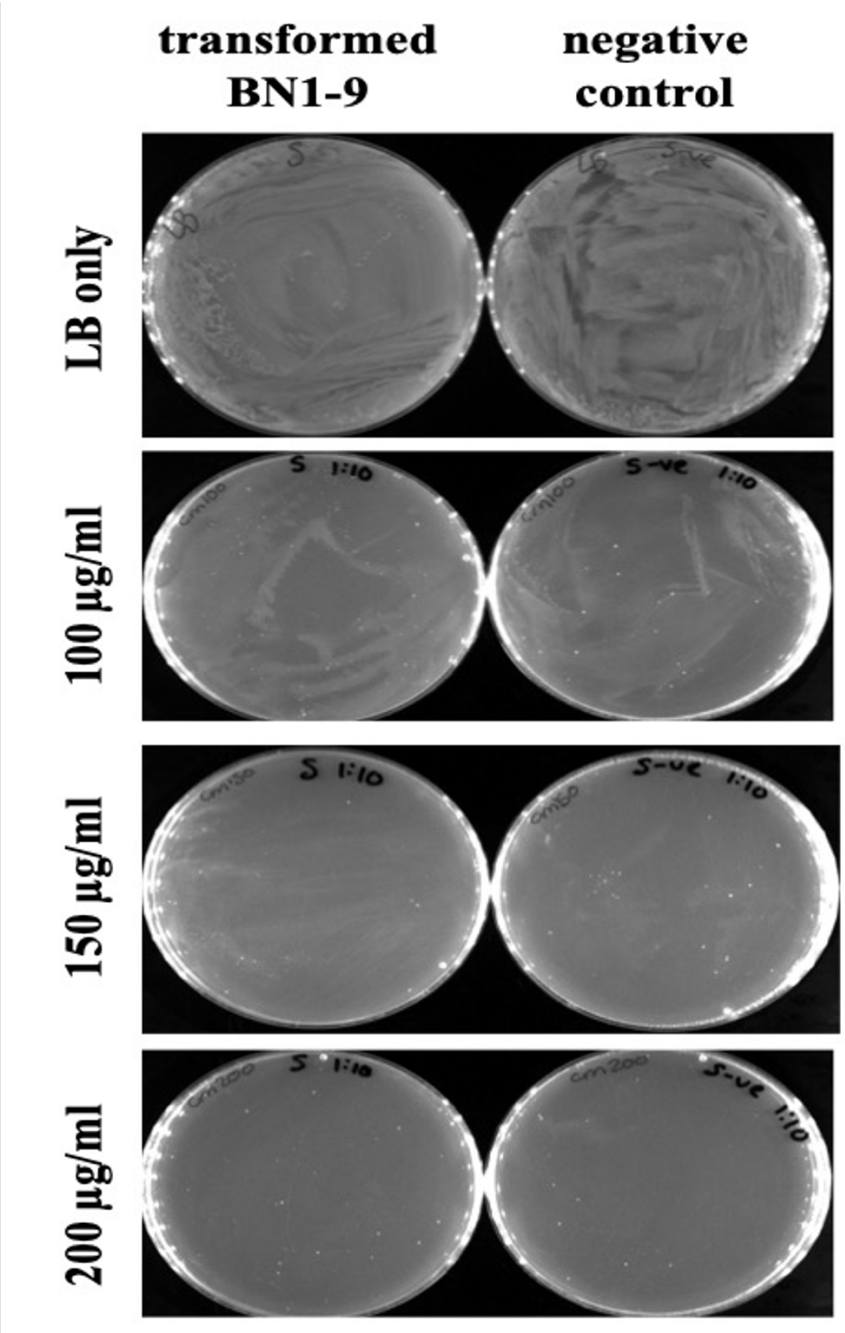


Figure 5.5 Test transformation of isolate BN1-9 with the mini-Tn5 chloramphenicol resistance transposome

Competent cells were prepared using overnight cultures of strain BN1-9 by washing cells twice with room temperature 300 mM sucrose and resuspending in a final volume of 100 µl of 300 sucrose. A volume of 0.2 µl of EZ-Tn5 chloramphenicol custom transposome was introduced into the cells by electroporation. No transposome was added to the negative control samples. Following a 2 h recovery period, transformed cells were selected for by spreading 100 µl of 1:10 diluted culture onto plates containing 0, 100, 150 or 200 µg/ml of chloramphenicol and incubated at 37°C for up to 24 h.

The amount of growth on each plate was categorised into 1 of 6 groups: very thick growth; thick growth; many single colonies; few single colonies; fine film; and no visible growth (Figure 5.6).

Both isolates B1 and B3 were more susceptible to trimethoprim when compared to isolate BN1-9. Isolate B1 was the most susceptible to trimethoprim and all isolates were susceptible at 200 μg/ml. Although isolate B1 was more susceptible to trimethoprim, reduced growth was observed on the LB only plate indicating that this isolate might have a growth defect (Figure 5.6). As all 3 *P. aeruginosa* isolates were susceptible to trimethoprim, the mini-Tn5 trimethoprim transposon was considered for further testing.

5.2.1.6 Transformation of isolates BN1-9, B1 and B3 with the mini-Tn5 trimethoprim transposome

In order to assess the suitability of the trimethoprim transposon for the construction of the *P. aeruginosa* transposon insertion library, a mini-Tn5 trimethoprim transposome was created as above by combining the EZ-Tn5™ transposon with the EZ-Tn5™ transposase (see section 5.2.2). Isolates BN1-9, B1 and B3 were made competent using the 'quick' method as described above and *E. coli* BW25113 was made competent using the standard method (see section 5.2.1.4). Following a 30 min incubation at room temperature with or without the trimethoprim transposome, cells were electroporated, recovered for 2 h in SOC medium and 100 μl of undiluted culture was spread onto LB plates containing 200 μg/ml of trimethoprim. The plates were incubated for up to 24 hours at 37°C and examined for colony formation.

Many colonies were observed on the *E. coli* BW25113 positive control plate indicating that the trimethoprim transposome had been constructed successfully (Figure 5.7). However, the

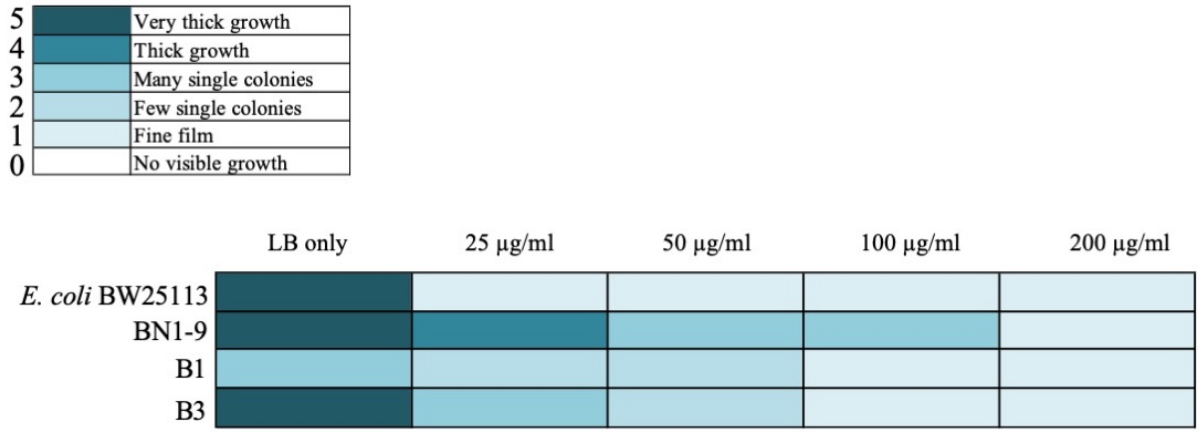


Figure 5.6 Testing the trimethoprim susceptibility of *P. aeruginosa* strains

Overnight cultures of isolates BN1-9, B1 and B3 were adjusted to an OD_{600} = 2 with LB broth and 10 µl of each isolate was streaked onto LB plates supplemented with 25, 50, 100 and 200 µg/ml of trimethoprim. The *E. coli* strain BW25113 was used as a positive control to indicate susceptibility to trimethoprim. The plates were incubated at 37°C for approximately 18 h and subsequently examined for growth. The growth of each strain was graded from 0-5, 0 indicating no visible growth and 5 indicating very thick growth.

efficiency of the transformation was approximately 10-fold lower than that of the chloramphenicol chloramphenicol transposome. Colonies were observed on the negative control plates for strains BN1-9 and B1 whereas no colonies were observed on the negative control plate for isolate B3 (Figure 5.7). This once again implied that strains BN1-9 and B1 had spontaneously developed an increase in resistance to trimethoprim and were therefore not good candidates for the parental library strain. As isolate B3 did not spontaneously become resistant, this isolate was selected for further assessment.

5.2.1.7 Identification of patient sera containing inhibitory antibody against strain B3

The primary purpose of creating a *P. aeruginosa* transposon library in a clinical strain isolated from a non-CF bronchiectasis patient was to be able to expose the library to patient serum that contained inhibitory antibodies, which specifically target the parental strain. Sequencing of the output library would indicate which genes are essential for the survival of the strain in the presence of inhibitory antibody and help to provide a mechanistic insight into the role of inhibitory antibodies in serum resistance.

As serum from patient P3 was not readily available, the available sera obtained from patients PN1, PN3, LATS, LAWC, LAPS and LANC were screened for the presence of inhibitory antibodies against isolate B3. As high titres of IgG antibodies are associated with the inhibitory phenotype. the relative titre of LPS-specific IgG in each of the serum samples was determined by ELISA and sera that contained elevated titres of IgG were screened for the inhibitory phenotype by serum bactericidal assay.

ELISA analysis revealed that sera obtained from patients PN1, PN3 and LAWC all contained high titres of LPS-specific IgG antibodies. Serum from patient LAWC contained the greatest

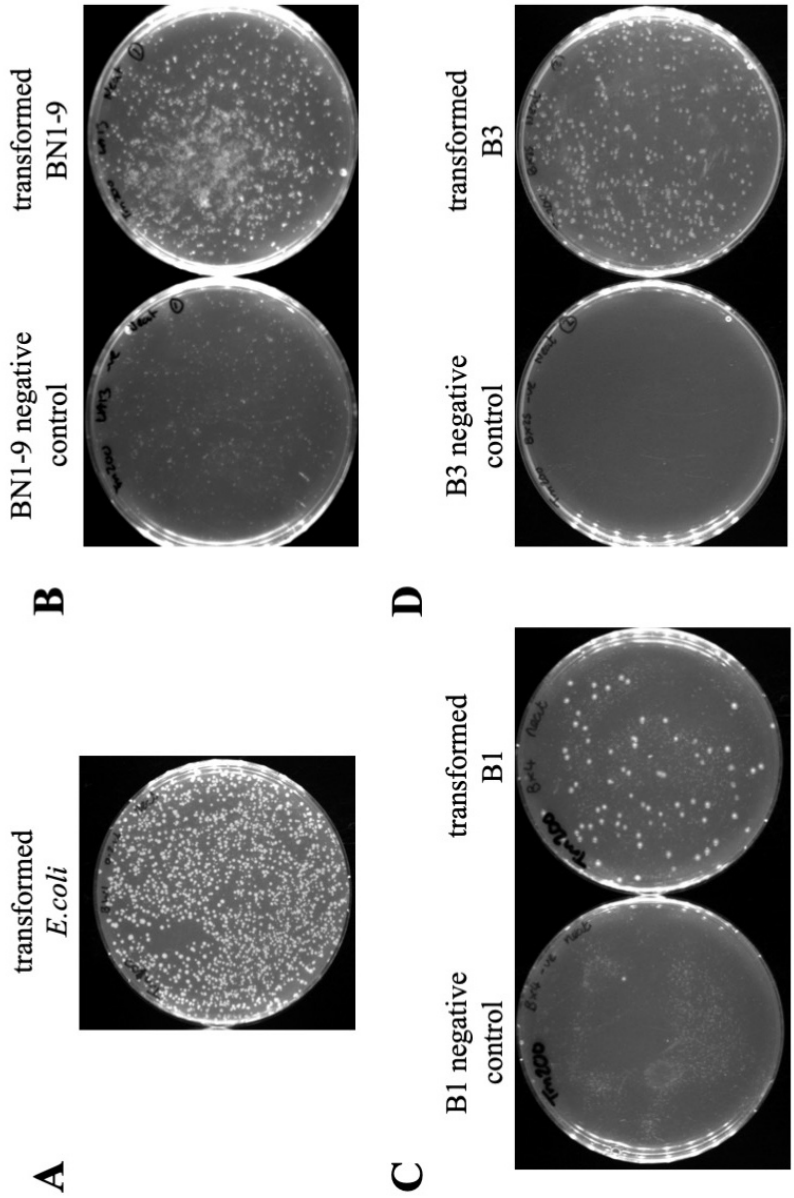


Figure 5.7 Trial transformation of *P. aeruginosa* strains with the trimethoprim resistance transposome

E. coli strain BW25113 cells were made competent by the standard method **(A).** Clinical strains of *P. aeruginosa* that were isolated from patient PN1 **(B)** P1 **(C)** and P3 **(D)** were made electrocompetent by the 'quick' method previously described (Choi *et al*., 2006). Competent cells were transformed with 0.2 µl of the EZ-Tn5 trimethoprim transposome and successfully transformed cells were selected by spreading 100 µl of undiluted culture onto plates containing 200 µg/ml of trimethoprim. Negative controls for each strain were electroporated without the presence of the transposome.

amount of antibody, which was over 3000-fold higher than the level of antibody present in pooled-HCS. The relative titres of IgG in sera from patients LATS, LAPS and LANC was approximately 500-fold greater than the level of antibody present in pooled-HCS (Figure 5.8A). The high titres of antibody in patients PN1, PN3 and LAWC indicated that these could be inhibitory. Therefore, PN1, PN3 and LAWC sera were screened for the ability to inhibit the serum-mediated killing of strain B3 by pooled-HCS.

Isolate B3 was susceptible to complement-mediated killing after a 45 min incubation in pooled-HCS and PN3 serum, which confirmed that the IgG antibodies present in PN3 serum were not inhibitory. Patient PN1 serum elicited a 1.5 log decrease in viable B3 cell number after 180 min. However, when mixed with pooled-HCS, PN1 serum was unable to block the bactericidal activity of pooled-HCS. Therefore, PN1 serum did not contain inhibitory antibody specific to strain B3. The serum from patient LAWC was unable to kill strain B3 and an increase in the viable B3 cell number was observed after 180 min. When LAWC serum was mixed with pooled-HCS, the bactericidal activity of pooled-HCS was blocked and an increase in the viable B3 cell number after 180 min was observed that was equivalent to that observed for patient serum alone (Figure 5.8B).

This confirmed that the serum from patient LAWC contained inhibitory antibody specific to strain B3. As strain B3 could be successfully transformed with the trimethoprim transposome and patient serum with inhibitory antibodies that were specific to the strain had been identified, this strain was used as the parental strain for the construction of a *P. aeruginosa* transposon insertion library.

5.2.2 Construction of the B3 transposon insertion library

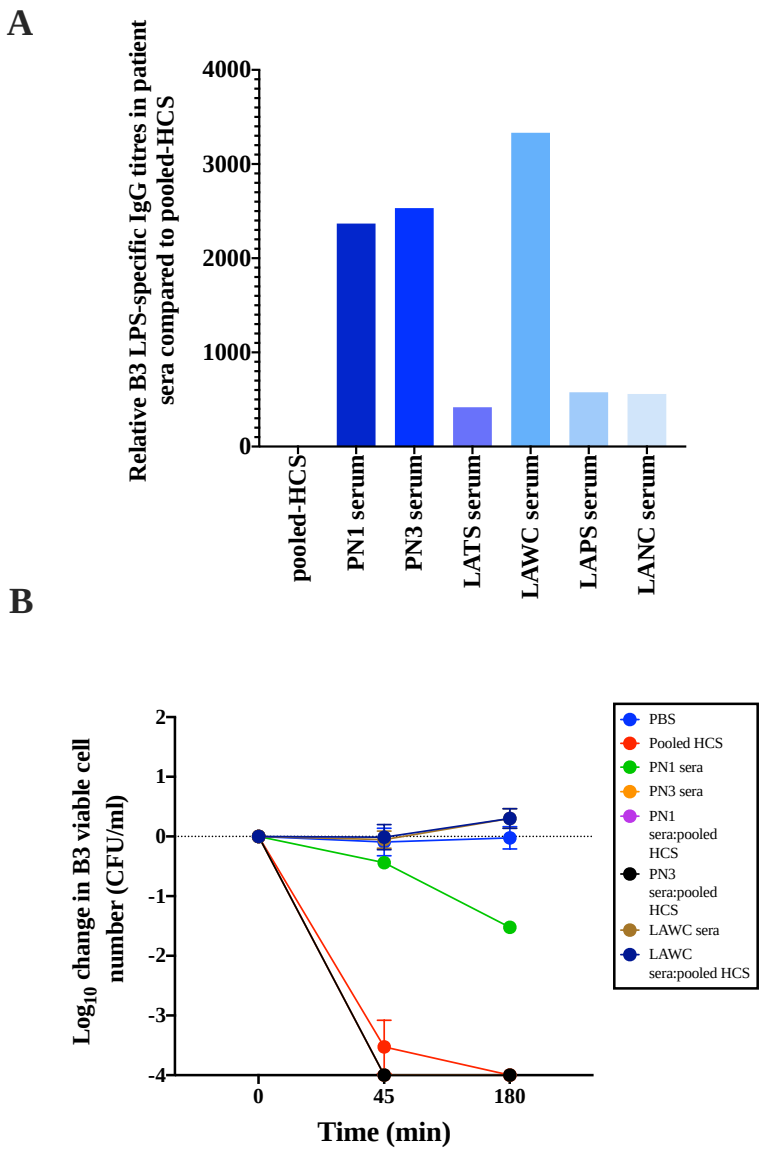


Figure 5.8 The screening of patient sera for inhibitory antibodies against strain B3

A) The relative titres of IgG antibodies in patient sera that were specific to the LPS purified from strain B3 were determined by ELISA. A 96-well immunoassay plate was coated with 1 mg/ml B3 LPS and kept overnight at 4°C. The plates were incubated at 37°C for 1.5 h in primary antibody (a 1:3 dilution series of patient sera ranging from 1:20 to 1:4860) and for 1.5 h in secondary antibody (1:10000 dilution of goat anti-human IgG-AP). Antibody binding was detected by the addition of *p*-nitrophenyl phosphate substrate solution and the absorbance at 405 nm was quantified after a 30 to 60 min incubation at room temperature. **B)** Serum bactericidal assay to confirm the inhibitory potential of the high titres of IgG in patient sera. Strain B3 was incubated with pooled-HCS, patient sera or a combination of pooled-HCS and patient sera for 180 min. The $\log_{10}$ change in viable cell number was calculated. Negative values indicate a decrease in viable cell number. Sera was considered inhibitory if the bactericidal activity of pooled-HCS was significantly decreased by the addition of patient sera. Data points and error bars are representative of the mean and standard deviation of 2 independent experiments.

5.2.2.1 Optimisation of the *P. aeruginosa* competent cell protocol for large-scale use

High-insertion density transposon libraries are required in order to confidently determine whether a gene can be classified as essential. As such, the effects of temperature and bacterial growth phase and number on the transformation efficiency were determined in order to optimise the method for making *P. aeruginosa* competent cells.

First, the effect of temperature was determined by preparing competent cells from overnight cultures of strain B3 as described above with modifications. The cells and 300 mM sucrose were either kept at 4°C or at room temperature throughout the process. To maintain the stability of the transposome, all cells were incubated with the transposome prior to electroporation at 4°C. Following electroporation and a 2 h recovery, 50 µl of undiluted cultures were spread onto LB plates containing 400 µg/ml of trimethoprim. A lower culture volume and higher antibiotic concentration were used to reduce the chance of background growth. After incubation for 24 h at 37°C, the numbers of colonies on each plate were compared.

At least a 10-fold increase in the number of colonies was observed for the cells that were kept at room temperature compared to the cells that were kept at 4°C (Figure 5.9A). This confirmed that the preparation of competent cells at room temperature increased the transformation efficiency of the cells. Incubation of the cells with the transposome at 4°C also resulted in a slight increase in the number of colonies compared to the previously transformed cells that had been incubated with the transposome at room temperature prior to electroporation (see Figure 5.7D). This indicated that incubation with the transposome at 4°C might also increase the transformation efficiency.

To assess the effect of cell number and bacterial growth phase and on transformation efficiency,

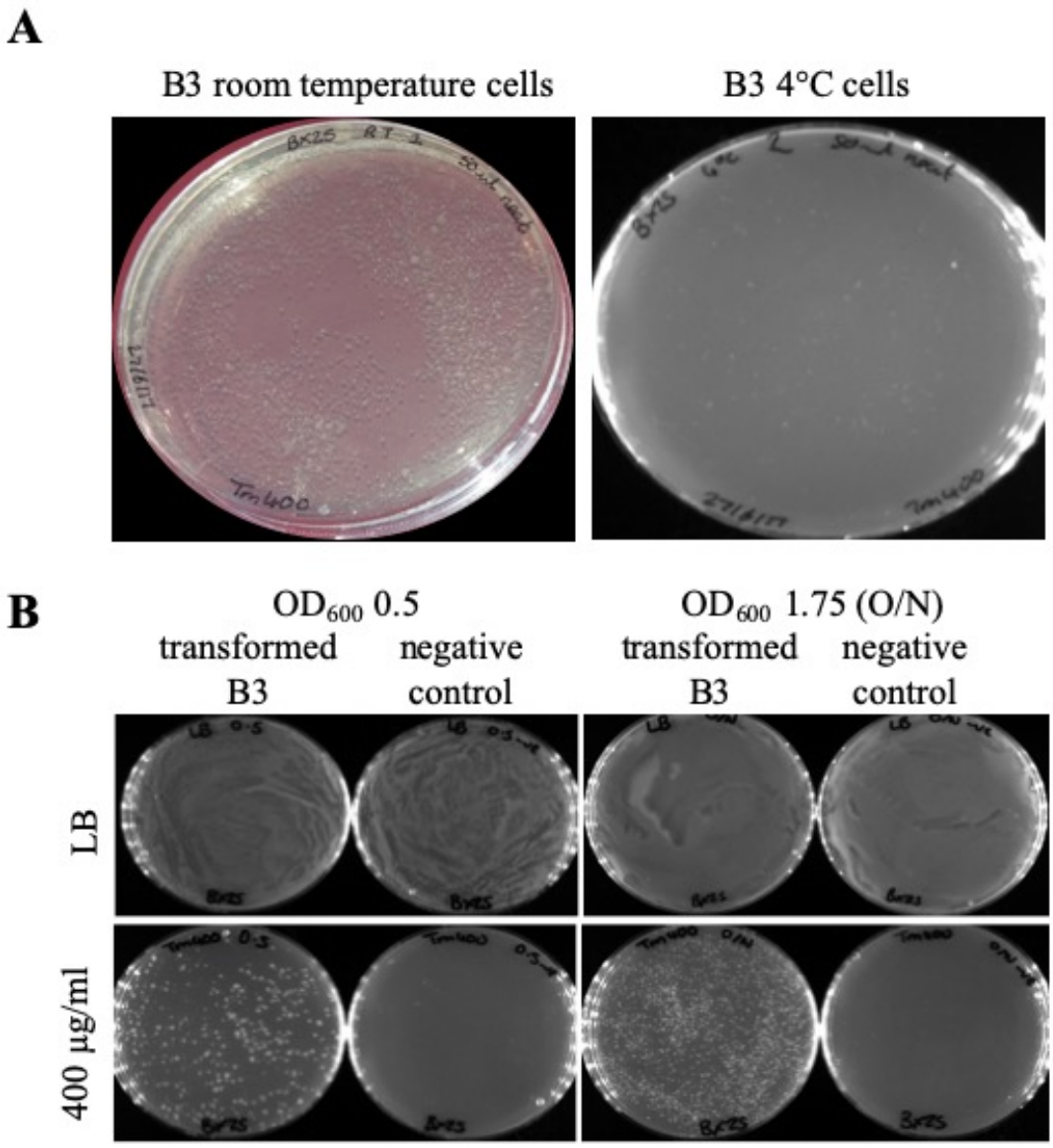


Figure 5.9 Optimisation of the *P. aeruginosa* competent cell protocol

A) The effect of temperature during the preparation of electrocompetent B3 cells on transformation efficiency was assessed by preparing electrocompetent cells with reagents and cells kept at room temperature or 4°C throughout the process. All cells were incubated on ice for 30 min prior to the addition of the transposome followed by a 30 min incubation on ice with the transposome prior to electroporation to maintain transposome stability. **B**) The effect of growth phase on the transformation efficiency of strain B3 cells. Electrocompetent cells were made from overnight cultures (stationary phase) or from cells grown to an OD_{600} of 0.5 (mid-logarithmic phase). Cells and reagents were kept at room temperature throughout the process and chilled prior to the addition of the transposome. Plates are representative of independent experiments.

overnight cultures of strain B3 were used directly or inoculated into fresh LB medium and grown to the mid-logarithmic phase (OD_{600} = 1.75 and 0.5, respectively). A 10 ml sample of each culture was used to make competent cells, keeping the cells and reagents at room temperature throughout the process. Cells were chilled at 4°C prior to the addition of the transposome. The rest of the transformation process remained the same as described above and the number of colonies were compared between the plates. An increase in the number of transformed colonies was observed when competent cells were prepared from the overnight cultures compared to the competent cells made from cells grown to the mid-logarithmic phase of growth (Figure 5.9B).

This confirmed that the use of overnight cultures of strain B3 would be suitable for the construction of the *P. aeruginosa* transposon insertion library. The library was subsequently constructed by three independent rounds of competent cell preparation and transformation. Each round consisted of the production of 1 ml of electrocompetent B3 cells from 100 ml of overnight culture and 10 independent transformation reactions by electroporation. Each round yielded approximately 400,000 colonies, which were pooled to obtain the final library consisting of an estimated 1.2 million mutants.

5.2.2.2 Sequencing of the transposon insertion library

In order to confirm that the transposon insertions had sufficiently covered the entire genome, the genomic position of the transposon insertions needed to be determined. High density transposon libraries are required to accurately assess gene essentiality. As the average gene length is approximately 1000 bp, an insertion on average every 20 bp would be deemed as sufficient coverage. Three independent genomic DNA extracts were prepared from the pooled library. The genomic DNA was fragmented and prepared for sequencing using a PCR-based

method. This method enriches for and amplifies the DNA fragments containing the transposon sequence whilst adding various adaptors that will enable the fragments to bind to the sequencing flow cell, stagger the transposon sequence start site to increase the nucleotide diversity between clusters on the flow cell, incorporate sequencing primer binding sites, and identify and collate sequencing reads that belong to the same sample. Sequencing libraries were prepared and then sequenced on two independent occasions using the Illumina MiSeq platform. The sequencing data were combined, processed and aligned to four *P. aeruginosa* reference genomes using custom scripts (Goodall *et al*., 2018). The genomes were as follows: PAO1 (GCF_000006765.1), PA14 (GCF_000014625.1), PA7 (GCA_000017205.1) and LESB53 (GCA_000026645.1.

The scripts produce a file containing the position of the transposon insertion and the insertion frequency at any given position in the genome. This file was viewed using Artemis, along with the four reference genomes in general feature format, and manually inspected to check that the insertions spanned the whole genome. The importance of having the correct reference genome for the analysis was revealed at this point as the number of insertions at each position of the genome varied and a large insertion free region was observed when the data were aligned to the alternative reference genomes (Figure 5.10). Upon manual inspection, these large insertion-free regions corresponded to several unannotated genes that were associated with the production of pyoverdine. The loss of pyoverdine production is commonly associated with strains isolated from chronic infections (O'Brien *et al*., 2017). All of the alternative reference genomes were from isolates obtained from acute infections whereas strain B3 was obtained from a chronic lung infection, which indicated that the large region associated with pyoverdine might not have been present in the B3 genome.

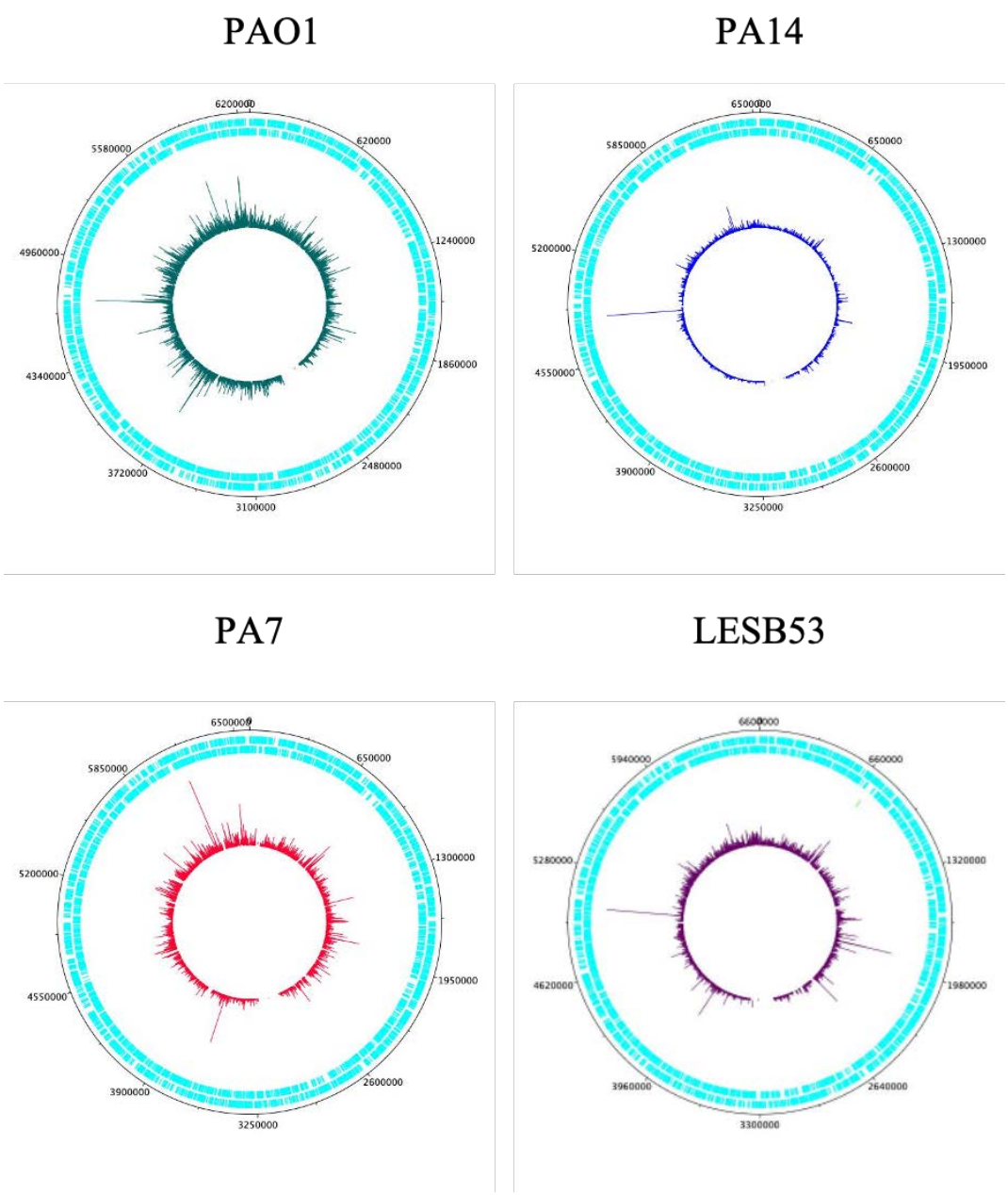


Figure 5.10 Variation in the frequency of mapped insertion sites between reference genomes

The positions of the transposons were mapped to four alternative reference genomes in order to determine the effect of reference genome selection on the bioinformatic analysis. The insertion sites and frequencies across the genomes were viewed with DNAplotter. The numbers around the outside of the circle represent the genomic position and the two blue tracks represent the sense and anti-sense strands, respectively. Transposon insertions are depicted in various colours with the height of the bars representing the insertion frequency at a given position. PAO1 (GCF_000006765.1); PA14 (GCF_000014625.1); PA7 (GCA_000017205.1) and LESB53 (GCA_000026645.1).

Had the analysis been completed using the alternative reference genomes, the region would have been incorrectly classified as an essential region. Therefore, the complete B3 reference genome was assembled by MicrobesNG using a combination of the Illumina short-read and Oxford Nanopore long-read technologies and the TraDIS data were aligned to this reference genome.

The insertion free region was no longer present when the data were aligned to the B3 reference genome. Manual inspection of the insertion sites revealed areas of both high-density and low-density (Figure 5.11A and B). However, insertions spanned the entirety of the genome indicating that the B3 transposon insertion library had been successfully constructed (Figure 5.10C). Further analysis of the transposon insertion sites using a custom R-script provided by PhD student Mathew Milner revealed that the library contained 577,494 unique insertion points with an average distance of 11 bp between each insertion across the genome.

5.2.3 The essential genome of clinical *P. aeruginosa* strain B3

5.2.3.1 The predicted essential genome of *P. aeruginosa* clinical isolate B3

As a library had been successfully constructed and transposon insertions spanned the length of the genome, the essential genes of strain B3 were predicted. Gene essentiality was predicted using an R-script provided by former PhD student Emily Goodall (Langridge *et al*., 2009; Goodall *et al*., 2018). The custom TraDIS script calculated the insertion index scores for each gene by calculating the number of insertions per coding sequence and normalises for gene length by dividing this number by the gene length. The R-script plots the frequency of the insertion index scores, which confirmed that the data followed a bimodal distribution (Figure 5.12), as previously reported (Langridge *et al*., 2009; Goodall *et al*., 2018). The R-script fits an exponential distribution to the first mode and a gamma distribution model to the second mode

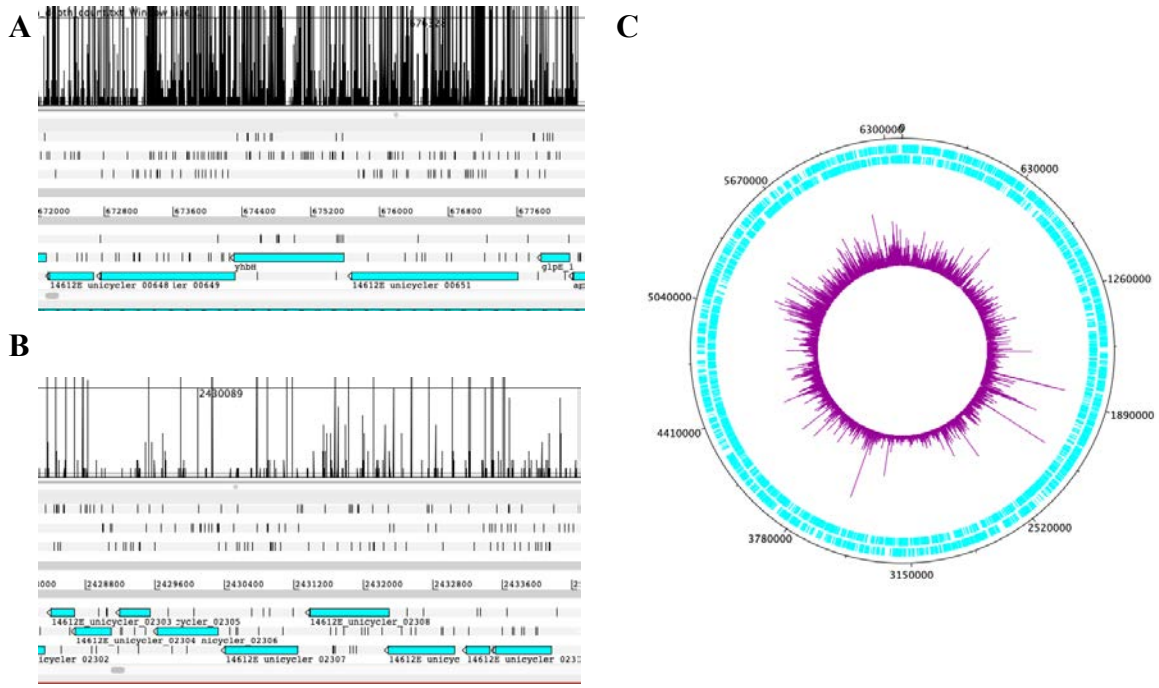


Figure 5.11 Transposon insertion density of the *P. aeruginosa* B3 transposon library

The position and frequency of each transposon insertion was determined bioinformatically using custom scripts (Goodall *et al*., 2018). The insertion positions and frequencies were visualised using the Artemis genome visualisation software. **A**) Representative genomic region with a high density of insertions. **B**) Representative genomic region with a lower density of insertions. The top track with black bars represents the transposon insertion sites. The height of the bar represents the frequency of insertions at a given position. The middle track represents the sense strand of DNA with the 3 open reading frames depicted. The bottom track represents the anti-sense strand of DNA with the 3 open reading frames depicted. The blue bars represent the annotated genes. **C**) The position and frequencies of all insertion sites within the B3 genome viewed with DNAplotter. The numbers around the outside of the circle represent the genomic position and the two blue tracks represent the sense and anti-sense strands, respectively. Transposon insertions are depicted in purple with the height of the bars representing the insertion frequency at a given position.

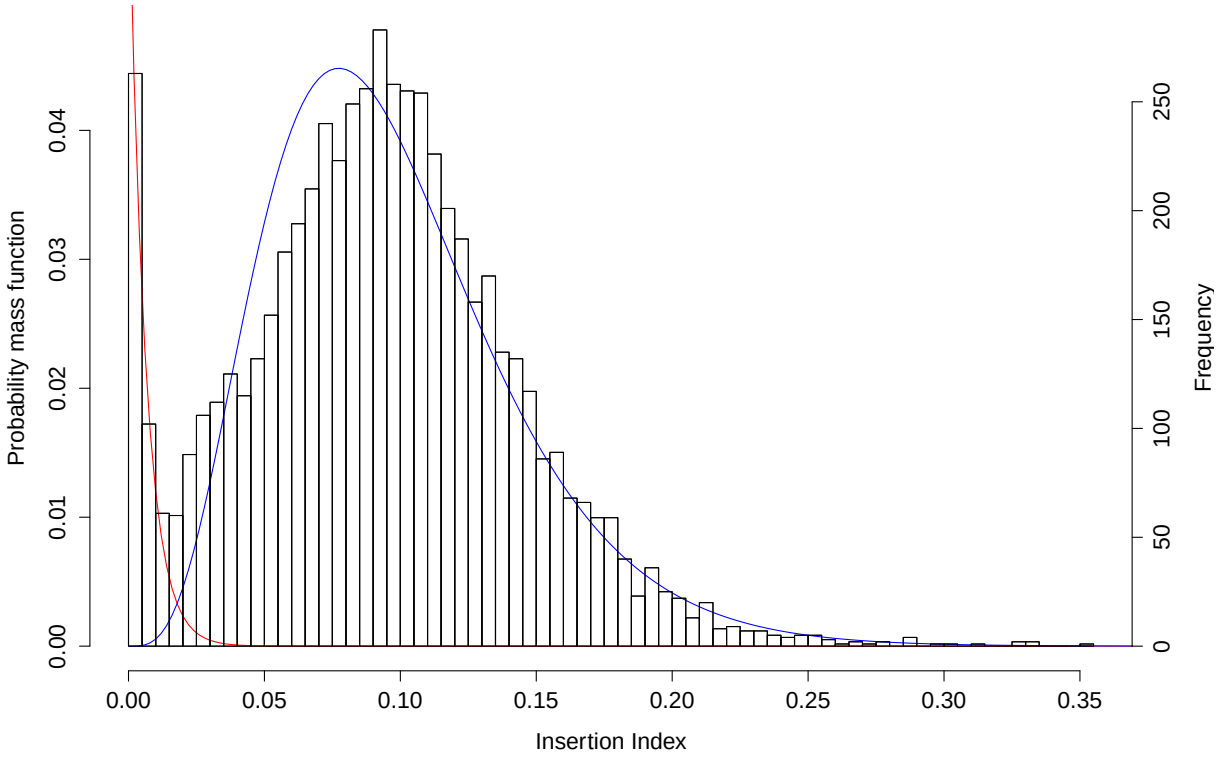


Figure 5.12 The frequency and distribution of insertion index scores used to predict gene essentiality

Custom scripts were used to produce insertion index scores for each gene by normalising the number and frequency of insertions per gene for gene length. This was calculated by dividing the total number of insertions per gene by the length of the gene. The frequency of each insertion index was plotted and produced a bimodal distribution. Statistical models were fitted to each mode (red and blue lines). Genes with an insertion index score under the red line were predicted as essential; genes with scores within the blue line were predicted as non-essential and genes with scores lying between the red and blue lines were unclear.

(Figure 5.12 red and blue lines, respectively). The script then predicted the probability of each insertion index score belonging to each distribution. Genes that were 12 times more likely to belong to the exponential distribution than the gamma distribution were classified as essential. Therefore, the first distribution included genes that had been predicted as essential whereas the second distribution included genes that were considered non-essential. Genes with an insertion index score located within both distributions cannot be confidently predicted as essential or non-essential and were classified as unclear.

This analysis suggested that the genome of strain B3 contains 5074 non-essential genes, 454 essential genes and 388 unclear genes. Genes that were classified as essential were manually inspected for insertions using Artemis and further sub-categorised into essential genes and genes with essential regions or insertion orientation-dependent essential genes. Genes with no insertions remained in the essential category. Genes with both insertion-free regions and areas with insertions, and genes which contained insertions in one orientation were moved to the subcategory. Examples of clear essential and non-essential genes are given in Figure 5.13. Following the sub-categorisation of the predicted gene list, 394 genes remained in the essential category (Appendix VI). This figure is in concurrence with previous estimates that suggest that the essential genome of *P. aeruginosa* consists of 300 to 800 genes (Jacobs *et al.*, 2003; Poulsen *et al*., 2019).

The function encoded by each essential gene was determined using the UniProtKB database and the genes were categorised into one or more of the following biological pathways for simplicity: metabolism, replication, protein biosynthesis, membrane biogenesis, stress response, and hypothetical proteins. The gene products and sub-categories belonging to each of the biological pathways can be found in Appendix VI and VII. The majority of the essential

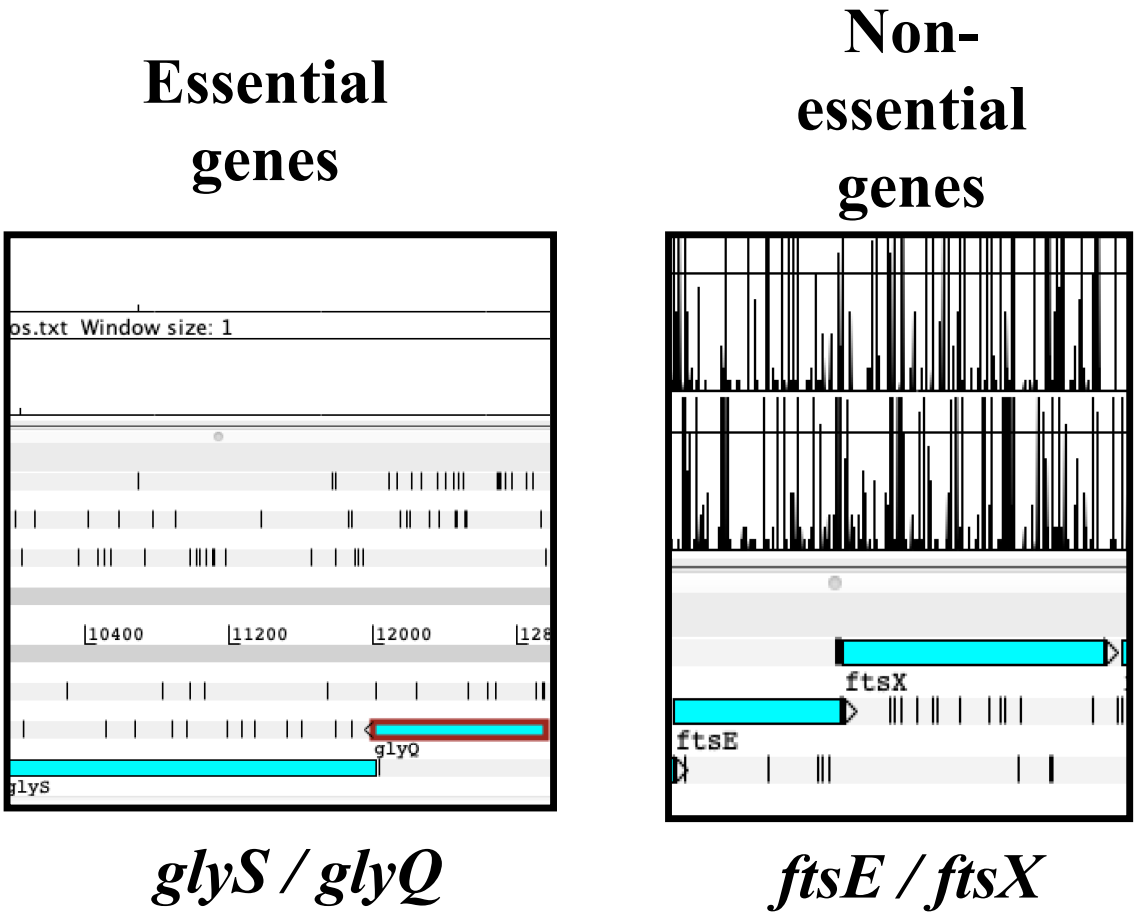


Figure 5.13 Examples of *P. aeruginosa* B3 essential and non-essential genes

A visual representation of mapped insertion for predicted essential genes, *glyS* and *glyQ* and predicted non-essential genes, *ftsE* and *ftsX*. Transposon insertions were viewed using the Artemis genome visualisation software. The top two tracks with black bars represent the transposon insertion sites in the anti-sense and sense strand orientation, respectively. The height of the bar represents the frequency of insertions at a given position. The middle track represents the sense strand of DNA with the 3 open reading frames depicted. The bottom track represents the anti-sense strand of DNA with the 3 open reading frames depicted. The blue bars represent the annotated genes.

genes were associated with protein biosynthesis and metabolism (36% and 35%, respectively) whereas 14% and 6% were associated with bacterial replication and membrane biosynthesis, respectively. Hypothetical genes represented 7% of the predicted essential gene list and only 2% of genes were associated with the stress response (Figure 5.14). Once again, these figures were in line with the previous reports for the *P. aeruginosa* species: metabolism, 36%; protein biosynthesis, 30%; replication, 7%; membrane biogenesis, 13%; stress response, 4%; and hypothetical proteins, 11% (Juhas, 2015). These data confirmed that the B3 transposon insertion library was comparable to existing *P. aeruginosa* transposon libraries and suitable for future conditional essential gene predictions.

5.2.3.2 Genes with essential regions or insertion orientation-dependent essential genes

The above sub-categorisation of essential genes and manual inspection of the insertions present on both the sense and anti-sense DNA strands revealed that 107 genes contained essential regions or essential genes dependent on the insertion orientation (Appendix VIII). Examples of genes with essential regions, orientation dependent insertions and low frequency insertions are described below.

The *ribBA* gene encodes a protein involved in the biosynthesis of riboflavin. This is an essential metabolite that is required for the production of flavin mononucleotide and flavin adenine dinucleotide, both of which have essential roles in cellular metabolism (Fassbinder *et* al., 2000; Gutiérrez-Preciado *et al*., 2015). Although insertion analysis classified this gene as non-essential, the 3' half of this gene contained insertions whereas the 5' half was insertion-free (Figure 5.15A). This implied that the 3' *ribA* region encoding the GTP cyclohydrolase-2 was dispensable whereas the *ribB* region encoding the synthase domain was essential for the production of riboflavin.

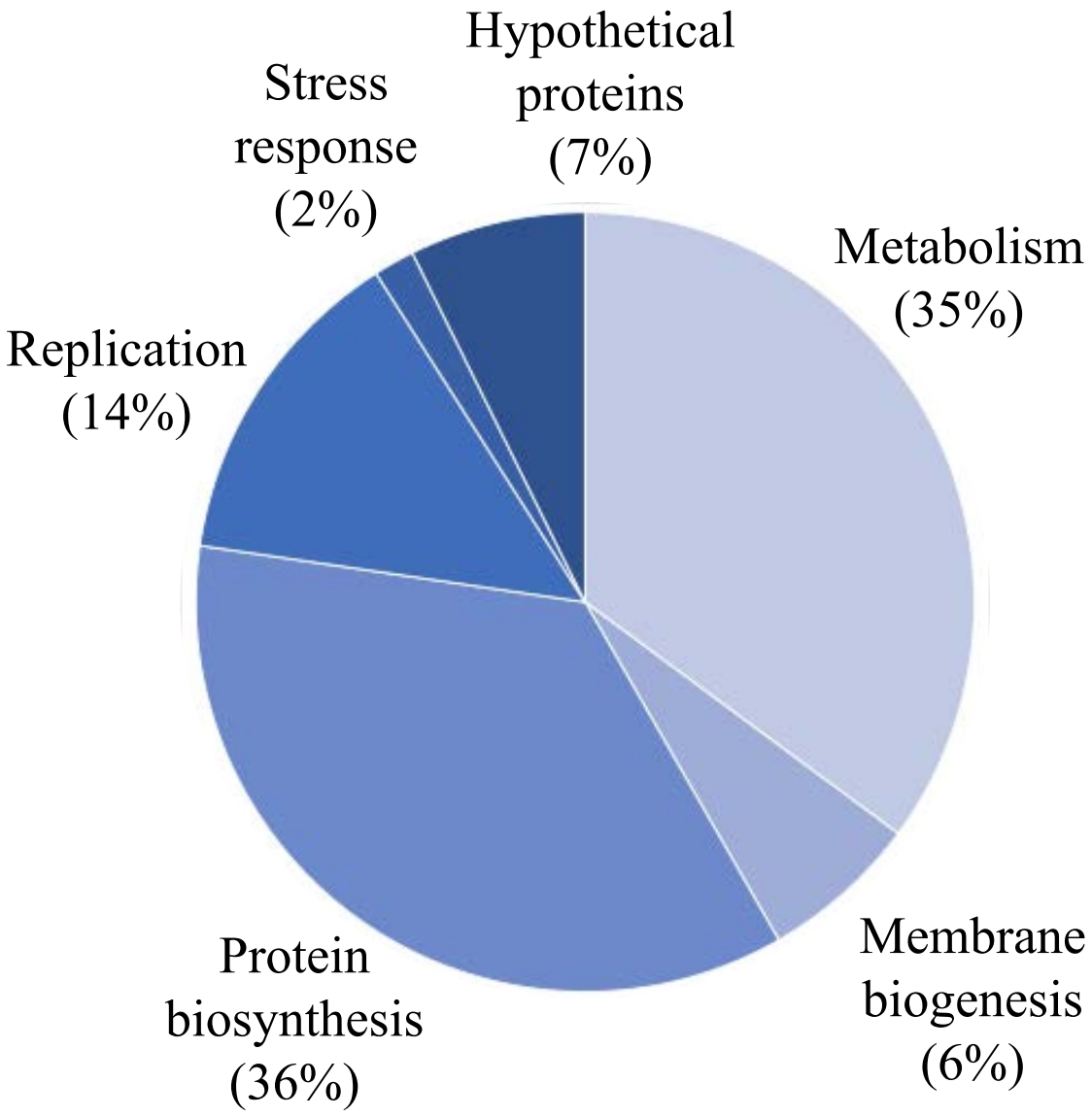


Figure 5.14 Categorisation of *P. aeruginosa* B3 essential genes

The functions of the essential genes were determined using the UniProtKB database and classified into the following general biological pathways for simplicity: metabolism; replication; protein biosynthesis; membrane biogenesis; stress response; and hypothetical proteins. The proportion of genes belonging to each pathway were compared.

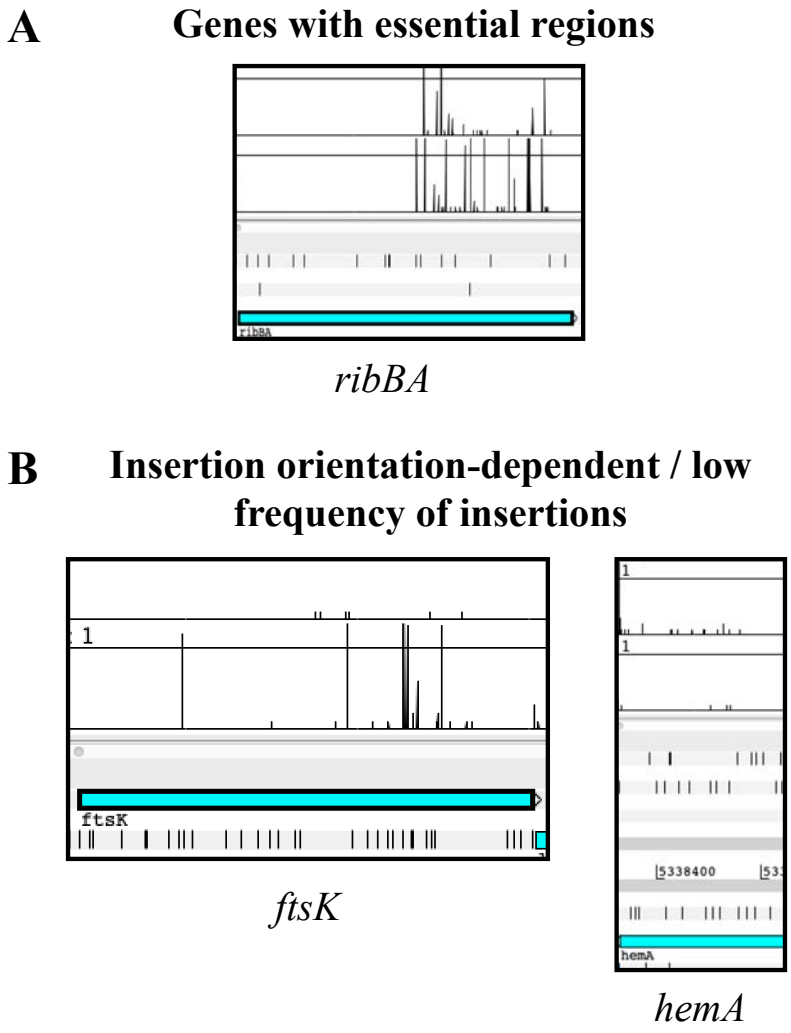


Figure 5.15 Manual inspection of predicted essential genes for transposon insertions

The genes that were predicted to be essential were manually inspected for insertions using the Artemis genome visualisation software. The top two tracks with black bars represent the transposon insertion sites in the anti-sense and sense strand orientation, respectively. The height of the bar represents the frequency of insertions at a given position. The blue rectangles represent the annotated gene. **A**) A representative example of a gene predicted as non-essential that contains an essential region. The *ribAB* gene contained insertions spanning half of the gene at the 3' end. However, no insertions were present in the 5' half of the gene. B) Representative examples of genes that contain a low frequency of insertions or insertion orientation-dependent insertions. The *ftsK* and *hemA* genes were predicted to be essential during the analysis but contain transposon insertions. Inspection of the *ftsK* gene (left) revealed transposon insertion was orientation-dependent as a higher frequency of insertions were observed in the sense orientation. Inspection of the *hemA* gene (right) revealed that only a very low frequency of insertions in this gene.

The *ftsK* gene encodes the DNA translocase that has been described as an essential protein required for cell division and chromosome segregation in *E. coli* (Berezuk *et al*., 2018). Although this gene was predicted to be essential, manual inspection for transposon insertions revealed that only 6 reads mapped to the gene in the anti-sense orientation whereas many more insertions mapped to the region in the sense orientation (Figure 5.15B). These insertions did not occur across the full length of the gene and were mainly located either at a specific position or in a localised cluster. This suggests that the *ftsK* gene contains non-essential regions.

Genes that are classified as essential can also contain a low frequency of transposon insertions, suggesting that disruption of such genes has a significant impact on the fitness of the bacterium. A low insertion frequency was observed in the *hemA* gene of strain B3 (Figure 5.15B). Similar to the observation for the *ftsK* gene, differences between the insertion frequencies in the sense and anti-sense strands were noted (4 and 15, respectively). This gene encodes a glutamyl tRNA reductase, which has an essential role in the production of 5-aminolevulinic acid, a precursor of the heme biosynthetic pathway (Verkamp and Chelm,1989). The gene is essential in the absence of an external source of this precursor, which is supported by the prediction and low transposon insertion frequency observed for this gene.

The presence of essential regions within genes and orientation-dependent transposon insertions have previously been described (Goodall *et al.*, 2018). Together these data indicated that the B3 transposon library could be used to locate functionally essential regions of genes and highlighted the importance of having a transposon library with a sufficient density of transposon insertions in order to predict essential genes. Manual inspection of transposon insertions during the analysis of predicted essential genes was crucial for revealing essential regions of genes.

5.3 Discussion

The work presented in this chapter outlines a new method for creating high-density transposon insertion libraries in *P. aeruginosa*. All *P. aeruginosa* transposon insertion libraries created to date have been constructed using the conjugation method to introduce plasmids containing the transposon and transposase sequences into the desired strain. This often involves the construction of a suitable donor plasmid, which needs to be transformed into a donor *E. coli* strain (Jacobs *et al*., 2003; Lawenza *et al*., 2005; Liberati *et al*., 2006; Skurnik *et al*., 2013; Lee *et al*., 2015). The *P. aeruginosa* B3 transposon insertion library described in this chapter was constructed using an electroporation-based technique to introduce an EZ-Tn5 transposome into the cells. The EZ-Tn5 transposome was constructed using a commercially available, high-efficiency transposon DNA and transposase enzyme (Lucigen, USA), which circumvents the need to use plasmid-incorporated transposon and transposase DNA. The method used to prepare efficient competent *P. aeruginosa* cells was adapted from a previously described method (Choi *et al*., 2006). This method bypasses the time-consuming step in standard methods that requires strains to be grown to mid-logarithmic phase of growth (Gonzalez *et al*., 2013).

Many of the previously constructed transposon insertion libraries are made up of a relatively small number of unique insertion points (Jacobs *et al*., 2003; Lawenza *et al*., 2005; Liberati *et al*., 2006; Skurnik *et al*., 2013). One PAO1 transposon library was comprised of 31,000 unique mutants, which corresponded to 5 insertions per gene (Jacobs *et al*., 2003). The library presented in this chapter contained 577,494 unique insertion points, which corresponds to an insertion on average every 11 bp. Data presented in this chapter highlighted the importance of sufficient transposon insertion coverage of the genome in order to predict the essentiality of genes more accurately. Without sufficient coverage of the genome, genes can be incorrectly predicted as essential.

Consistent with the analysis of other TraDIS datasets, the transposon insertion-orientation also impacts gene essentiality (Goodall *et al*., 2018). Transposon insertion in the anti-sense orientation into a non-essential gene containing the promotor region of an essential gene can result in a gene becoming essential. However, if the insertion occurs in the sense direction, the promotor contained within the transposon allows the transcription of the essential gene and the gene remains non-essential (Goodall *et al*., 2018). Therefore, this must be taken into account during the analysis of TraDIS data.

Until very recently, all of the described *P. aeruginosa* transposon insertion libraries were constructed in the laboratory reference strains PAO1 and PA14. The library presented in this chapter is therefore one of the first transposon insertion libraries to be constructed in a clinical strain that was isolated from a chronic infection. This library could therefore be useful for the elucidation of how the essential genome of *P. aeruginosa* varies between acute and chronic strains.

Unfortunately, due to time constraints, the transposon library created was not used to identify conditionally essential genes in the presence of serum that contained inhibitory antibodies. In order to test the hypothesis that inhibitory antibodies in non-CF bronchiectasis patient sera target the O-antigen of the corresponding *P. aeruginosa* isolate, the library would be incubated in 50% pooled HCS or 50% patient LAWC serum followed by overnight incubation on LB-trimethoprim plates. The genomic DNA of the output libraries would then be sequenced and the position of the transposon insertions would be mapped to the B3 reference genome. These positions would be compared to the transposon insertions present in the original input library. Genes that had insertions in the input library but no longer have insertions following incubation in pooled HCS would be considered as essential for the survival in human serum. Genes that

contain insertions in the input library and the pooled HCS output library but no longer have insertions following incubation in patient LAWC serum would be considered as genes essential for the survival in the presence of inhibitory antibody and undergo further validation. This technique has the potential to identify novel targets of inhibitory antibodies other than the O-antigen and to identify the antigen modification pathways that produce the specific epitopes that are targeted by these antibodies.

In conclusion, this chapter provides a novel method for creating high-density transposon insertion libraries in *P. aeruginosa.* This work highlights the importance of reference genome selection for the analysis of TraDIS data. Analysis of the essential genome of strain B3 identified 394 essential genes and 107 genes with essential regions or low frequency of insertion. This was consistent with the previous reported figures for the essential genomes of other *P. aeruginosa* strains (Juhas, 2015). Finally, the library constructed in strain B3 is suitable for future analysis of the conditionally-essential genome of this strain to provide a mechanistic insight into the survival of *P. aeruginosa* in the presence of inhibitory antibodies.

CHAPTER 6

FINAL DISCUSSION

6.1 General summary

Overall, the general theme of this thesis focuses on the relationship between antibody and *P. aeruginosa* in human infection. Previous work has demonstrated that the overproduction of *P.* aeruginosa-specific inhibitory antibodies by patients with chronic *P. aeruginosa* lung infections can protect the bacterium from the bactericidal activity of human sera and the removal of these antibodies from circulation enables the immune system of the patients to control the infection (Wells *et al*., 2014; Wells *et al*., 2017). As this treatment had never been used as a therapy to treat patients with inhibitory antibodies, the effect of removing these antibodies on the subsequent *P. aeruginosa* population in the lung was unknown and initially explored in chapter 3. Phenotypic and genotypic analyses of the isolates obtained from these patients at various points throughout the course of treatment confirmed that the strains isolated from the patients following treatment were all derived from a clonal lineage that was present prior to treatment and therefore plasmapheresis did not result in strain replacement. Rather, a dominant strain persisted within a specific niche in the lung, remaining dormant and undetected in the sputum for several months and re-emerging in the presence of high titres of inhibitory antibody.

The patients received healthy donor antibody to replace the antibodies removed during plasmapheresis. As such, the role of antibody in the protection against *P.* aeruginosa by healthy sera was explored further in chapter 4. In the absence of cell-mediated killing, functional complement proteins were essential for the bactericidal activity elicited by human sera. Killing in an antibody-independent manner was represented by a decrease in viable cell number for 6 out of the 7 *P. aeruginosa* strains tested. However, total killing was not elicited. Purified IgG antibodies were not able to cause a bactericidal effect against the strains. However, the combination of complement and purified IgG enhanced the killing of 4 strains and reduced the

killing of 2 strains. The presence of inhibitory antibody in healthy sera was confirmed, which suggested that the production of inhibitory antibodies could occur with sufficient environmental exposure to bacterial antigens and was not limited to having an active infection.

As the bacterial antigens that are targeted by inhibitory antibodies and the mechanism of protection against serum-mediated killing are not completely understood, a transposon library in a clinically relevant strain of *P. aeruginosa* was constructed (chapter 5). The library consisted of 577,494 unique mutants and was the first library constructed in a multi-drug resistant respiratory isolate of *P. aeruginosa.* This library was constructed to provide a tool that could be used to identify genes that are essential for the survival of *P. aeruginosa* in the presence of inhibitory antibody. These genes could reveal the specific LPS modifications or novel bacterial epitopes that are targeted by inhibitory antibodies.

6.2 The role of antibody in the control of *P. aeruginosa* infections

The role of neutrophils in the protection against *P. aeruginosa* infection has been widely studied. These cells primarily clear the bacteria through phagocytosis (Rada, 2017). However, neutrophils can prevent the dissemination of the bacterium from biofilms by producing neutrophil extracellular traps (NETs) that are able to contain the biofilm. The production of these NETs has been associated with the secretion of exotoxins by the *P. aeruginosa* type 3 secretion system (Thanabalasuriar *et al*., 2019; Skopelia-Gardner *et* al., 2019). In humans, the complement protein C5a has been shown to promote the recruitment of neutrophils (Hair *et al*., 2017). However, the presence of *P. aeruginosa* in the airway can stimulate the recruitment of neutrophils in the absence of a functional complement system through the release of chemotactins such as IL-8 by epithelial cells (Sordelli *et al*., 1985; Rada, 2017). The release of chemokines by lung epithelial cells or *P. aeruginosa* secreted factors including pyocyanin and

quorum sensing molecules can cause an increased influx of neutrophils to the site of infection (Rada, 2017). However, this can have a negative effect on the host as a higher number of neutrophils leads to increased damage to lung tissue caused by an excess of neutrophil elastase and reactive oxygen species production during chronic infection (Jasper *et al*., 2019).

This thesis has demonstrated an important relationship between antibody and *P. aeruginosa* in the control of infection in the absence of such cell-mediated killing. Healthy sera contained *P. aeruginosa* specific antibody with IgG as the most abundant isotype. The activation of the complement system by antibody resulted in the successful killing of some *P. aeruginosa* strains, irrespective of the level of O-antigen expression by the bacteria. The length and density of the O-antigen produced by strains BN1-1, BN3-6 and B1 was not quantitively determined. The production of long-chain O-antigen rather than very long-chain O-antigen has been associated with an increase in serum resistance (Kintz *et al*., 2008). An increase in LPS density has also been associated with increased resistance to serum (Grossman *et al*., 1987). Therefore, the production of low-density, very-long chain LPS by these isolates could permit the access of antibody and complement to the surface associated antigens, resulting in serum sensitivity.

This study only evaluated the role of antibody and complement in the killing of *P. aeruginosa* strains isolated from chronic lung infections. However, as healthy individuals will normally only encounter *P. aeruginosa* originating from the environment, the role of antibody and complement in the protection against environmental strains should also be considered. Studies have revealed that environmental and clinical isolates do not vary greatly at the genomic level and the level of virulence remains constant between the two groups (Vives-Flórez and Garnica, 2006; Grosso-Becerra *et al*., 2014). The down-regulation of virulence factors during chronic infection could have an impact on the antibody response to these strains. Therefore, repeating

the experiments with environmental isolates or early stage clinical isolates could provide more of an insight into the role of antibodies and complement in the prevention of colonisation establishment.

The importance of the regulation of IgG production has also been highlighted. An increase in the titre of IgG antibodies in healthy sera, specific to the O4 serotype of *P. aeruginosa*, resulted in the inhibition of serum-mediated killing. This is in concurrence with the current hypothesis that high titres of O-antigen specific antibodies in the sera of patients with chronic *P. aeruginosa* infection can inhibit serum-mediated killing (Wells *et al*., 2014). The production of inhibitory antibody by healthy individuals has been demonstrated previously against *Salmonella* Typhimurium. However, these LPS-specific antibodies belonged to the IgM class and were not produced in excessive titres (Trebicka *et al*., 2013). IgM antibodies are produced by naïve B-cells, which then switch to producing IgG, IgA or IgE antibodies once activated following exposure to a foreign antigen after infection of vaccination (Stavnezer and Schrader, 2014). The production of inhibitory antibodies belonging to the IgG class by HCS-1 suggests that environmental exposure to *P. aeruginosa* is sufficient to trigger this class switching. *P. aeruginosa* can be found as part of the normal bacterial flora of the skin and throat (Cogen *et* al., 2008; Islam *et* al., 2011). As the length of time this donor was exposed to *P. aeruginosa* is unknown, longitudinal analysis of serum inhibitory antibody titres and confirming if this donor was colonised by a commensal *P. aeruginosa* strain could reveal a correlation between the commensal microbiome and inhibitory antibody production in heathy individuals.

The discovery of inhibitory antibody in healthy sera could have an impact on future vaccine development and implications for the use of healthy donor sera during therapeutic treatments such as plasmapheresis. As vaccination strategies often involve the stimulation of antibody

production towards specific target antigens or the administration of pre-formed antibodies, the inhibitory potential of the antibodies produced should also be taken into consideration (Baxter, 2007; Sharma *et al*., 2011). The presence of inhibitory antibody in healthy sera following environmental exposure suggests that inhibitory antibodies could also be produced after exposure to target antigens following vaccination. However, the inhibitory effect would only be evident once a person becomes infected. Therefore, during the clinical trial period of development, the prevalence of inhibitory antibody following vaccination should be determined in order to assess the efficacy of the vaccine. Similarly, this highlights the importance of screening the healthy donor immunoglobulin used in plasmapheresis for the inhibition of serum-mediated killing of each patients' corresponding strain prior to treatment.

6.3 Potential factors affecting the mechanism of inhibitory antibodies

The current hypothesis for the mechanism of inhibitory antibodies suggests that the binding of these antibodies to long-chain O-antigen blocks the access of bactericidal serum components to the cell surface and that inhibitory antibodies are serotype specific (Wells *et al*., 2014). Following the removal of inhibitory antibodies by plasmapheresis, 2 out of the 6 *P. aeruginosa* isolates obtained from patient PN1 were sensitive to patient serum although they belonged to the same serotype as the remaining post-plasmapheresis isolates and were genetically nearly identical to the pre-plasmapheresis isolate BN1-1. These isolates no longer produced O-antigen, which led to the increased sensitivity to patient PN1 serum. This suggests that the presence of inhibitory antibody in sera could create a selection pressure for *P. aeruginosa* to retain the production of long-chain O-antigen, even during the chronic stages of infection. Following the removal of these antibodies, this selection pressure was eliminated, thus potentially allowing the down-regulation of O-antigen production by these two isolates as usually occurs during chronic infection (Cullen and McClean, 2015). Previous work has

demonstrated that mucoid strains have increased expression of the transcription factor AmrZ, which inhibits the *wzz2* promotor and results in the downregulation of very long-chain O-antigen production (Cross and Goldberg, 2019). Although BN1-5 and BN1-7 did not have the mucoid phenotype, the level of AmrZ expression by these isolates could be quantified to reveal if this is also the case for these isolates. Alternatively, the DNA sequence of the O-antigen clusters could be compared to those of O-antigen producing isolates to determine whether a mutation has occurred, for example in the *waaL* O-antigen ligase gene, resulting in the lack of O-antigen attachment to the LPS core.

The work presented in chapter 3 has demonstrated that the production of long-chain O-antigen does not necessarily guarantee protection against serum-mediated killing by inhibitory antibody. All of the strains isolated from patient PN3 produced long-chain O-antigen that was recognised by the IgG and IgA antibodies produced by patient PN3; yet the majority of these strains were sensitive to the bactericidal activity of PN3 serum. Although they belonged to the same O-antigen serotype, clear structural variations were observed by Western immunoblot. In order to determine why the isolates varied in their susceptibility to PN3 serum, the density of the O-antigen produced by each isolate should be compared. If the density of the O-antigen produced by the serum-sensitive isolates is lower than that of the serum-resistant isolates, this could still permit the access of bactericidal serum components to the cell surface (Figure 6.1A and B).

The ratio of O-specific antigen and common polysaccharide antigen produced by each isolate could also be compared. Studies have revealed that during chronic infection in CF patients, *P. aeruginosa* no longer expressed OSA antigen but still retained the expression of the CPA (Lam *et al*., 1989). The CPA and OSA can be produced simultaneously in *P. aeruginosa*. However,

OSA is more immunogenic than CPA, which can create a selective advantage for the production of CPA during chronic infections (Lam *et al*., 2011). Previous work has implied that CPA-specific antibodies can prolong the duration of a *P. aeruginosa* infection and are associated with poor lung function (Lam *et al*., 1989). Inhibitory antibodies that target the OSA have been shown to have the same effect (Wells *et al*., 2014). The role of CPA is yet to be defined and therefore variations in the production of CPA could explain the variation in the resistance to serum mediated killing between isolates.

Patient sera contained elevated titres of IgG2 and IgA specific to the LPS purified from *P. aeruginosa* isolates that were resistant to serum-mediated killing when compared to the LPS from sensitive strains. IgG2 and IgA antibodies are produced in response to polysaccharide antigens, such as the O-antigen of LPS. However, IgG2 antibodies are poor activators of the complement system and IgA antibodies do not activate the complement system via the classical pathway (Hoffman *et al*., 2015). IgA antibodies have a role in activating effector cells and there is some evidence to support IgA activation of the lectin pathway (Woof and Kerr, 2006). Serum IgA can also inhibit the release of pro-inflammatory cytokine release and inhibit phagocytosis by polymorphonuclear leukocytes or the chemotaxis of neutrophils when it forms complexes with other serum components (Leong and Ding, 2014). IgG2 antibodies are the least effective of all IgG subtypes at initiating the antibody-dependent cell-mediated cell cytotoxicity, antibody-dependent cellular phagocytosis and complement-dependent cytotoxicity effector functions (Irani *et al*., 2015).

The IgG1 subclass mainly reacts to protein antigens and can activate complement system by the classical pathway following the interaction with C1q and can promote phagocytic uptake (Goh *et al.,* 2011). The retention of O-antigen production by *P. aeruginosa* strains in chronic

infections perhaps confers a selective advantage by stimulating the production of IgG2 antibodies, leading to immune evasion. *P. aeruginosa* can secrete alkaline proteases and elastases that are able to cleave IgG antibodies (Döring *et al*, 1984). Previous work has demonstrated that the ability to cleave IgG antibodies is associated with invasive disease and the IgG2 subclass is the most resistant to cleavage by proteases (Breszski and Jordan, 2010). The retention of IgG2 stimulating antigens and the production of proteases that cleave bacterial clearance-promoting antibodies would confer a selective survival advantage. Comparative analysis of the genomes of the longitudinal isolates could be used to identify the presence of genes encoding such proteases. Comparison of the level of expression of the proteases between these strains could explain the variation observed in the serum resistance profiles of the isolates.

The resistant and sensitive BN3 isolates belonged to the same O-antigen serotype but Western blot analysis revealed variations in the LPS structure. This suggested that the variation in LPS-specific titres in patient sera might be due to a modified LPS epitope, to which inhibitory antibody could have a higher affinity towards than protective antibody (Figure 6.1C). Therefore, long-chain O-antigen production and the elevated titres of IgG2 and/or IgA with increased affinity to the LPS modification could form a secondary barrier to prevent the insertion of the membrane attack complex (Figure 6.1). As well as outcompeting protective antibodies for this epitope, the high titre of inhibitory IgG2 and IgA present at the site of infection might also prevent the recruitment of neutrophils to the site due to the poor neutrophil-attracting properties of IgG2 and the blocking of cytokine release by IgA (Leong and Ding, 2014). This could be investigated further by comparing the level of neutrophil uptake of IgG2/IgA opsonised bacteria and level of cytokine release between patients that are chronically

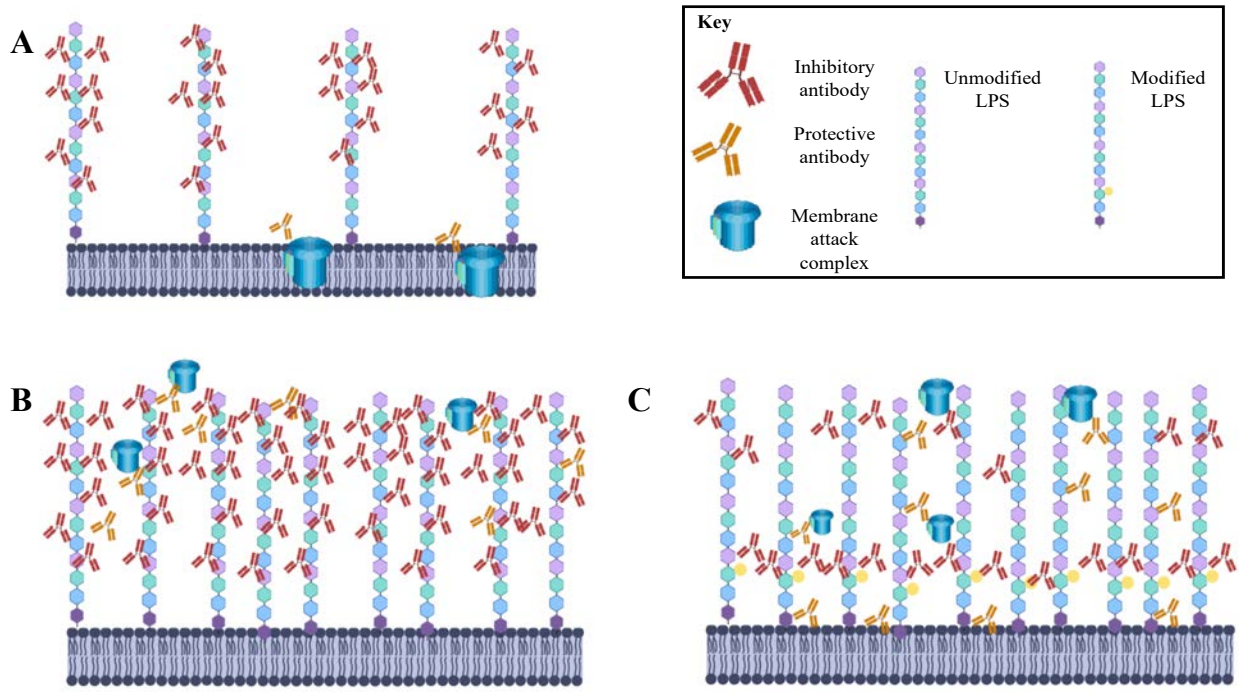


Figure 6.1 Alternative hypotheses for the mechanism of inhibitory antibodies

A) Strains that produce long-chain O-antigen at low density are still sensitive to complement-mediated killing even in the presence of high titres of inhibitory antibody as protective antibodies can still access the bacterial cell surface allowing the insertion of the membrane attack complex. **B)** Strains that produce long-chain O-antigen at high density remain resistant to complement-mediated killing as the inhibitory antibodies as the special arrangement of inhibitory binding blocks the access of complement proteins to the cell surface. **C)** The increased affinity of inhibitory antibody to a specific LPS modification allows inhibitory antibodies to outcompete protective antibodies and provides an addition barrier to prevent the access of complement proteins to the cell surface.

colonised with *P. aeruginosa* with the production of inhibitory antibody to those without inhibitory antibody production.

Transposon sequencing has previously revealed the LPS IgM epitope that is required for the clearance of *Haemophilus influenzae* by neutrophils (Langereis and Weiser, 2014). Therefore, comparison of the TraDIS datasets obtained following the incubation of the transposon library described in chapter 5 in pooled-HCS and in sera containing inhibitory antibodies could reveal the LPS epitope or modification required for survival of *P. aeruginosa* in the presence of inhibitory antibody. The identification of a specific target or pathway using this tool could provide a novel drug target for the control of *P. aeruginosa* infections in patients that produce inhibitory antibody and potentially lead to improved diagnostics and treatments for these patients. In the absence of matched isolates for the patients, the current diagnostic test involves screening the sera for high titres of antibodies against 4 common O-antigen serotypes followed by SBA confirmation. This process is time consuming and will not identify inhibitory antibodies that are specific to O-antigen serotypes not included in the assay. Identifying the specific epitopes that are targeted by inhibitory antibodies will improve the accuracy and efficiency of this test. Furthermore, identifying the target of inhibitory antibody could enable the plasmapheresis protocol to be improved through the addition of a filter that specifically removes inhibitory antibody from the serum rather than all immunoglobulin. The use of healthy donor immunoglobulin would no longer be required, reducing the risk to patients.

6.4 Limitations and future directions

6.4.1 The effect of plasmapheresis on the *P. aeruginosa* population in the lung

The major limitation for the work described in chapter 3 was the lack of multiple isolates per sample time over the course of treatment. Therefore, definitive conclusions about how the *P.*

aeruginosa population dynamics change over time cannot be made. Without the increased sampling of the population at each time point, it can only be proposed that plasmapheresis resulted in the disappearance of strains BN1-2 and BN1-3 and any genetic variations between the pre- and post-plasmapheresis cannot be confirmed as the result of a selective pressure created by the removal of inhibitory antibody without increased sampling of the sputum and analysis of the sputum microbiome. *P. aeruginosa* isolates that were sensitive to killing by patient sera were able to be cultured throughout the course of treatment. This suggests that the serum-resistant variant could have also been present in the same sample, which possibly provided protection for the serum-sensitive variant against the host immune response.

Genetic variations observed between strains isolated prior to plasmapheresis and after treatment can not solely be attributed to the treatment alone. Many other environmental variables can influence how a population changes over time and these factors should also be taken into consideration when assessing the genomic variation between isolates. These include: nutrient availability, oxygen availability, antibiotic usage and the presence of other microbial species (Winstanley *et al*., 2016). Further in-depth analysis of the variation between strains for genes involved in metabolism could provide an insight into how the lung environment changes over time whereas comparing the presence of antibiotic resistance genes with the antibiotic treatment regime could reveal how antibiotics impact the population. Furthermore, comparing the DNA sequences of the *mutL and mutS* genes could identify mutations that confer the hypermutator phenotype (Maciá *et al*., 2005; Cramer *et al*., 2011). The gene presence/absence data obtained from the Roary output could be analysed further using Scoary to try and link this data with the observed phenotypic variations (Brynildsrud *et al*., 2016).

Unfortunately, as there was no access to patient sputum during this study, the sputum

microbiome was not analysed. Previous work has found that *P. aeruginosa* accounts for approximately 87% of the lung microbiota in patients with a *P. aeruginosa* infection. However, in the absence of a *P. aeruginosa* infection, the microbiota is more diverse and mainly consists of bacterial species including: *Prevotella, Viellonella* and *Streptococcus* (31.9, 20.8 and 13.2%, respectively) (Rogers *et al*., 2015). If plasmapheresis causes the clearance of the dominating *P. aeruginosa* population, the other microbial species could then re-populate the lung. This would create competition for *P. aeruginosa* during the early stages of proliferation once the high inhibitory antibody titres return and could therefore explain the genetic variations between isolates.

6.4.2 The origin of inhibitory antibodies

The presence of inhibitory antibody in healthy sera indicates that an active *P. aeruginosa* infection is not required for the stimulation of inhibitory antibody production. In order to confirm that this healthy donor does not have an underlying immune abnormality, the concentration of antibody should be confirmed to fall within the normal reference concentrations: IgA 70–400 mg/dl; IgG 700–1600 mg/dl; and IgM 40–230 mg/dl (Gonzalex-Quintela *et al*., 2008).

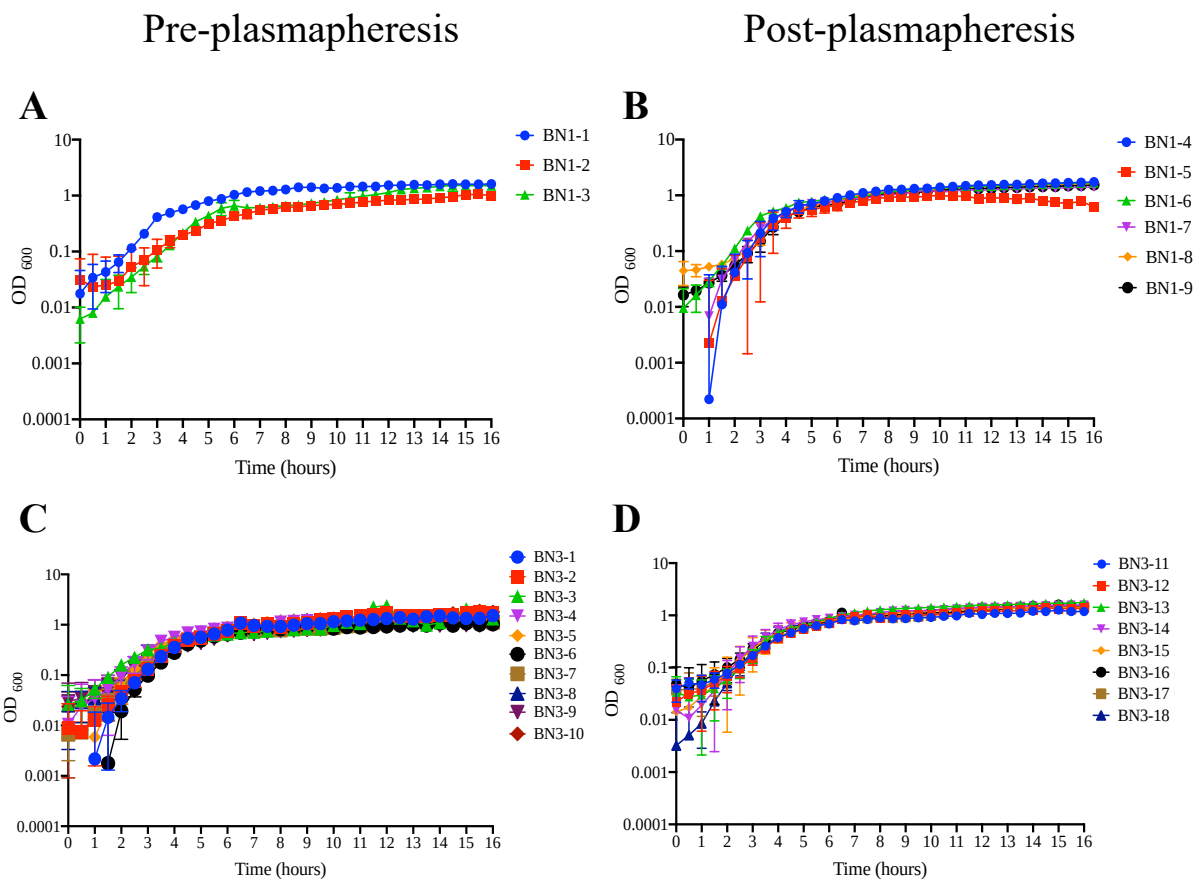
B-cells are responsible for the production of antibodies and the levels of B-cells are mainly influenced by environmental stimuli rather than genetic variables (Aguirre-Gamboa *et al*., 2016). The production of inhibitory antibodies could be linked to an increase in the production of B-cells. Therefore, the number of antibody secreting B-cells that secrete LPS-specific inhibitory antibody could be determined and compared to the number of B-cells present in donors without inhibitory antibodies using an ELISpot assay (Carter *et al*., 2017). Alternatively, the level of expression of the B lymphocyte-induced maturation protein-1

(BLIMP-1) could be assessed. This protein plays a role in down-regulating B-cell proliferation and promotes B-cell differentiation into long lived-antibody secreting plasma cells (Shaffer *et al,* 2002). These cells have an increased rate of antibody secretion and therefore an increase in B-cell differentiation in response to the inhibitory antibody target could explain the elevated antibody titres observed in inhibitory antibody patients (Tellier and Nutt, 2019). Finally, a specific B-cell receptor might be responsible for the production of inhibitory antibody. Recently a B-cell receptor sequencing technique has been described that allows B-cell sequences to be mapped to specific antigens (Setliff *et al*., 2019). This technique could be utilized to identify the B-cell receptor responsible for the production of inhibitory antibody.

Understanding the origin of inhibitory antibodies could lead to the development of a therapeutic treatment to control the production of these antibodies. This would be beneficial for patients with inhibitory antibodies as the effect of plasmapheresis is only temporary and the concentration of inhibitory antibody increases over time, which means patients need to receive the treatment repeatedly (Wells *et al*., 2017). A treatment that prevents the production of inhibitory antibody would circumvent the need for regular treatment with plasmapheresis.

APPENDICES

Appendix I Log scale growth curves for BN1(A and B) and BN3 (C and D) isolates

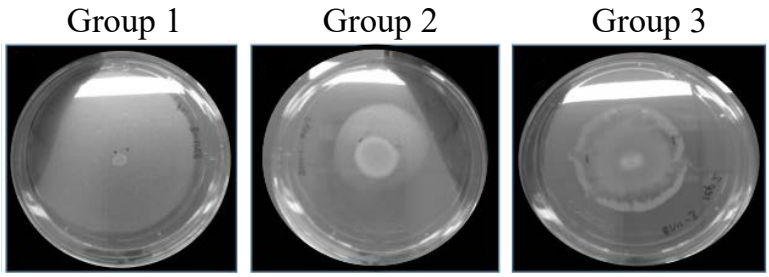


*missing data points for 30 min and 1 h are due to negative OD_{600} Clariostar readings

Appendix II Individual swimming motility results for BN1 (A) and BN3 (B) isolates with representative plate images

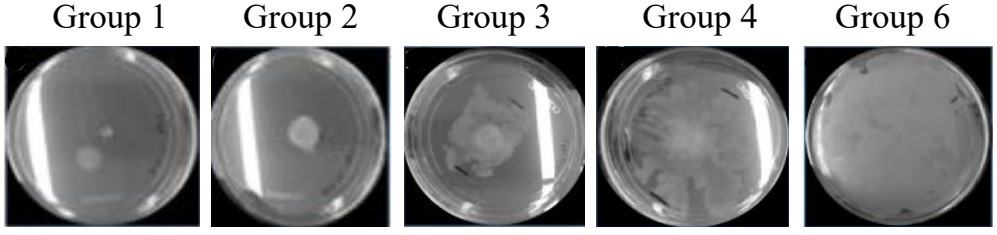
A

Strain	Diameter (mm)	Group
BN1-1	17 ± 2	2
BN1-2	5	1
BN1-3	34.7 ± 9.7	3
BN1-4	21 ± 5	2
BN1-5	19.7 ± 2.3	2
BN1-6	25 ± 1	2
BN1-7	24.3 ± 9.7	2
BN1-8	30 ± 10	2
BN1-9	26.3 ± 2.3	2



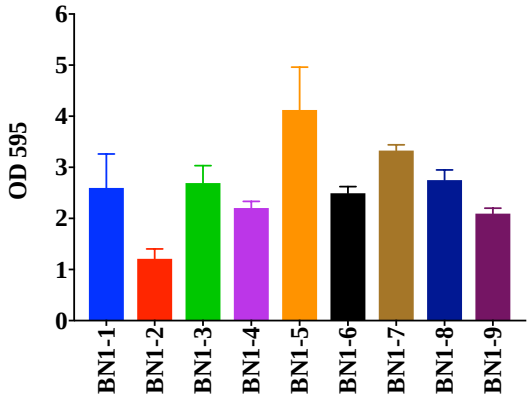
B

Strain	Diameter (mm)	Group	Strain	Diameter (mm)	Group
BN3-1	20.7 ± 6.7	2	BN3-10	31 ± 19	3
BN3-2	19.7 ± 10.3	2	BN3-11	32.7 ± 7.7	3
BN3-3	5	1	BN3-12	31 ± 11	3
BN3-4	90	6	BN3-13	22.5 ± 2.5	2
BN3-5	90	6	BN3-14	90	6
BN3-6	22.7 ± 15.3	2	BN3-15	85 ± 5	6
BN3-7	22.7 ± 17.3	2	BN3-16	51.7 ± 9.7	4
BN3-8	85 ± 10	6	BN3-17	39.7 ± 5.7	3
BN3-9	18 ± 2	2	BN3-18	90	6



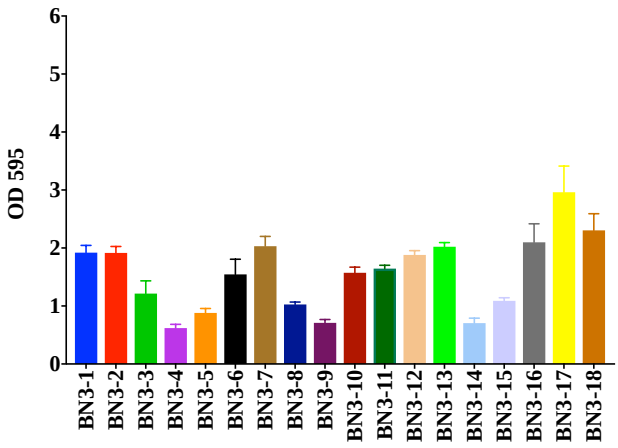
Appendix III Individual biofilm results for BN1 isolates (A) and BN3 isolates (B). Significant variations between isolates were determined by one-way ANOVA with Tukey multiple comparisons test ($p < 0.05$)

A



M9	BN1-1	BN1-2	BN1-3	BN1-4	BN1-5	BN1-6	BN1-7	BN1-8	BN1-9
BN1-1		****	-	-	****	-	*	-	-
BN1-2	****		****	***	****	****	****	****	**
BN1-3	-	****		-	****	-	-	-	-
BN1-4	-	***	-		****	-	****	-	-
BN1-5	****	****	****	****		****	*	****	****
BN1-6	-	****	-	-	****		**	-	-
BN1-7	*	****	-	****	*	**		-	****
BN1-8	-	****	-	-	****	-	-		-
BN1-9	-	**	-	-	****	-	****	-	

B



M9	BN3-1	BN3-2	BN3-3	BN3-4	BN3-5	BN3-6	BN3-7	BN3-8	BN3-9	BN3-10	BN3-11	BN3-12	BN3-13	BN3-14	BN3-15	BN3-16	BN3-17	BN3-18
BN3-1		-	****	****	****	*	-	****	****	-	-	-	-	****	****	-	****	*
BN3-2	-		****	****	****	*	-	****	****	-	-	-	-	****	****	-	****	*
BN3-3	****	****		****	-	-	****	-	***	*	**	****	****	***	-	****	****	****
BN3-4	****	****	****		****	****	****	**	-	****	****	****	****	-	***	****	****	****
BN3-5	****	****	-	-		****	****	-	-	****	****	****	****	-	-	****	****	****
BN3-6	*	*	-	****	****		***	****	****	-	-	-	***	****	**	****	****	****
BN3-7	-	-	****	****	****	***		****	****	**	*	-	-	****	****	-	****	-
BN3-8	****	****	-	**	-	****	****		-	****	****	****	****	-	-	****	****	****
BN3-9	****	****	***	-	-	****	****	-		****	****	****	****	-	*	****	****	****
BN3-10	-	-	*	****	****	-	**	****	****		-	-	**	****	***	****	****	****
BN3-11	-	-	**	****	****	-	*	****	****	-		-	*	****	****	**	****	****
BN3-12	-	-	****	****	****	-	-	****	****	-	-		-	****	****	-	****	**
BN3-13	-	-	****	****	****	***	-	****	****	**	*	-		****	****	-	****	-
BN3-14	****	****	***	-	-	****	****	-	-	****	****	****	****		*	****	****	****
BN3-15	****	****	-	***	-	**	****	-	*	***	****	****	****	*		****	****	****
BN3-16	-	-	****	****	****	****	-	****	****	****	**	-	-	****	****		****	-
BN3-17	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****		****
BN3-18	*	*	****	****	****	****	-	****	****	****	****	**	-	****	****	-	****	

Appendix IV Individual group allocations for BN1 and BN3 *P. aeruginosa* isolates

Isolate	Swarm Group *	Biofilm Group **	Total killing by patient sera?	Inhibitory antibody?	LPS group ***	Serotype	MLST	acsA	aroE	guaA	mutL	nuoD	ppsA	trpE
BN1-1	2	3	N	N	1	O6	ST-179	36/205	27	28	3	4	13	7
BN1-2	1	2	N	N	2	O1	unknown (nearest: ST-1995, 2265, 17, 2894)	11	132	1	7	9	4	7
BN1-3	3	3	Y	N	2	O1	unknown (nearest: ST-1995, 2265, 17, 2894)	11	132	1	7	9	4	7
BN1-4	2	3	N	Y	1	O6	ST-179	36/205	27	28	3	4	13	7
BN1-5	2	5	Y	N	3	O6	ST-179	36/205	27	28	3	4	13	7
BN1-6	2	3	N	Y	1	O6	ST-179	36/205	27	28	3	4	13	7
BN1-7	2	4	Y	N	3	O6	ST-179	36/205	27	28	3	4	13	7
BN1-8	3	3	N	Y	1	O6	ST-179	36/205	27	28	3	4	13	7
BN1-9	2	3	N	Y	1	O6	ST-179	36/205	27	28	3	4	13	7
BN3-1	2	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-2	2	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-3	1	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-4	6	1	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-5	6	1	N	Y	2	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-6	2	2	N	Y	2	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-7	2	3	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-8	6	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-9	2	1	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-10	3	2	N	Y	2	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-11	3	2	N	Y	3	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-12	3	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-13	2	3	N	Y	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-14	6	1	Y	N	2	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-15	6	2	Y	N	1	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-16	4	3	N	Y	3	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-17	3	3	N	Y	3	probable O3	ST-1328	118	104	85	86	42	54	90
BN3-18	6	3	N	Y	2	probable O3	ST-1328	118	104	85	86	42	54	90

Red lines indicate the border between pre-plasmapheresis and post-plasmapheresis isolates

* 1) 0-15 mm, 2) 16-13 mm, 3) 31-45 mm, 4) 46-60 mm, 5) 61-75 mm, 6) 76-90 mm

** OD_{545} 1) 0 <= OD < 1, 2) 1 <= OD < 2, 3) 2 <= OD < 3, 4) 3 <= OD < 4, 5) 4 <= OD < 5, 6) 5 <= OD < 6

*** Visual representation of LPS groups are presented in Figure 3.9.

Appendix V

The pan-genome of BN1 *P. aeruginosa* isolates as determined using Roary

Core genes	**99% ≤ to ≤ 100% genomes**	**5634**
Soft core genes	95% ≤ to < 99% genomes	0
Shell genes	15% ≤ to < 95% genomes	1192
Cloud genes	0% ≤ to < 15% genomes	110
Total		6936

The pan-genome of BN3 *P. aeruginosa* isolates as determined using Roary

Core genes	**99% ≤ to ≤ 100% genomes**	**5736**
Soft core genes	95% ≤ to < 99% genomes	0
Shell genes	15% ≤ to < 95% genomes	163
Cloud genes	0% ≤ to < 15% genomes	76
Total		5975

Appendix VI List of *P. aeruginosa* clinical isolate B3 predicted essential genes

Gene name	Gene product	Pathway
accA	Acetyl-coenzyme A carboxylase carboxyl transferase subunit	Lipid metabolism
accB_2	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	Lipid metabolism
accC_2	Biotin carboxylase	Lipid metabolism
accD	Acetyl-coenzyme A carboxylase carboxyl transferase subunit	Lipid metabolism
acpP_2	Acyl carrier protein	Lipid metabolism
adk	Adenylate kinase	Energy metabolism
ahcY	Adenosylhomocysteinase	Amino acid metabolism
alaS_2	Alanine--tRNA ligase	Protein biosynthesis-translation
algL	Alginate lyase	Glycan biosynthesis and metabolism
amiC_3	N-acetylmuramoyl-L-alanine amidase	Glycan biosynthesis and metabolism
apaH	diadenosine tetraphosphatase	Amino acid metabolism
apbC	Iron-sulfur cluster carrier protein	Energy metabolism
argS	Arginine--tRNA ligase	Protein biosynthesis-translation
aroC	Chorismate synthase	Amino acid metabolism
asd	Aspartate-semialdehyde dehydrogenase	Amino acid metabolism
aspS	Aspartate--tRNA(Asp/Asn) ligase	Protein biosynthesis-translation
atpA	ATP synthase subunit alpha	Energy metabolism
atpB	ATP synthase subunit a	Energy metabolism
atpD	ATP synthase subunit beta	Energy metabolism
atpE	ATP synthase subunit c	Energy metabolism
atpF	ATP synthase subunit b	Energy metabolism
atpG	ATP synthase gamma chain	Energy metabolism
atpH	ATP synthase subunit delta	Energy metabolism
bamA	Outer membrane protein assembly factor	Outer membrane biogenesis
bamB	Outer membrane protein assembly factor	Outer membrane biogenesis
can	Carbonic anhydrase 2	Energy metabolism
cc4	Cytochrome c4	Energy metabolism

ccmA	Cytochrome c biogenesis ATP-binding export protein	Energy metabolism
ccmB	Heme exporter protein B	Energy metabolism
ccmC	Heme exporter protein C	Energy metabolism
ccmE	Cytochrome c-type biogenesis protein	Energy metabolism
ccmF	Cytochrome c-type biogenesis protein	Energy metabolism
ccmH	Cytochrome c-type biogenesis protein	Energy metabolism
cdsA_1	Phosphatidate cytidylyltransferase	Lipid metabolism
cmk	Cytidylate kinase	Nucleotide metabolism
coaBC	Coenzyme A biosynthesis bifunctional protein	Energy metabolism
coaD	Phosphopantetheine adenylyltransferase	Energy metabolism
coaE	Dephospho-CoA kinase	Energy metabolism
coaX	Type III pantothenate kinase	Biosynthetic metabolism
coq7	3-demethoxyubiquinol 3-hydroxylase	Biosynthetic metabolism
csrA_2	translation regulator	Protein biosynthesis-translation
cysS	Cysteine--tRNA ligase	Protein biosynthesis-translation
dapA_4	4-hydroxy-tetrahydrodipicolinate synthase	Amino acid metabolism
dapB	4-hydroxy-tetrahydrodipicolinate reductase	Amino acid metabolism
dapD	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	Amino acid metabolism
dapE	Succinyl-diaminopimelate desuccinylase	Amino acid metabolism
dapF	Diaminopimelate epimerase	Amino acid metabolism
dcd	dCTP deaminase	Nucleotide metabolism
def_1	Peptide deformylase	Protein biosynthesis-Folding, sorting and degradation
degP	Periplasmic serine endoprotease	Protein biosynthesis-Folding, sorting and degradation
der	GTPase	Protein biosynthesis-translation
dksA_3	RNA polymerase-binding transcription factor	Protein biosynthesis-transcription
dnaA	Chromosomal replication initiator protein	DNA replication
dnaB_3	Replicative DNA helicase	DNA replication
dnaE	DNA polymerase III subunit alpha	DNA replication
dnaG	DNA primase	DNA replication

dnaN	DNA polymerase III subunit beta	DNA replication
dnaQ	DNA polymerase III subunit epsilon	DNA replication
dnaX	DNA polymerase III subunit tau	DNA replication
dpp5	Dipeptidyl-peptidase 5	Protein biosynthesis-Folding, sorting and degradation
dsbE	Thiol:disulfide interchange protein	Energy metabolism
dsrE	Putative sulfurtransferase	Protein biosynthesis-translation
dsrF	Intracellular sulfur oxidation protein	Protein biosynthesis-translation
dut	Deoxyuridine 5'-triphosphate nucleotidohydrolase	Nucleotide metabolism
dxr	1-deoxy-D-xylulose 5-phosphate reductoisomerase	Terpenoid metabolism
dxs	1-deoxy-D-xylulose-5-phosphate synthase	Metabolic intermediate biosynthesis
engB	putative GTP-binding protein	Cell division
eno	Enolase	Carbohydrate metabolism
entD	Enterobactin synthase component D	Energy metabolism
era	GTPase Era	Protein biosynthesis-translation
erpA	Iron-sulfur cluster insertion protein	Energy metabolism
etfA_1	Electron transfer flavoprotein subunit alpha	Fatty acid metabolism
etfB	Electron transfer flavoprotein subunit beta	Fatty acid metabolism
exbB_3	Biopolymer transport protein	Protein transport
exbD_2	Biopolymer transport protein	Protein transport
fabA	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase	Fatty acid metabolism
fabB	3-oxoacyl-[acyl-carrier-protein] synthase 1	Fatty acid metabolism
fabD_2	Malonyl CoA-acyl carrier protein transacylase	Fatty acid metabolism
fabG_4	3-oxoacyl-[acyl-carrier-protein] reductase	Fatty acid metabolism
fabV	Enoyl-[acyl-carrier-protein] reductase [NADH]	Fatty acid metabolism
fabY	Beta-ketoacyl-[acyl-carrier-protein] synthase	Fatty acid metabolism
fabZ	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	Fatty acid metabolism
fbcH	Cytochrome b/c1	Energy metabolism
fbp_2	Fructose-1,6-bisphosphatase class 1	Carbohydrate metabolism

fda	Fructose-bisphosphate aldolase	Carbohydrate metabolism
fdx_2	2Fe-2S ferredoxin	Energy metabolism
ffh	Signal recognition particle protein	Transport
fis	DNA-binding protein	Protein biosynthesis-transcription
fmt	Methionyl-tRNA formyltransferase	Protein biosynthesis-translation
folB	Dihydroneopterin aldolase	Cofactor and vitamin metabolism
folC	Dihydrofolate synthase/folylpolyglutamate synthase	Cofactor and vitamin metabolism
folD	Bifunctional protein	Cofactor and vitamin metabolism
folK	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	Cofactor and vitamin metabolism
folP	Dihydropteroate synthase	Cofactor and vitamin metabolism
fpr_1	Ferredoxin--NADP reductase	Energy metabolism
frr	Ribosome-recycling factor	Protein biosynthesis-translation
ftsA_1	Cell division protein	Cell division
ftsB	Cell division protein	Cell division
ftsI	Peptidoglycan D,D-transpeptidase	Cell wall biogenesis / cell division
ftsL	Cell division protein	Cell division
ftsQ	Cell division protein	Cell division
ftsW	Probable peptidoglycan glycosyltransferase	Cell wall biogenesis / cell division
ftsY	Signal recognition particle receptor	Inner membrane biogenesis
ftsZ	Cell division protein	Cell division
fur	Ferric uptake regulation protein	Protein biosynthesis-transcription, translation
fusA_1	Elongation factor G 1	Protein biosynthesis-translation
gap2	Glyceraldehyde-3-phosphate dehydrogenase-like protein	Carbohydrate metabolism
gatA	Glutamyl-tRNA(Gln) amidotransferase subunit A	Carbohydrate metabolism, transport
gatB	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	Carbohydrate metabolism, transport
gatC	Glutamyl-tRNA(Gln) amidotransferase subunit C	Carbohydrate metabolism, transport
glmM	Phosphoglucosamine mutase	Carbohydrate metabolism
glmS_2	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	Carbohydrate metabolism
glmU	Bifunctional protein	Carbohydrate metabolism

glnA_1	Glutamine synthetase	Energy metabolism
glnK	Nitrogen regulatory protein P-II 2	Energy metabolism
glnS	Glutamine--tRNA ligase	Protein biosynthesis-translation
gltA_2	Citrate synthase	Carbohydrate metabolism
gltX	Glutamate--tRNA ligase	Protein biosynthesis-translation
glyA_2	Serine hydroxymethyltransferase	Amino acid metabolism
glyQ	Glycine--tRNA ligase alpha subunit	Protein biosynthesis-translation
glyS	Glycine--tRNA ligase beta subunit	Protein biosynthesis-translation
gmhA	Phosphoheptose isomerase	LPS biosynthesis
gmhB	D-glycero-beta-D-manno-heptose-1,7-bisphosphate 7-phosphatase	LPS biosynthesis
gpmI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Energy metabolism
groL	60 kDa chaperonin	Protein biosynthesis-Folding, sorting and degradation / cell division
groS	10 kDa chaperonin	Protein biosynthesis-Folding, sorting and degradation / cell division
grpE	Protein GrpE	Protein biosynthesis-Folding, sorting and degradation / stress response
guaA_2	GMP synthase [glutamine-hydrolyzing]	Nucleotide metabolism
guaB_1	Inosine-5'-monophosphate dehydrogenase	Nucleotide metabolism
gyrA	DNA gyrase subunit A	DNA replication
gyrB	DNA gyrase subunit B	DNA replication
hda	DnaA regulatory inactivator	DNA replication
hemH	Ferrochelatase	Cofactor and vitamin metabolism
hisS	Histidine--tRNA ligase	Protein biosynthesis-translation
hldE	Bifunctional protein	LPS biosynthesis
holA	DNA polymerase III subunit delta	DNA replication
holB	DNA polymerase III subunit delta	DNA replication
hscA	Chaperone protein	Protein biosynthesis-Folding, sorting and degradation / energy metabolism
hscB	Co-chaperone protein HscB homolog	Protein biosynthesis-Folding, sorting and degradation
hupB_1	DNA-binding protein HU-beta	Stress response

ibaG	Acid stress protein	Stress response
ileS	Isoleucine--tRNA ligase	Protein biosynthesis-translation
infA	Translation initiation factor IF-1	Protein biosynthesis-translation
infB	Translation initiation factor IF-2	Protein biosynthesis-translation
infC	Translation initiation factor IF-3	Protein biosynthesis-translation
iscA	Iron-binding protein	Cofactor and vitamin metabolism
iscS_1	Cysteine desulfurase	Cofactor and vitamin metabolism
iscU	Iron-sulfur cluster assembly scaffold protein	Cofactor and vitamin metabolism
ispA	Farnesyl diphosphate synthase	Terpenoid metabolism
ispB	Octaprenyl diphosphate synthase	Terpenoid metabolism
ispD_1	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Terpenoid metabolism
ispE	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	Terpenoid metabolism
ispF	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	Terpenoid metabolism
ispG	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	Terpenoid metabolism
ispH	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Terpenoid metabolism
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase	LPS biosynthesis
kdsB	3-deoxy-manno-octulosonate cytidylyltransferase	LPS biosynthesis
kdsD	Arabinose 5-phosphate isomerase	LPS biosynthesis
lepB_2	Signal peptidase I	Protein biosynthesis-Folding, sorting and degradation
leuS	Leucine--tRNA ligase	Protein biosynthesis-translation
lgt	Prolipoprotein diacylglyceryl transferase	Protein biosynthesis-Folding, sorting and degradation
ligA	DNA ligase	DNA replication / DNA repair
lipA	Lipoyl synthase	Protein modification
lipB	Octanoyltransferase	Protein modification
lnt	Apolipoprotein N-acyltransferase	Protein modification
lolA	Outer-membrane lipoprotein carrier protein	Protein transport
lolB	Outer-membrane lipoprotein	Protein transport
lolD	Lipoprotein-releasing system ATP-binding protein	Protein transport

lolE_1	Lipoprotein-releasing system transmembrane protein	Protein transport
lolE_2	Lipoprotein-releasing system transmembrane protein	Protein transport
lpd	Dihydrolipoyl dehydrogenase	Energy metabolism
lptA	Lipopolysaccharide export system protein	LPS transport
lptB_2	Lipopolysaccharide export system ATP-binding protein	LPS transport
lptC	Lipopolysaccharide export system protein	LPS transport
lptD	LPS-assembly protein	LPS transport
lptF	Lipopolysaccharide export system permease protein	LPS transport
lptG	Lipopolysaccharide export system permease protein	LPS transport
lpxA	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	Lipid metabolism
lpxB	Lipid-A-disaccharide synthase	Lipid metabolism
lpxC	UDP-3-O-acyl-N-acetylglucosamine deacetylase	Lipid metabolism
lpxD	UDP-3-O-acylglucosamine N-acyltransferase	Lipid metabolism
lpxH_2	UDP-2,3-diacylglucosamine hydrolase	Lipid metabolism
lpxK	Tetraacyldisaccharide 4'-kinase	Lipid metabolism
lspA	Lipoprotein signal peptidase	Protein biosynthesis-Folding, sorting and degradation
lysC	Aspartate kinase Ask_LysC	Amino acid metabolism
lysS	Lysine--tRNA ligase	Protein biosynthesis-translation
metG	Methionine--tRNA ligase	Protein biosynthesis-translation
metK	S-adenosylmethionine synthase	Amino acid metabolism
mnmA	tRNA-specific 2-thiouridylase	Protein biosynthesis-translation
mnmC_2	tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein	Protein biosynthesis-translation
mraY	Phospho-N-acetylmuramoyl-pentapeptide-transferase	Cell wall biogenesis
mraZ	Transcriptional regulator	Protein biosynthesis-translation
mrdA	Peptidoglycan D,D-transpeptidase	Cell wall biogenesis
mrdB	Peptidoglycan glycosyltransferase	Cell wall biogenesis
mreB	Rod shape-determining protein	Cell wall biogenesis

mreC	Cell shape-determining protein	Cell wall biogenesis
mreD	Rod shape-determining protein	Cell wall biogenesis
mrpD	Na(+)/H(+) antiporter subunit D	Nucleotide metabolism
msbA_4	Lipid A export ATP-binding/permease protein	LPS transport / energy metabolism
murA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Cell wall biogenesis
murB	UDP-N-acetylenolpyruvoylglucosamine reductase	Cell wall biogenesis
murC	UDP-N-acetylmuramate--L-alanine ligase	Cell wall biogenesis
murD	UDP-N-acetylmuramoylalanine--D-glutamate ligase	Cell wall biogenesis
murE	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	Cell wall biogenesis
murF	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	Cell wall biogenesis
murG	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	Cell wall biogenesis
murI	Glutamate racemase	Cell wall biogenesis
murJ	Probable lipid II flippase	Cell wall biogenesis
nadE	NH(3)-dependent NAD(+) synthetase	Cofactor and vitamin metabolism
nadK	NAD kinase	Cofactor and vitamin metabolism
ndk	Nucleoside diphosphate kinase	Nucleotide metabolism
nrdA	Ribonucleoside-diphosphate reductase 1 subunit alpha	DNA replication
nrdB	Ribonucleoside-diphosphate reductase subunit beta	DNA replication
nusA	Transcription termination/antitermination protein	Protein biosynthesis-transcription
nusB	N utilization substance protein B	Protein biosynthesis-transcription
nusG	Transcription termination/antitermination protein	Protein biosynthesis-transcription
orn	Oligoribonuclease	Protein biosynthesis-transcription
pal_2	Peptidoglycan-associated lipoprotein	Cell division
parC	DNA topoisomerase 4 subunit A	DNA replication / cell division
parE	DNA topoisomerase 4 subunit B	DNA replication / cell division
petA	Ubiquinol-cytochrome c reductase iron-sulfur subunit	Energy metabolism

pgk	Phosphoglycerate kinase	Carbohydrate metabolism
pglJ	N-acetylgalactosamine-N,N'-diacetylbacillosaminyl-diphospho-undecaprenol 4-alpha-N-acetylgalactosaminyltransferase	Protein modification
pgsA	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Lipid metabolism
pheS	Phenyalanine--tRNA ligase alpha subunit	Protein biosynthesis-translation
pheT	Phenyalanine--tRNA ligase beta subunit	Protein biosynthesis-translation
phoU	Phosphate-specific transport system accessory protein	Stress response
pnp	Polyribonucleotide nucleotidyltransferase	Protein biosynthesis-translation
ppa	Inorganic pyrophosphatase	Energy metabolism
prfA	Peptide chain release factor RF1	Protein biosynthesis-translation
prfB_1	Peptide chain release factor RF2	Protein biosynthesis-translation
priA	Primosomal protein N'	DNA replication
proS	Proline--tRNA ligase	Protein biosynthesis-translation
prtR_2	HTH-type transcriptional regulator	Secondary metabolite biosynthesis
prtR_3	HTH-type transcriptional regulator	Secondary metabolite biosynthesis
psd	Phosphatidylserine decarboxylase proenzyme	Lipid metabolism
pth	Peptidyl-tRNA hydrolase	Protein biosynthesis-translation
purB_2	Adenylosuccinate lyase	Nucleotide metabolism
pyrG	CTP synthase	Nucleotide metabolism
pyrH	Uridylate kinase	Nucleotide metabolism
qorA_2	Quinone oxidoreductase 1	Cofactor and vitamin metabolism
ratA	Ribosome association toxin	Protein biosynthesis-translation
rfaC	Lipopolysaccharide heptosyltransferase 1	Outer membrane biogenesis
rfaF	ADP-heptose--LPS heptosyltransferase 2	Outer membrane biogenesis
rfaG	Lipopolysaccharide core biosynthesis protein	Outer membrane biogenesis
rfaP_1	Lipopolysaccharide core heptose(I) kinase	Outer membrane biogenesis
rfaP_2	Lipopolysaccharide core heptose(I) kinase	Outer membrane biogenesis
rho	Transcription termination factor	Protein biosynthesis-transcription

ribD	Riboflavin biosynthesis protein	Cofactor and vitamin metabolism
ribE	Riboflavin synthase	Cofactor and vitamin metabolism
ribF	Riboflavin biosynthesis protein	Cofactor and vitamin metabolism
ribH	6,7-dimethyl-8-ribityllumazine synthase	Cofactor and vitamin metabolism
rimM	Ribosome maturation factor	Protein biosynthesis-translation
rimP	Ribosome maturation factor	Protein biosynthesis-translation
rnhA	Ribonuclease HI	DNA replication
rnpA	Ribonuclease P protein component	DNA replication
rpe	Ribulose-phosphate 3-epimerase	Carbohydrate metabolism
rpiA	Ribose-5-phosphate isomerase A	Carbohydrate metabolism
rplA	50S ribosomal protein L1	Protein biosynthesis-translation
rplB	50S ribosomal protein L2	Protein biosynthesis-translation
rplC	50S ribosomal protein L3	Protein biosynthesis-translation
rplD	50S ribosomal protein L4	Protein biosynthesis-translation
rplE	50S ribosomal protein L5	Protein biosynthesis-translation
rplF	50S ribosomal protein L6	Protein biosynthesis-translation
rplJ	50S ribosomal protein L10	Protein biosynthesis-translation
rplK	50S ribosomal protein L11	Protein biosynthesis-translation
rplL	50S ribosomal protein L7/L12	Protein biosynthesis-translation
rplM	50S ribosomal protein L13	Protein biosynthesis-translation
rplN	50S ribosomal protein L14	Protein biosynthesis-translation
rplO	50S ribosomal protein L15	Protein biosynthesis-translation
rplP	50S ribosomal protein L16	Protein biosynthesis-translation
rplQ	50S ribosomal protein L17	Protein biosynthesis-translation
rplR	50S ribosomal protein L18	Protein biosynthesis-translation
rplS	50S ribosomal protein L19	Protein biosynthesis-translation
rplT	50S ribosomal protein L20	Protein biosynthesis-translation
rplU	50S ribosomal protein L21	Protein biosynthesis-translation
rplV	50S ribosomal protein L22	Protein biosynthesis-translation
rplW	50S ribosomal protein L23	Protein biosynthesis-translation
rplX	50S ribosomal protein L24	Protein biosynthesis-translation
rpmB	50S ribosomal protein L28	Protein biosynthesis-translation

rpmC	50S ribosomal protein L29	Protein biosynthesis-translation
rpmD	50S ribosomal protein L30	Protein biosynthesis-translation
rpmH	50S ribosomal protein L34	Protein biosynthesis-translation
rpmI	50S ribosomal protein L35	Protein biosynthesis-translation
rpmJ	50S ribosomal protein L36	Protein biosynthesis-translation
rpoA	DNA-directed RNA polymerase subunit alpha	Protein biosynthesis-transcription
rpoB	DNA-directed RNA polymerase subunit beta	Protein biosynthesis-transcription
rpoC	DNA-directed RNA polymerase subunit beta	Protein biosynthesis-transcription
rpoD	RNA polymerase sigma factor	Protein biosynthesis-transcription
rpoH	RNA polymerase sigma factor	Protein biosynthesis-transcription
rpoN	RNA polymerase sigma-54 factor	Protein biosynthesis-transcription
rpsA_1	30S ribosomal protein S1	Protein biosynthesis-translation
rpsB	30S ribosomal protein S2	Protein biosynthesis-translation
rpsC	30S ribosomal protein S3	Protein biosynthesis-translation
rpsD	30S ribosomal protein S4	Protein biosynthesis-translation
rpsE	30S ribosomal protein S5	Protein biosynthesis-translation
rpsF	30S ribosomal protein S6	Protein biosynthesis-translation
rpsG	30S ribosomal protein S7	Protein biosynthesis-translation
rpsH	30S ribosomal protein S8	Protein biosynthesis-translation
rpsI	30S ribosomal protein S9	Protein biosynthesis-translation
rpsJ	30S ribosomal protein S10	Protein biosynthesis-translation
rpsK	30S ribosomal protein S11	Protein biosynthesis-translation
rpsL	30S ribosomal protein S12	Protein biosynthesis-translation
rpsM	30S ribosomal protein S13	Protein biosynthesis-translation
rpsN	30S ribosomal protein S14	Protein biosynthesis-translation
rpsO	30S ribosomal protein S15	Protein biosynthesis-translation
rpsP	30S ribosomal protein S16	Protein biosynthesis-translation
rpsQ	30S ribosomal protein S17	Protein biosynthesis-translation
rpsR	30S ribosomal protein S18	Protein biosynthesis-translation
rpsS	30S ribosomal protein S19	Protein biosynthesis-translation
rpsU	30S ribosomal protein S21	Protein biosynthesis-translation

rsmH	Ribosomal RNA small subunit methyltransferase H	Protein biosynthesis-translation
rssB	Regulator of RpoS	Protein biosynthesis-translation
sdhA	Succinate dehydrogenase flavoprotein subunit	Carbohydrate metabolism
sdhB	Succinate dehydrogenase iron-sulfur subunit	Carbohydrate metabolism
sdhC	Succinate dehydrogenase cytochrome b556 subunit	Carbohydrate metabolism
sdhD	Succinate dehydrogenase hydrophobic membrane anchor subunit	Carbohydrate metabolism
secA	Protein translocase subunit	Protein export
secY	Protein translocase subunit	Protein export
serS	Serine--tRNA ligase	Protein biosynthesis-translation
sodB_1	Superoxide dismutase [Fe]	Stress response
ssb	Single-stranded DNA-binding protein	DNA replication / DNA repair
sucA	Succinate dehydrogenase iron-sulfur subunit	Carbohydrate metabolism
sucB	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Carbohydrate metabolism
surA	Chaperone	Outer membrane biogenesis / protein biosynthesis-Folding, sorting and degradation
tadA	tRNA-specific adenosine deaminase	Protein biosynthesis-translation
tatA	Sec-independent protein translocase protein	Protein export
thiL	Thiamine-monophosphate kinase	Cofactor and vitamin metabolism
thrS	Threonine--tRNA ligase	Protein biosynthesis-translation
thyA	Thymidylate synthase	Nucleotide metabolism
tilS	tRNA(Ile)-lysidine synthase	Protein biosynthesis-translation
tktA	Transketolase 1	Carbohydrate metabolism / stress response
tmk_2	Thymidylate kinase	Nucleotide metabolism
tobZ	nebramycin 5' synthase	Secondary metabolite biosynthesis
topA_1	DNA topoisomerase 1	DNA replication
tpiA	Triosephosphate isomerase	Carbohydrate metabolism
trmD	tRNA (guanine-N(1)-)-methyltransferase	Protein biosynthesis-translation
trpS	Tryptophan--tRNA ligase	Protein biosynthesis-translation

trxA_3	Thioredoxin 1	Energy metabolism
tsaB	tRNA threonylcarbamoyladenosine biosynthesis protein	Protein biosynthesis-translation
tsaC	Threonylcarbamoyl-AMP synthase	Protein biosynthesis-translation
tsaD	tRNA N6-adenosine threonylcarbamoyltransferase	Protein biosynthesis-translation
tsaE	tRNA threonylcarbamoyladenosine biosynthesis protein	Protein biosynthesis-translation
tsf	Elongation factor Ts	Protein biosynthesis-translation
tusA_1	Sulfurtransferase	Protein biosynthesis-translation
tusB	polypeptide sulfurtransferase complex subunit	Protein biosynthesis-translation
tusE	Sulfurtransferase	Protein biosynthesis-translation
ubiA	4-hydroxybenzoate octaprenyltransferase	Cofactor and vitamin metabolism
ubiB	Probable protein kinase	Cofactor and vitamin metabolism
ubiD	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	Cofactor and vitamin metabolism
ubiE_3	Ubiquinone/menaquinone biosynthesis C-methyltransferase	Cofactor and vitamin metabolism
ubiG_2	Ubiquinone biosynthesis O-methyltransferase	Cofactor and vitamin metabolism
ubiH	2-octaprenyl-6-methoxyphenol hydroxylase	Cofactor and vitamin metabolism
ubiI	2-octaprenylphenol hydroxylase	Cofactor and vitamin metabolism
uppS	Ditrans,polycis-undecaprenyl-diphosphate synthase ((2E,6E)-farnesyl-diphosphate specific)	Cell wall biogenesis / cell division
valS	Valine--tRNA ligase	Protein biosynthesis-translation
waaA	3-deoxy-D-manno-octulosonic acid transferase	Outer membrane biogenesis / LPS biosynthesis
wbgU	UDP-N-acetylglucosamine 4-epimerase	Outer membrane biogenesis / LPS biosynthesis
ybeY	Endoribonuclease	Protein biosynthesis-translation
ygfZ	tRNA-modifying protein	Protein biosynthesis-translation
yhdN_4	General stress protein 69	Stress response
yidC	Membrane protein insertase	Inner membrane biogenesis
yqgF	Putative pre-16S rRNA nuclease	Protein biosynthesis-translation
zipA	Cell division protein	bacterial replication

Appendix VII Sub-categories of the biological processes included in the essential genome analysis

Biological process	Sub-category
Metabolism	Amino acid metabolism (12)
	Carbohydrate metabolism/transport (22)
	Cofactor and vitamin metabolism (26)
	Energy metabolism (36)
	Fatty acid metabolism (9)
	Glycan biosynthesis and metabolism (2)
	Lipid metabolism (14)
	Nucleotide metabolism (12)
	Secondary metabolite biosynthesis (3)
	Terpenoid metabolism (8)
Replication	Cell division (16)
	Cell wall biogenesis (18)
	DNA replication / repair (22)
Protein biosynthesis	Protein biosynthesis-transcription (14)
	Protein biosynthesis-translation (105)
	Protein biosynthesis-Folding, sorting and degradation (12)
	Protein modification (4)
	Protein transport (11)
Membrane biogenesis	Inner membrane biogenesis (2)
	LPS biosynthesis (8)
	LPS transport (7)
	Outer membrane biogenesis (10)
Stress	Stress response (7)
Hypothetical	Hypothetical proteins (30)

Appendix VIII List of *P. aeruginosa* clinical isolate B3 genes that have essential regions or orientation-dependent insertions

Gene name	Gene product
aceF	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex
algC	Phosphomutase/phosphoglucomutase
anoR	Transcriptional activator protein
aroQ1_2	3-dehydroquinate dehydratase
bamD	OM protein assembly factor
bamE	OMP assembly factor
bedC2	Benzene 1,2-dioxygenase subunit beta
birA	Bifunctional ligase/repressor
bsaA	Glutathione peroxidase homologue
bvgA_2	Virulence factor putative positive transcription regulator
ccoN1_4	Cbb3-type cytochrome c oxidase subunit
ccoP2	Cbb3-type cytochrome c oxidase subunit
copA_4	Probable copper-importing P-type ATPase A
ctpB	Carboxy-terminal processing protease
dnaK_1	Chaperone protein
efp	Elongation factor P
epd_1	D-erythrose-4-phosphate dehydrogenase
exsA_2	Exoenzyme S synthesis regulatory protein
fdx_1	Ferredoxin
fdxA_1	Ferredoxin
ftsK	DNA translocase
ftsN_2	Cell division protein
gmk	Guanylate kinase
gpr_2	L-glyceraldehyde-3-phosphate reductase
hemA	Glutamyl-tRNA reductase
hin	DNA-invertase
holC	DNA polymerase III subunit chi
hpxO_1	FAD-dependent urate hydroxylase
hrp1_2	Hypoxic response protein 1
ilvE	Branched chain amino acid aminotransferase

lecA	PA-I galactophilic lectin
lon_1	Lon protease
lppL	Lipopeptide
lptE	LPS assembly lipoprotein
maeB	NADP-dependent malic enzyme
minE	Cell division topological specificity factor
mnhC1	Na(+)/H(+) antiporter subunit C1
mnmG	tRNA uridine 5-carboxymethylaminomethyl modification enzyme
mqo_2	Malate:quinone oxidoreductase
mrpA	Na(+)/H(+) antiporter subunit A
mrpA	Na(+)/H(+) antiporter subunit A
nrfG	Formate-dependent nitrite reductase complex subunit
obg	GPTase
ompR_4	Transcriptional regulatory protein
oprD_5	Porin D
paaF_2	2,3-dehydroadipyl-CoA hydrolase
petB	Cytochrome b
pgdA	Peptidoglycan deacetylase
pgi	Glucose-6-phosphate isomerase
phzB2_4	Phenazine biosynthesis protein 2
phzB2_5	Phenazine biosynthesis protein 2
phzB2_7	Phenazine biosynthesis protein PhzB 2
phzD_5	Putative isochorismatase
polA	DNA polymerase I
ppiB	Peptidyl-prolyl cis-trans isomerase B
ptsN	Nitrogen regulatory protein
purA	Adenylosuccinate synthetase
ramA	(R) - selective amidase
regA	Photosynthetic apparatus regulatory protein
ribBA	Riboflavin biosynthesis protein
rnc	Ribonuclease 3
rne	Ribonuclease E
rodZ	Cytoskeleton protein
rpmA	50S ribosomal protein L27

rpoE	ECF RNA polymerase sigma-E factor
rsgA	Small ribosomal subunit biogenesis GTPase
sigW	ECF RNA polymerase sigma factor
smc_1	Chromosome partition factor
spoT_2	Bifunctional (P)ppGpp synthase/hydrolase
tatB	Sec-independent protein translocase protein
tatC	Sec-independent protein translocase protein
yadV_6	Putative fimbrial chaperone
yciB	Intracellular septation protein A
ydcV_2	Inner membrane ABC transporter permease protein
yfkJ	Low molecular weight protein-tyrosine-phosphatase
zapE_1	Cell division protein
zraR_5	Transcriptional regulatory protein

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