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Contribution of Glucose to Increased Respiratory Bacterial Burden in Hyperglycaemia

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Abstract

It has recently been proposed that uncontrolled hyperglycaemia in people with diabetes increases lung glucose, so providing a richer growth medium facilitating bacterial infection. Where diabetes is controlled, there is a reduced associated risk of bacterial infection. The aim of this thesis was to determine the effect of glucose on bacterial growth *in vivo* and directly link bacterial glucose metabolism with increased respiratory tract bacterial load in hyperglycaemia. *P. aeruginosa*, a Gram-negative opportunistic pathogen, which is a major cause of respiratory infections, was used as chronic *P. aeruginosa* infections are most commonly associated with people with cystic fibrosis (CF) and chronic obstructive pulmonary diseases (COPD), but importantly *P. aeruginosa* is increasingly diagnosed in diabetic patients with pneumonia/lung infection. To test the hypothesis, *P. aeruginosa* mutants were generated by deleting *oprB*, *gltK*, *gtrS* and *glk* genes and these mutant strains were used in *in vitro* and *in vivo* infection models developed during this thesis. The mutants had drastically reduced growth in minimal medium containing glucose as the sole carbon source, whereas they were unaltered when grown in rich medium. In order to explore the effect of elevated glucose with minimal effects on the immune response, streptozocin was used to induce diabetes, instead of genetically obese mice, which have a complex phenotype and impaired immune responses. Streptozocin induced hyperglycaemia also led to increased bacterial load in the airways when mice were infected with wild type PAO1 but not with the glucose uptake and metabolism mutants. To further support this hypothesis when metformin was used to lower glucose levels it resulted in a reduced bacterial burden.

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Declaration of Originality

The work presented in this thesis is my own and where appropriate all references are cited.

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Abbreviations

ACK Buffer Ammonium-Chloride-Potassium Buffer

ASL Airway surface liquid

BAL/BALF Bronchoalveolar Lavage Fluid

CF Cystic Fibrosis

CFU Colony Forming Units

COPD Chronic Obstructive Pulmonary Disorder

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphate

ELISA Enzyme-linked immunosorbent assay

HPI Hours Post infection

IFN Interferon

IL Interleukin

IN Intranasal

IP Intraperitoneal

KO Knock Out

LB Luria Broth

LPS Lipopolysaccharide

mRNA Messenger RNA

MRSA Methicillin-resistant *Staphylococcus aureus*

MyD88 Myeloid Differentiation primary response gene 88

NF-κB Nuclear Factor kappa-light-chain-enhancer of activated B cells

NOD Nucleotide-binding Oligomerization Domain

OD Optical Density

PAO1 Pseudomonas strain

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PRR Pseudomonas Isolation Agar

PRR Pattern Recognition Receptor

RPM Revolutions Per Minute

S.O.C. Super Optimal Broth

STZ Streptozocin

TLR Toll Like Receptor

TNF Tumour Necrosis Factor

WHO World Health Organisation

WT Wild Type

1. Introduction

In an age of rising antibiotic resistance there is a growing need to identify novel alternatives to current treatment regimens and there is increasing pressure on a global scale for the need to develop new drugs to combat antibiotic resistant pathogens (Braine, 2011).

The overuse of antibiotics has led to resistance in a range of pathogenic bacteria and according to recently published reports by the World Health Organisation (WHO) there is increasing prevalence of infections in susceptible patients (Organization, 2014)

Current approaches to combat the rise in resistance involve research to understand how bacteria evolve and develop resistance, better approaches to diagnose bacterial infections, improved surveillance and prescription practices and the development of new drugs and vaccines (Coates and Hu, 2007). In order to develop novel strategies against bacterial pathogens a detailed understanding of host pathogen interactions is required and one area of interest, the main focus of this study, is the role of airway glucose in respiratory bacterial infections.

1.1. Respiratory Bacterial Infections

Respiratory infections can be caused by both bacteria and viruses and can be split into upper and lower respiratory tract infections depending on the location of the pathogen (Figure 1.1). Upper respiratory infections are most commonly caused by viruses but are often caused by bacteria and bacteria are the more common cause

of lower respiratory tract infections (Dasaraju and Liu, 1996) . Infection can be exacerbated by smoking, environmental factors and co-infection. Infection through inhalation is typically the result of an impaired immune response or co-infection where healthy individuals would usually clear infection, those susceptible become infected.

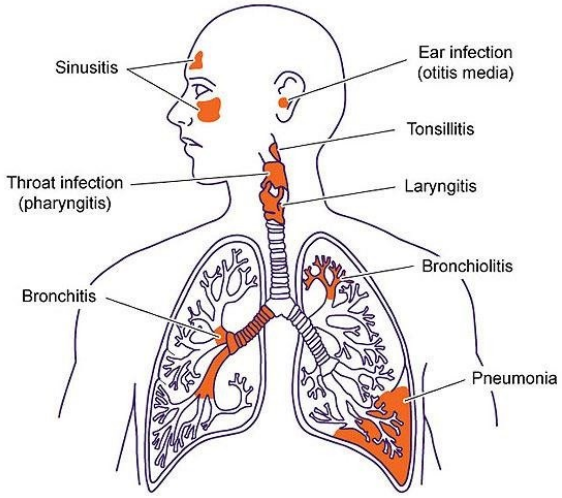


Figure 1.1 Location of upper and lower respiratory tract infections. Upper respiratory infections above the chest include the common cold, throat infections and ear infections. Lower respiratory infections include more serious conditions such as bronchitis, pneumonia and can cause lung damage. Adapted from NPS Medicine Wise 2016.

1.2. Signs and symptoms

Upper respiratory tract infections are usually mild compared to lower respiratory tract infections and are self-limiting in healthy adults. Lower respiratory tract infections lead to bronchitis, bronchiolitis and pneumonia (Dasaraju and Liu, 1996).

Disease following respiratory infection is driven by narrowing or blockage of the airways due to inflammation or direct destruction of lung tissue by the pathogen (Dasaraju and Liu, 1996). Additionally lower respiratory tract infections can result in pneumonia, which is excess fluid in the lungs, which can be caused by infection of the alveolar and surrounding tissue and is commonly caused by bacterial

pathogens. Symptoms of infection include a cough, shortness of breath and chest pains as well as excess sputum production. Combined, these factors contribute to respiratory failure which can be fatal.

Treatment of respiratory tract infections depends on the infectious agent, and can vary as many disease causing bacteria are resistant to current antibiotics adding to the concern of increasing antibiotic resistance on a global scale.

1.3. Clinical Infections with P. aeruginosa

Whilst immunocompromised patients are most likely to be at risk of *P. aeruginosa* infection, individuals with existing lung damage or impaired mucus clearance are also at risk. Systemic infection and entry into the blood stream is uncommon in *P. aeruginosa* infection, highlighting the importance of the progression of infection from acute to chronic in the lungs. Common causes of Pseudomonas infection include susceptibility due to burn wounds, patients with underlying immune deficiencies, diabetes – particularly patients with diabetic foot ulcers, chronic obstructive pulmonary disorder (COPD), and existing pneumonia (Gellatly and Hancock, 2013). Acute infection is usually the result of hospital acquired infection due to susceptibility of at risk patients as well as the high antibiotic resistance of *P. aeruginosa* which is also associated with increased mortality (Zhuo et al., 2008).

1.4. Antimicrobial Resistance

Since the discovery of antibiotics, morbidity and mortality had decreased through prevention and treatment of infections. Antimicrobial resistance is an increasing threat to global health and spans from resistance to antibiotics in isolated areas to resistance to antibiotics in hospitals which can spread. Resistance is a global threat

and the lack of development of novel drugs means that with increasing resistance there are fewer effective antibiotics against bacteria increasing the severity of the consequences of infection. Figure 1.2 demonstrates the spread of one genetic element causing resistance of bacteria to a specific class of antibiotics, metallo-beta-lactamases which was first identified in India in 2008 and has since spread across the world.

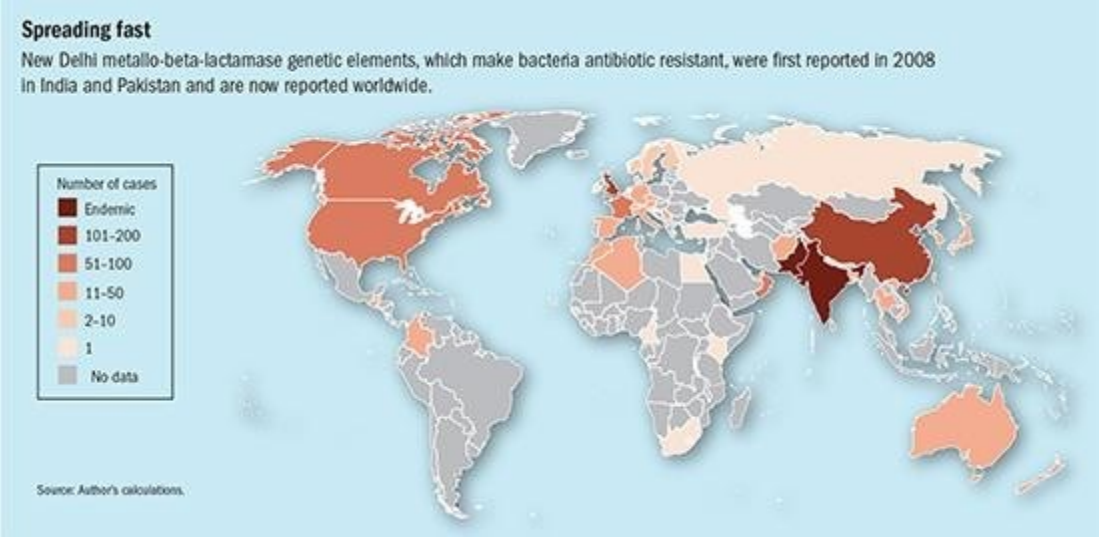


Figure 1.2 The spread of one type of resistance over 6 years.

Metallo beta lactamase spread from India in 2008 to the rest of the world. Taken from a report published by the International Monetary Fund in 2014 (Laxminarayan, 2014).

Strategies to combat this have been implemented such as policy changes on antibiotic use and also public education on adherence to prescriptions of antibiotic courses however the development of novel drugs would accelerate progress against antibiotic resistant bacteria (Pinder et al., 2015).

There are numerous estimates on the cost of infections caused by antibiotic resistant bacteria and according to a recently published report on antimicrobial resistance by the Commercial Secretary to the Treasury in the United Kingdom Government, Lord O'Neill in 50 years there will be up to 10 million annual deaths due to antimicrobial resistant bacteria compared to a current figure of 50 000 annual deaths costing as much as $100 trillion, also contributing to the economic burden of antibiotic resistance (O'Neill, 2014).

Due to the rapidly increasing spread of antibiotic resistance caused by a number of factors including overuse of antibiotics, improper use as well as the natural evolution of bacteria against drugs summarised in table 1.1 there is an urgent need for the development of new treatment strategies.

In light of recent estimates, policy changes are now being implemented globally. For example, a risk factor for increased antibiotic resistance is the intensive use of antibiotics in the farming industry in the United Stated of America to promote growth and profit. This leads to increased resistance as there is greater exposure of bacteria to drugs and once developed, resistance can spread by human to animal contact or vice versa, contact with excrement and spread of resistant strains into the environment in water for example. The Food and Drug Administration (FDA) in the United States of America is promoting the phasing out of antibiotics in farming to stop antibiotics from being used to increase production with tighter controls on medically important antibiotics which are used to treat human infections. Increased use of antibiotics contributes to resistance by inducing environmental stress on bacteria which leads to a change in gene expression particularly in genes that can promote resistance.

Additionally, Public Health England developed a campaign called ‘Antibiotic Guardian’ to increase public knowledge of antimicrobial resistance and the correct use of antibiotics to prevent unnecessary use which contributes to resistance (England, 2016).

Host Factors	Infection Site Factors	Bacteria Factors
Poor adherence	Low pH	High infectious dose
Immune deficiency	Low oxygen	Intrinsic resistance
Lack of delivery to infectious site		Spontaneous mutation

Table 1.1 Factors contributing to antibiotic resistance

Host factors which can contribute to increased antibiotic resistance are varied and can range from individuals not finishing the course of antibiotics which can contribute to persistence to the fact that the antibiotic simply does not reach the site of infection. The site of infection could also prevent antibiotics from working effectively which could expose other bacterial species to antibiotics causing mutations and antibiotic resistance. Bacterial factors are most commonly discussed in the context of antimicrobial resistance and include considerations such as a high initial infectious dose, which would not be cleared by drugs leading to adaptation caused by mutation as well as intrinsic resistance to drugs. Adapted from Aeschlimann, 2003.

1.5. Pseudomonas

One of the key species of bacteria associated with antimicrobial resistance is *Pseudomonas aeruginosa* which belongs to the genus *Pseudomonas,* a broad genus of Gram negative bacteria which includes a wide range of species capable of occupying a broad range of environmental niches. *Pseudomonas* are ubiquitous environmental bacteria commonly found in soil and contaminated water supplies where the conditions promote the formation of biofilms (Stover et al., 2000). Most species of *Pseudomonas* are not a threat to humans and infections with disease causing species such as *P. aeruginosa* are primarily associated with immune compromised humans (discussed in detail below).

1.5.1. Pseudomonas aeruginosa

The most common cause of human *Pseudomonas* infections is due to the Gram negative rod *bacteria P. aeruginosa,* which is typically 0.5 by 1.5 μm and a commonly used laboratory strain, PAO1 was first isolated from an infected wound (Holloway, 1955). Often associated with pneumonia in Cystic fibrosis (CF) patients, *P. aeruginosa* is a leading cause of morbidity and mortality in these patients and is the most frequently isolated pathogen from their lungs (Davies, 2002). Patients with other severe inflammatory conditions such as chronic obstructive pulmonary disorder (COPD), compromised immune systems, such as cancer patients undergoing treatment and patients with burn wounds admitted to hospital are also susceptible to *P. aeruginosa* infection all of which are discussed in further detail.

Published data from the Centre for Disease Control (CDC) suggest that *P. aeruginosa* infections account for 0.4 % of overall infections in the United States of

America and that the pathogen is the fourth most common hospital pathogen, isolated from 10 % of patients. The CDC also reports that *P. aeruginosa* is the second most common cause of nosocomial pneumonia as well as being the most common multi drug resistant Gram negative pathogen in pneumonia (Goossens, 2003). A contributing factor to disease and persistence is the transition from planktonic to biofilm stages of growth (discussed in detail below).

1.5.2. Pseudomonas Genome

The ability of *P. aeruginosa* to utilise a wide range of nutrients and adapt and survive in a range of different environments is due to the large genome which consists of 5,770 open reading frames making *P. aeruginosa* one of the largest bacterial genomes (Stover et al., 2000). This supports growth in a wide range of niches environmentally including immuno-compromised hosts. Adaptation to the environment to utilise a wide range of available nutrients is supported by non-specific porins, redundant pathways to consume nutrients and gene expression of virulence factors and porins promoting environmental adaptation including to antibiotics (Balloy et al., 2015). In support of this, gene expression analysis has identified that there is a clear change in gene expression depending on planktonic or biofilm growth with half of the genes changed are either over expressed or under expressed.

1.5.3. P. aeruginosa Virulence Mechanisms During Acute infection

The contribution of virulence factors to pathology and host damage is discussed in detail in the sections on host response to *P. aeruginosa*, below the major virulence factors of *P. aeruginosa* are summarised with respect to acute infection and increased virulence.

The characteristics of *P. aeruginosa* change during acute and chronic infection and pathogenicity can be attributed to 4 main mechanisms during acute infection – adhesion, invasion, dissemination and immune evasion. There are many contributing factors to virulence and a hallmark of acute infection is the Type III secretion system (T3SS) which is characteristic of Gram negative bacteria where bacteria inject effector proteins into host cells (Gaytan et al, 2016).

P. aeruginosa has several Type III toxins which have been identified as virulence factors and approximately 80 % of isolates from acute infections secrete type III proteins and the T3SS is associated with disease severity (Hauser et al., 2002). Each secreted toxin has different host targets and roles as well as different expression levels which are thought to be reduced during chronic infection.

The T3SS toxins are encoded by the *exo* gene products, , ExoS, ExoT, ExoU and ExoY. Epithelial cells are targeted by ExoS (Kudoh et al., 1994) and to a lesser extent ExoT which is delivered directly into the host cells. 70 % of clinical isolates produce ExoS (Feltman et al., 2001) and it has been demonstrated that ExoS and ExoT target epithelial cells by modulating small GTPases, which are required for maintenance of tight junctions and epithelial repair (Kazmierczak et al., 2004) as well as invasion of the basolateral epithelium layer (Shaver and Hauser, 2004).

Rangel et al. (2015) used an acute murine infection model with *P. aeruginosa* to demonstrate that upon infection, neutrophils recruited to the lung are injected with ExoS. This prevents pathogen clearance leading to lung damage as epithelial cells are

also injected eventually compromising the epithelial barrier leading to dissemination. The suggestion that ExoS plays a role in dissemination has previously been demonstrated where colonisation was not dependent on the presence of ExoS but dissemination was (Nicas et al., 1985) further highlighting the importance of secreted toxins during infection.

Another Type III secreted toxin of *P. aeruginosa* is ExoU which causes the greatest damage to the host and is the most toxic by activating pro-apoptotic pathways and loss of plasma membrane integrity leading to greater damage to the epithelial barrier. Once injected into host cells, ExoU is activated and cleaves membrane phospholipids which results in cell lysis (Finck-Barbancon et al., 1997). However this toxin is the least prevalent and is rarely isolated from clinical samples (Shaver and Hauser, 2004). Finally ExoY enters the host cell cytosol and causes cell rounding, detachment as well as altering tight junctions between cells further contributing to lung damage.
Taken together, the toxins secreted via the type III system during acute infection directly contribute to disruption of the epithelial barrier and could lead to basolateral damage as well as dissemination in extreme cases, the role of which is discussed in greater detail later (Sayner et al., 2004, Chung et al., 2013).

The significance of barrier disruption is that an increase in paracellular permeability could contribute to an increase in nutrients, such as glucose, in the lung which would normally be tightly regulated leading to favourable conditions for bacterial growth. In addition to toxins directly injected into host cells, previous studies have demonstrated the severity of secreted proteases and their role in disease, for example strains secreting Exotoxin A, by the type II secretion system have increased virulence which

causes host cell death by apoptosis (Miyazaki et al., 1995). Exotoxin A targets the host cells, and after internalisation, conformational changes result an inhibition of protein synthesis by targeting translation mechanisms. Exotoxin A has also been thought to contribute to a suppression of the immune response as absence of Exotoxin A leads to a reduced cytokine response in vitro (Schultz et al., 2000).

Additionally, secreted toxins such as alkaline proteases and elastase can also lead to direct tissue damage through the degradation of lung surfactant and epithelia tight junctions (Kipnis et al., 2006). The secreted elastase, LasB contributes to virulence via the degradation of lung surfactant proteins A and D (Mariencheck et al., 2003) and mutants lacking the *lasB* gene have attenuated virulence. Furthermore, protease IV, secreted by *P. aeruginosa* is also known to degrade lung surfactant proteins as well as contributing to increased survival by preventing uptake of *P. aeruginosa* by macrophages in *in vitro* studies (Malloy et al., 2005).

In the context of lung infections, pyocyanin, a blue-green pigment produced by *P. aeruginosa,* contributes to virulence as it has been shown *in vitro* to cause neutrophil apoptosis and inhibit phagocytosis (Lau et al., 2004). Additionally, pyocyanin has been shown to directly effect host defence by suppressing cilia beating, the role of which in host defence is discussed elsewhere, and increasing the expression of interleukin-8 leading to increased tissue damage (Denning et al., 1998).

On top of secreted toxins, surface factors also contribute to virulence. For example, lipopolysaccharide (LPS) on the surface of *P. aeruginosa* is a potent stimulator of the immune response, details of which are discussed elsewhere in the thesis and there is

evidence, albeit controversial, to suggest that in healthy individuals the cystic fibrosis transmembrane receptor binds to *P. aeruginosa* leading to epithelial internalisation and killing.

LPS is composed of lipid A, attached to the outer membrane, a polysaccharide core and a O-polysaccharide which is the variable region (Gellatly and Hancock, 2013). Lipid A is the glycolipid that maintains LPS within the outer membrane is known to stimulate an immune response through TLR4 binding (Akira et al., 2005). It has been demonstrated that changes in the composition if lipid A can alter the immune response elicited by different strains, the most striking difference can be observed between lab strains and clinical isolates (King et al., 2009). The O-polysaccharide region of LPS, also known as the O-antigen is used to serologically identify different strains of *P. aeruginosa* and there are differences in the types of expression of the O-antigen depending on the origin of the isolate. During *P. aeruginosa* infection, LPS is shed and this shedding is thought to contribute to mucus production through the expression of the mucin genes activated by NF-κB dependent pathways which also leads to the expression of pro-inflammtory cytokines (Rastogi et al., 2001).

P. aeruginosa virulence is multifactorial and there are many other contributing virulence factors, in acute infection as well as chronic infection, which are not discussed in this section. In the context of this thesis, many of the virulence factors and host response to infection are discussed with relation to lung infections and the innate immune response which can be found in section 1.10.

1.5.4. Chronic Infection and Biofilm Formation

P. aeruginosa can be acquired in the community or in hospital, with the latter being most commonly associated with ventilator acquired pneumonia (Barbier et al., 2013).

Colonisation in the lung during acute infection leads to the development of biofilms which in turn leads to persistence and patients with ventilator acquired pneumonia can become infected with *P. aeruginosa* growing in biofilms on plastic surfaces highlighting the wide range of environmental niches *P. aeruginosa* can occupy (Williams et al., 2010).

Chronic infection with *P. aeruginosa* is strongly associated with biofilm formation and was first linked to chronic infections in people with cystic fibrosis (Hoiby, 1977). During this time, *P. aeruginosa* undergoes extensive genetic diversification, particularly in the CF lung (Ashish et al., 2013). This adaptation is due to the variety of environmental niches *P. aeruginosa* can occupy and contributes to the difficulty in treatment. Figure 1.3 illustrates biofilm formation in chronically infected patients, such as those with CF, where bacteria are able to transition from free floating planktonic bacteria which are susceptible to host responses and antibiotics to bacteria embedded in mucus to persist in the lungs. The same pathway also occurs on surfaces such as catheters which can lead to infections in ventilated patients and importantly it has been estimated that 80 % of persistent bacterial infections involve biofilms (National Institutes of Health. Minutes of the National Advisory Dental and Craniofacial Research Council–153rd Meeting. 1997. Report.)

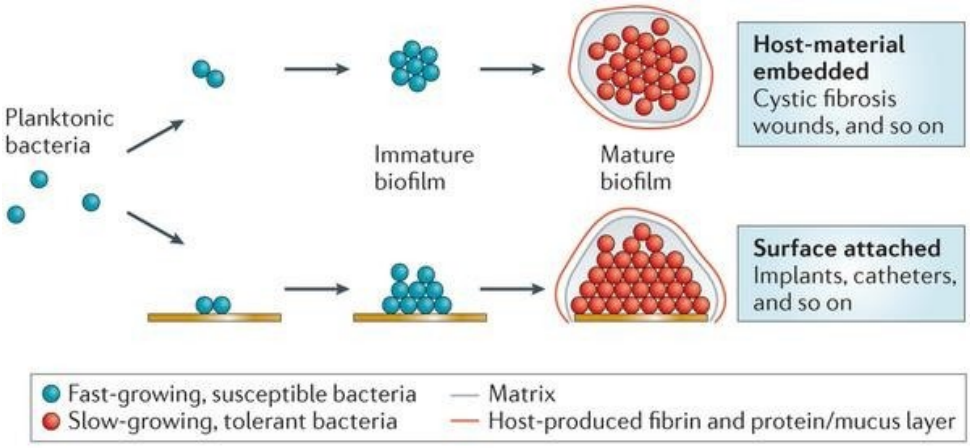


Figure 1.3 Biofilm formation in human hosts and on surfaces. Planktonic bacteria are capable of forming biofilms which are free floating and also attached to artificial surfaces. Persistence of *P. aeruginosa* is due to the formation of biofilms composed of protein, DNA and polysaccharides. Adapted fromBjarnsholt et al. (2013).

The impact of chronic infection has been demonstrated *in vitro* where the formation of biofilms protects *P. aeruginosa* from neutrophils during host defence (Bjarnsholt et al., 2005) leading to impaired clearance. During chronic infection, it has been suggested that bacteria form biofilms or colonies which are encased in a polysaccharide which shelters the bacteria from the innate host response and also potentially drives a neutrophil response which aggravates lung damage. Additionally, *P. aeruginosa* in biofilms demonstrate a slower rate of growth which leads to adaptation to the environment and an increased tolerance to antibiotics (Fux et al.,2005). The significance of this is that biofilms formed in the lungs are more difficult to treat as they are more resistant to antibiotics and host responses therefore if methods to

prevent the establishment of acute infection are developed this can prevent the formation of biofilms which leads to chronic infection.

1.6. Acute and Chronic Infection

Published data on the consequences of infection on host response are conflicting due to the multifactorial nature of *P. aeruginosa* growth therefore identification of risk factors is an essential part of developing therapies against *P. aeruginosa* infection. Additionally the acute and chronic stages of infection affect the host in different ways and activate different pathways further adding to the complexity of *P. aeruginosa* infection (Figure 1.4) suggesting that if *P. aeruginosa* is able to establish infection due to a risk factor such as excess glucose, then it becomes more difficult to treat as chronic infection is more resistant to antibiotics and the host immune response.

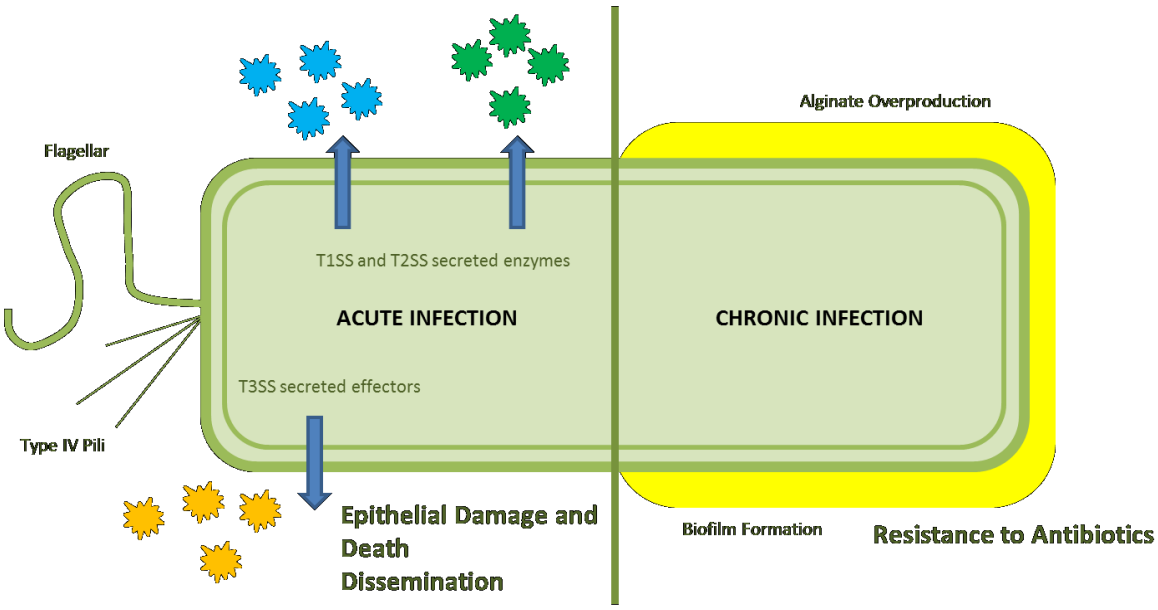


Figure 1.4 Features of *P. aeruginosa* during acute and chronic infection

The 2 stages of *P. aeruginosa* infection – acute and chronic are characterised by expression and secretion of toxins during acute infection promoting host cell damage and initiation of an immune response. Chronic infection is typically characterised by alginate production which provides increased resistance to antibiotics as well as increased immune evasion.

1.7. Carbon Utilisation by P. aeruginosa

One of the determining factors for pathogenicity is the availability of metabolites to support the growth of infectious bacteria, which need to utilise host derived metabolites to grow and survive. This dependence on the host makes a promising target for intervention, but there is a need to understand more about it, in order to develop effective strategies.

P. aeruginosa occupies a wide range of environmental niches such as marine environments, soil, marshes in addition to the aforementioned animal and human

tissues (Hardalo and Edberg, 1997). As such, *P. aeruginosa* uses multiple pathways for the uptake of nutrients and the selective pressure of the host environment and competing bacteria in the lung drives diversification (Jiricny et al., 2014, Smith et al., 2006).

Figure 1.5 summarises the various pathways of glucose utilisation in *P. aeruginosa. Pseudomonas* breaks down glucose via the Entner-Doudoroff Pathway (ED) which catabolises glucose to pyruvic acid and ATP (Entner and Doudoroff, 1952). In addition to passive transport *Pseudomonas sp* actively transport glucose into the cell for glycolysis, which does not require oxygen and results in the production of pyruvate and ATP (Entner and Stanier, 1951).

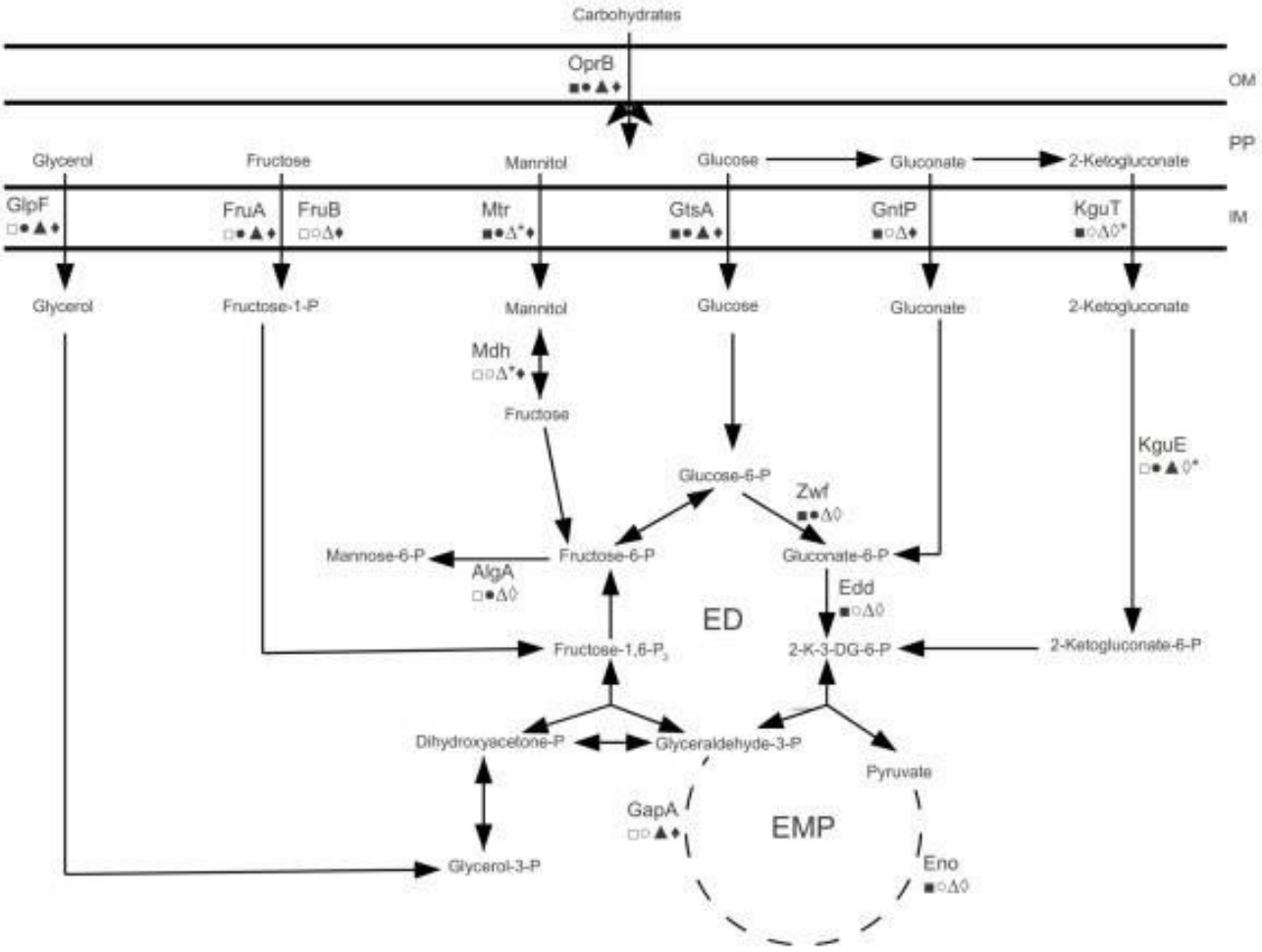


Figure 1.5 *P. aeruginosa* carbohydrate metabolism

Taken from Carbohydrate Catabolism in *P. aeruginosa* (Temple *et al*) the complexity of carbohydrate uptake and entry into a central cycle is detailed. Transport is via by outer membrane porins as well as intermediate metabolic pathways.

The various pathways of glucose uptake and utilisation in *P. putida*, which can also be found in *P. aeruginosa* are summarised in Figure 1.6 (Ebert et al., 2011). Glucose can be transported into the cell and oxidised by Glk, alternatively, glucose is oxidised in the periplasm to gluconate and transport into the cell. Gluconate can also be converted into 2-ketogluconate for conversion in the cell. Fig.6 summarises the complex uptake pathways in *Pseudomonas sp* and supports the highly adaptive nature of *Pseudomonas.*

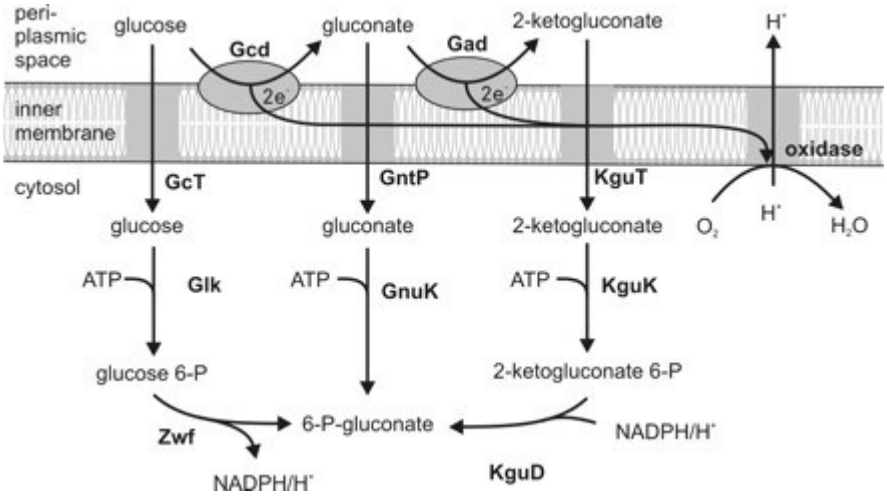


Figure 1.6 Glucose uptake in *P. putida*

The numerous alternative pathways involved in the conversion of glucose in *P. putida* by phosphorylation. The conversion of glucose leads to a proton gradient which drives ATP generation via the ATP synthase which highlights how *P.sp* are well adapted to utilise glucose (Ebert et al., 2011).

As demonstrated in Figures 1.5 and 1.6, there is a degree of redundancy with respect to the uptake of glucose. The details of the various routes of uptake and utilisation of glucose suggest that *Pseudomonas* is well adapted to utilise glucose in the environment. This could be important in a clinical infection where excess glucose could support growth of *P. aeruginosa* leading to increased bacterial growth.

1.8. Risk Factors for infection

P. aeruginosa is opportunistic and susceptibility to *P. aeruginosa* is mainly due to defective mechanical or innate immune defences and there are clear co-morbidities which increase susceptibility to bacterial infection, including cystic fibrosis, chronic obstructive pulmonary disease (COPD), diabetes, obesity and obesity-associated hyperglycaemia. Identifying risk factors for colonisation could prevent establishment of chronic infection as well as reduce damage caused to the epithelial lining of the lungs caused during acute infection (Hodgson et al., 2014). These co-morbidities are all similar because they all cause damage to the lungs contributing to impaired barrier function potentially leading to increased susceptibility to infection.

1.8.1. Cystic Fibrosis (CF)

P. aeruginosa is the most common pathogen isolated from the lungs of adult cystic fibrosis patients (Harrison, 2007). It is associated with the final stages of bacterial colonisation as *P. aeruginosa* is isolated from 80 % of CF patients over the age of 18 (Treggiari et al., 2007). CF is a genetic disease caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene which affects an estimated 100 000 people globally and is characterised by inflammation, compromised airflow and eventually chronic bacterial infection (Davies et al., 2014). Mutations in CFTR lead to high levels of sodium in cells of the mucosal epithelial

membrane which leads to a thicker mucus layer limiting effective mechanical clearance by flattening the cilia (Buchanan et al., 2009, Mall et al., 1996). It has therefore been hypothesised that an increase in *P. aeruginosa* infection in CF patients is due to the lack of lipopolysaccharide (LPS) detection by CFTR, a host response which is discussed in detail later on (Schroeder et al., 2002) however this remains unclear leading to conflicting reasons as to why *P. aeruginosa* is the most commonly isolated pathogen

Chronic infection in CF individuals is dominated by *P. aeruginosa* and the majority of these strains produce a polysaccharide capsule composed of alginate. This is thought to contribute to immune evasion as overexpression of alginate leads to a reduction in extracellular markers on *P. aeruginosa* which are detected by the immune system (Lyczak et al., 2002). Additionally changes in composition of mucus and the airway surface liquid in CF could contribute to a failure to kill *P. aeruginosa* and progressive lung damage (Govan and Deretic, 1996), Pezzulo et al. (2012).

1.8.2. Chronic Obstructive Pulmonary Disorder (COPD)

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death in the world (Lozano et al., 2012) and according to the World Health Organisation in 2012 there were over 3 million deaths due to COPD. According to guidelines by the National Institute for Health and Care Excellence COPD is diagnosed by measuring the ratio between the forced expiratory volume (FEV) which is the maximum volume of air exhaled in the 1 second and the forced vital capacity (FVC) which is the greatest volume of air exhaled in a single breath. A ratio of below 70 % leads to a positive COPD diagnosis. In addition to reduced lung function, the risk of infection and secondary infection increases

with disease severity. Bacterial exacerbations are responsible for hospitalisation and it has been demonstrated that the risk of infection increased with decreased lung function (Groenewegen and Wouters, 2003).

The main cause of acute exacerbations of COPD is bacterial infection with *P. aeruginosa* and *Haemophilus influenzae* which are commonly isolated from patients and bacterial infections are responsible for approximately 50 % of exacerbations (Sethi and Murphy, 2001). More recently Gallego et al. (2014) demonstrated that COPD patients with severe bronchiectasis (widening of the airways) corresponds with chronic *P. aeruginosa* infection and colonisation similar to the observations in *P. aeruginosa* infected CF patients. Another characteristic of COPD is lung remodelling which can lead to further lung damage as a result of impaired barrier function and complications which are potentially fatal.

1.8.3. Obesity

There is an established link between neutrophils and obesity caused by excess nutrients and the build-up of adipose tissue (Herishanu et al., 2006, Zaldivar et al., 2006) suggesting that obesity causes an aberrant immune response. Chronic low grade inflammation has been identified in conditions such as obesity and diabetes without pathogen involvement and is termed sterile activation triggered by Danger Associated Molecular Patterns (DAMPs) as well as by the release of pro-inflammatory cytokines by stressed adipocytes and adipose tissue macrophages (Hodgson et al., 2014, Nieto-Vazquez et al., 2008, Lumeng et al., 2007). For example, obese mice sustained on a high fat diet have elevated levels of active IL-1β which acts as a pro-inflammatory cytokine and contributes to the chronic low

grade inflammation observed in people with type 2 diabetes**.** One important consideration is that obese individuals also have elevated levels of glucose and are not diagnosed as being diabetic. This can be an associated risk factor as people with elevated levels of glucose but without diabetes may lack the clinical care people with diabetes have.

1.8.4. Diabetes

Diabetes and the associated hyperglycaemia is a major risk factor for respiratory bacterial infection (Brennan et al., 2007). There are two main types of diabetes, type 1 and type 2 diabetes. Type 1 diabetes is an autoimmune disease and is treated by insulin administration as the pancreas cannot produce insulin. Type 2 diabetes is characterised by chronic hyperglycaemia and is caused by a resistance to insulin which can be caused by obesity, poor diet, ethnicity and a genetic history of diabetes (Jin and Patti, 2009) . Type 2 diabetes is more prevalent and accounts for 85-95 % of diabetic cases (Sethi and Murphy, 2001, 2013) and in the United States of America alone, over 26 million people have been diagnosed with diabetes. In addition to this, to 79 million more are classed as pre-diabetic which could eventually contribute to kidney failure, heart disease (Wild et al., 2004).

P. aeruginosa is a leading cause of infection in diabetics with foot ulcers (Yoga et al., 2006). In addition to infection, the formation of biofilms contributes to complications in wound healing in people with diabetes as biofilms can act as a physical barrier against host defences (Edwards and Harding, 2004, James et al., 2008). *In vivo* studies have demonstrated that diabetic mice had increased bacterial loads and biofilm formation in a chronic wound model of infection which was not observed in

non-diabetic mice suggesting that diabetes is a risk factor for chronic wound infection (Watters et al., 2013).

Diabetes also causes increased levels of inflammation and there is evidence of defects in the immune response in people with poorly controlled diabetes where glucose levels are not tightly regulated which is less consistent in people with well controlled diabetes where glucose level fluctuations are more similar to people without diabetes highlighting complications associated with diabetes.

1.8.5. Hyperglycaemia

Although diabetes alone does not contribute to increased infection and the mortality associated with Diabetes Mellitus (Vardakas et al., 2007), hyperglycaemia is linked to an increase in mortality. For example Kornum et al. (2007) showed that in a population based cohort study, people classed as non-diabetic, due to condition such as obesity, with glucose values above 14 mmol/l had an increased risk of 30 day mortality of 26.1 % compared to patients with glucose values below 6 mmol/l with a 30 day mortality of 13.9 %. The same study also showed that elevated glucose on admission correlates with an increased risk of mortality and the association between hyperglycaemia and infection is discussed in greater detail later in the thesis.

Hyperglycaemia has been associated with increased risk of bacterial infection particularly in patients who have been intubated. Hyperglycaemia increases glucose

concentration in the airway surface liquid (ASL) in both non-diabetic people and diabetic people (Baker et al., 2007) and the evidence of increased bacterial growth in glucose strongly supports the link between bacterial infections in hyperglycaemic individuals. There is evidence that hyperglycaemia is associated with an increased risk of nasal *Staphylococcus aureus* colonisation in CF individuals. (Garnett et al., 2013). Although the association between CF and bacterial infection with *P. aeruginosa* is well documented (Jones et al., 2001), the mechanisms of infection in CF related diabetes is unclear (Merlo et al., 2007)

The significance of the presence of glucose in infection has been shown where hyperglycaemia increases the levels of in airway secretions (Wood et al., 2004) suggesting that an increase in available nutrients in the airway secretions can lead to an increase in colonisation of bacteria.

1.9. Therapies against P. aeruginosa

Most current antibiotics active against *P. aeruginosa* belong to 3 classes, beta-lactams, quinolones and aminoglycosides. Beta-lactams inhibit cells wall synthesis and are the most effective antibiotics against *P. aeruginosa* (Giamarellou, 2002). Quinolones are synthetic antimicrobials which block DNA replication through the inhibition of DNA gyrase (Hooper, 1993) and are also used to treat *P. aeruginosa* infections. Aminoglycosides target protein synthesis of *P. aeruginosa* but are rarely administered due to adverse side effects (Pagkalis et al., 2011).

More recent effective antibiotics classes against antibiotics include cephalosporins, fluoroquinolones and carbapenems, however carbapenems are associated with resistance and therefore are used only when other antibiotics do not work (Pillar et al., 2008) further highlighting the need for novel drug development.

1.9.1. Antimicrobial resistance in P. aeruginosa

P. aeruginosa is listed as a serious threat by the Centre for Disease Control and Prevention which suggests that prompt action is required against this pathogen. This is due to multi drug resistant strains which are commonly isolated from hospitalised patients. *P. aeruginosa* infections are difficult to treat do the intrinsic resistance to antibiotics courtesy of low membrane permeability and efflux systems (Hancock, 1998). The large array of efflux pumps produced by *P. aeruginosa* contributes to the intrinsic resistance which actively transport drugs out of the cell to promote survival and are effective against most antibiotics (Bagkeri, 2013).

The genetic complexity of *P. aeruginosa* also contributes to resistance by changing outer membrane porins and pumps in response to antibiotics. Gram negative efflux pumps were first identified in *P. aeruginosa* strains (Poole et al., 1993) and overexpression of outer membrane pumps increases the minimum inhibitory concentration of antibiotics required to be effective against *P. aeruginosa* which contributes to the multi-drug resistance observed with *P. aeruginosa* (Lomovskaya et al., 1999).

The outer membrane of *P. aeruginosa* contributes to the intrinsic resistance due to the low permeability as well as selective porins which restrict the uptake of molecules such as antibiotics (Poole and Srikumar, 2001) In addition to porins, *P. aeruginosa* possess efflux pumps which eject antibiotics such as beta-lactams, chloramphenicol and tetracycline to name a few out of the cell (Livermore, 2002).

As discussed previously, biofilm formation is a feature of chronic infections and it has been demonstrated that biofilms in the host protect against antibiotics and host

defences (Bjarnsholt et al., 2009). In addition to the intrinsic resistance of *P. aeruginosa,* acquired resistance can be passed to from one bacterium to others with greater ease in biofilms contributing to wider antibiotic resistance in infection patients. With such a complex cell envelope, large genome and easy acquisition of resistance *P. aeruginosa* is emerging as a major pathogen in the class of multi drug resistance bacteria.

Resistance to the most common classes of antibiotics used to treat *P. aeruginosa* has increased (Figure 1.7) and levels range from 20 to 30 % resistance in intensive care with most patients carrying isolates resistant to β-lactams (Lister et al., 2009). Importantly even though resistance is clearly increasing the rise of multidrug resistance is a serious concern. Additionally, isolates from lower respiratory tract infections carries the highest prevalence of multidrug resistance (Obritsch et al., 2004).

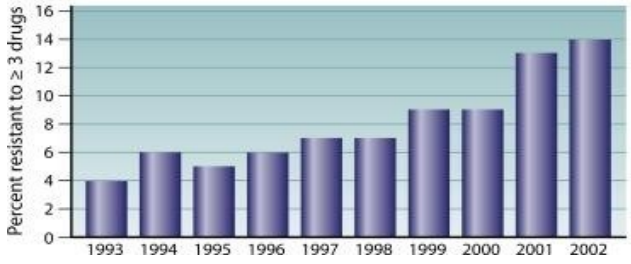


Figure 1.7 Increasing resistance of *P. aeruginosa* to antibiotics

Increasing incidence of multidrug resistance in patients in intensive care unit in the United States of America, Figure taken from Lister et al. (2009).

1.9.2. Potential Alternative Therapies

Due to the acquired resistance from horizontal gene transfer and intrinsic resistance of *P. aeruginosa* to current antibiotics, alternative therapies have been developed. For example, the flagellum of *P. aeruginosa* has been tested as a vaccine candidate through the generation of an immunoglobulin response however despite progression

into clinical trials the vaccine candidate was unsuccessful (Doring et al., 2007, Doring et al., 1995).

The vaccines developed with the intention of reducing *P. aeruginosa* infection in the lungs of CF patients have been unsuccessful. In addition to this, the lack of development of novel antimicrobials means that targeting the host and associated risk factors could be a potential strategy.

1.10. Host Defence

During acute infection the interaction between the lung epithelium and the host immune response determines the outcome of infection and the establishment of chronic infection. The host defence against *P. aeruginosa* involves mucociliary clearance, epithelial defence and an innate immune response triggered by epithelial and immune cells.

1.10.1. Airway Surface Liquid

The airway surface liquid (ASL) is a thin layer of liquid lining the surface of the lungs and acts as a mucosal barrier to clear pathogens and is composed of water, ions and mucin macromolecules (Tarran, 2004). The composition is tightly regulated and abnormal ASL composition, such as changes in ion concentrations, antimicrobial peptides or glucose concentration are common in CF patients and can promote bacterial colonisation in addition to chronic low grade inflammation (Dubin et al., 2004).

The ASL serves as a protective barrier by trapping inhaled pathogens but it also contains lipids and amino acids which *P. aeruginosa* and other respiratory pathogens could use to support colonisation (Widdicombe et al., 1997).

Carbon sources such as glucose in the ASL have an impact on inflammatory immune cells and also bacterial growth influencing the establishment of infection. Glucose levels are under tight control and are normally up to 12 times lower in the ASL than circulating levels (Baker et al., 2006). Glucose homeostasis is an essential function of the ASL and is regulated by a number of mechanisms including paracellular diffusion, glucose uptake by epithelial cells and intracellular metabolism (Kalsi et al., 2009a). An increase in the gradient of glucose across the epithelial barrier caused by hyperglycaemia, increased paracellular diffusion caused by inflammation or a shift in glucose uptake and expression proteins could all increase the levels of glucose in the ASL and in turn increase the risk of respiratory bacterial infection due to an increase in available nutrients. Increases in ASL glucose have been observed in diabetic patients and patients with chronic lung inflammation and elevated ASL glucose also correlates with an increased risk of bacterial infection (Brennan et al., 2007).

In addition to acting as a barrier to invading pathogens, the airway surface liquid is composed of antimicrobial compounds such as the cationic defensin which contributes to the host response to invading pathogens. The importance of epithelial cell secretions in host defence was highlighted by Smith et al. (1996) who demonstrated in vitro killing of small inoculum doses of *P. aeruginosa* by secretions from healthy cell lines but not with CF cell lines suggesting that alterations in the composition of the ASL impacts bacterial killing.

1.10.2. Physical Defence mediated by Epithelial Cells

The epithelial lining of the lung is composed of morphologically different cells interspaced with mucus producing cells. A primary host defence function of the airway is to act as a barrier to prevent pathogen entry. Increased damage to the epithelium due to factors other than invading pathogens such as host susceptibility suggests a role for chronic low-grade inflammation as a possible contributor to susceptibility to invading bacterial pathogens such as *P. aeruginosa.* Physical defence is also influenced by the ASL covering the airway epithelium (Garnett et al., 2012) and its role in clearance of inhaled pathogens. Mechanical movement of the cilia on cells acts to clear pathogens trapped in the mucus and damage to the cilia reduces mucocillary clearance (Knight and Holgate, 2003). Additionally, it has been demonstrated previously that *P. aeruginosa* destroys tight junctions in the airway epithelium and cell death mediated in part by T3SS toxins, as indicated by the release of lactate dehydrogenase, in a *P. aeruginosa* dose dependent manner suggesting that tight junction regulation is important during infection (Rejman et al., 2007).

1.10.3. Pattern Recognition of P. aeruginosa

In addition to acting as a physical barrier, the epithelium is important in triggering an innate immune response to invading pathogens by producing chemokines and cytokines which lead to the recruitment of phagocytic cells as part of the innate immune response. The innate immune response is triggered by recognition of pathogens by pathogen recognition receptors (PRR) (Mogensen, 2009).

Pattern recognition receptors are important in the early response to pathogens. One family, the Toll like receptors (TLRs) are particularly important in recognition of *P. aeruginosa* ligands (Barton and Medzhitov, 2003). TLR are involved in sensing bacterial products such as lipopolysaccharide (LPS), flagellin and peptidoglycan and trigger an inflammatory immune response. The main TLRs associated with an immune response to *P. aeruginosa* are TLR2, TLR4 and TLR5 and they are located on both inflammatory and epithelial cells.

TLR4 and TLR5 are specific for LPS and flagellin respectively (McIsaac et al., 2012, Hayashi et al., 2001). The role of TLR4 was identified using TLR4 knockout mice which after infection with *P. aeruginosa* had reduced neutrophil recruitment and increased bacterial burden (Skerrett et al., 2007, Power et al., 2004) as well as a reduction in TNF-α and IL-6 *in vitro* (Raoust et al., 2009). TLR5 on epithelial cells detects *P. aeruginosa* flagella to stimulate a NF-κB mediated response (Zhang et al., 2005) and airway epithelial cells exposed to flagella from *P. aeruginosa* express TLR5 on the apical surface (Adamo et al., 2004) demonstrating the importance of apical detection of flagella to induce an immune response. TLR2, which recognises peptidoglycan, plays a role in the recognition of many components of mucoid strains of *P. aeruginosa* which are commonly isolated from chronically infected CF patients (Firoved et al., 2004) suggesting that TLR2 plays a role during the late stages of infection. TLR9 is located in the lysosome and is involved in the recognition of foreign DNA particularly CpG motifs which is important for *P. aeruginosa* recognition due to the high GC content (Vollmer, 2006). Binding of foreign DNA rich in cytosine and guanine to TLR9 signals through MyD88 leading to an IL-8 dependent neutrophil recruitment (Hacker et al., 2000).

Another key PRR involved in recognition of *P. aeruginosa* is nucleotide oligomerisation domain -2 (NOD-2) which is located in epithelial cells, macrophages and dendritic cells and activates NF-κB (Travassos et al., 2005). Internalisation of *P. aeruginosa* has been hypothesised to be mediated by the CFTR protein which in turn triggers the activation and translocation of NF-κB via MyD88 and IL-1β/IL-1R (Reiniger et al., 2007). This is also supported by studies using MyD88$^{-/-}$ mice which displayed reduced or lack of neutrophil recruitment in response to *P. aeruginosa.* As MyD88 knockout mice are more susceptible to infection this suggests that Myd88 is essential for a robust innate response against *P. aeruginosa* (Skerrett et al., 2004) and the recruitment of neutrophils, which is crucial during acute infection (Hajjar et al., 2005).

1.10.4. Cytokine Release by Airway Epithelial Cells

An important step in host defence is the recognition of bacterial pathogens which leads to the release of cytokines and chemokines initiating an influx of neutrophils to the site of infection (Whitsett, 2002). Epithelial cells are capable of producing a large number of cytokines in response to infection which are released after NF-κB stimulation and are characteristic of an acute host response to infection (Lavoie et al., 2011) and clearance (Power et al., 2004)

The innate immune response triggered by the epithelium is through the production of type 1 interferon (IFN) (Decker et al., 2005) which is known to be activated by *P. aeruginosa* and is important for bacterial clearance. Type 1 IFN production also leads to a decrease in the production of the detrimental cytokines IL-1β and IL-18 suggesting that type 1 IFN regulates the inflammasome response to *P. aeruginosa* to limit pulmonary damage (Cohen and Prince, 2013).

In response to *P. aeruginosa* IL-8 is released by epithelial cells and in murine infections mice without the IL-8 analogue KC/CXCL1 display decreased neutrophil responses and bacterial clearance (Tsai et al., 2000) highlighting the importance of IL-8 during acute infection. In healthy individuals, recruited immune cells are able to clear invading pathogens with little damage to the host however when the inflammatory response excessive and prolonged as in CF patients, the result is tissue damage and a reduction in lung function

Inflammation during acute infection is thought to increase disease severity which is supported by levels of IL-1β, IL-8 and TNF-α in the lungs of CF patients infected with *P. aeruginosa* (Tiringer et al., 2013). Elevated levels of these cytokines and chemokines contribute to the disruption of tight junctions and integrity of the airway epithelium due to inflammation leading to an increase in paracellular permeability which contributes to inflammation.

The innate immune response during the early stages of infection triggers an influx of cells to the site of infection. This is characterised by an influx of neutrophils and the airway surface liquid acts as viscous mucus which traps inhaled particles and is mechanically cleared by cilia which sweep pathogens by mucocilliary clearance.

1.10.5. Macrophages

Macrophages play a crucial role in the innate response to infection and are located at various sites in the body. Alveolar macrophages are a lung airway resident cell type that has a critical role in the surveillance and response to an infection (Davies et al., 2013). Additionally on infection, further inflammatory macrophages will be

recruited into the lungs, potentiating the immune response. This leads to a dichotomy in the function of macrophages, on one hand they can control an infection early in the response, but they can also fuel inflammation and local damage.

The protective function of macrophages is mediated during lung infection. Macrophages located between the air and lung tissue in the lower airways are activated by PRRs can detect and phagocytose *P. aeruginosa* (Ahrens, 1992). Additionally, Wangdi et al. (2010) demonstrated the importance of early recognition of *P. aeruginosa* as macrophages are able to discriminate between type 3 secretion positive and negative strains and deletion of T3SS failed to induce a strong neutrophil response suggesting that during acute infection, macrophage recognition is crucial for an effective host response through the release of IL-1β, IL-6, IL-8 and TNF-α (Benabid et al., 2012).

In contrast to this, Cohen and Prince (2013) demonstrated that *P. aeruginosa* flagella activates the inflammasome which results in the release of IL-1β and IL-18 primarily from alveolar macrophages (AMs) adding to lung pathology, damage and increased mortality suggesting the presence of AMs during infection is of little benefit as inhibition of these inflammasome activated products improves bacterial clearance. In support of the limited role of macrophages during *P. aeruginosa* infection, in a murine model of infection, depletion of macrophages using liposome-encapsulated dichloromethylene diphosphonate (LDMDP) resulted in decreased phagocytic activity of macrophages as expected but did not result in increased susceptibility to *P. aeruginosa* infection suggesting that macrophages play a limited role in defence against *P. aeruginosa* (Cheung et al., 2000).

Taken together this suggests that during the early stages of acute infection, macrophages are activated and recruit neutrophils to the site of infection for phagocytosis but when maintained in the lungs, activation could lead to greater lung damage and as described before inflammation induced lung damage can lead to a vicious cycle by damaging the epithelial barrier, increasing the leakiness of the lung and enabling the movement of glucose to support the growth of airway bacteria.

1.10.6. Neutrophils

Neutrophils are polymorphonuclear cells and are the first leukocytes recruited to the sites of inflammation (Kolaczkowska and Kubes, 2013). Neutrophil recruitment to the lungs is essential for clearance of *P. aeruginosa.* As one of the main immune cells involved in tissue destruction, neutrophil activation is tightly regulated to limit further damage and dysregulation due to hyperglycaemia is discussed in detail later.

After activation, the lifespan of neutrophils increases during inflammation which ensures that neutrophils are present at the site of infection and inflammation (Summers et al., 2010, Colotta et al., 1992). A characteristic of *P. aeruginosa* infection is a robust neutrophil response during acute infection and neutrophils are required to clear infection as knockout studies using mice have demonstrated that neutrophil depletion results in an increased susceptibility to infection with *P. aeruginosa* (Koh et al., 2009)

1.10.7. Eosinophils

Eosinophils are rarely explored in the context of acute infection with *P. aeruginosa* but there is evidence to suggest that eosinophils play a role in the innate immune

response.- *In vitro*, eosinophils are capable of killing *P. aeruginosa* and this was replicated *in vivo* where IL-5 transgenic mice with eosinophilia have improved bacterial clearance in a peritoneal infection model (Linch et al., 2009). However, as with most published data on the role of specific immune responses to *P. aeruginosa* there are conflicting results in lung infection models which have demonstrated the opposite suggesting that their role may not be essential for bacterial clearance from the lungs (Beisswenger et al., 2006).

1.11. Adaptive Immune Response

Despite a robust innate immune response to *P. aeruginosa* during acute infection, there is evidence that the adaptive immune response is also involved, particularly in chronic infections in response to the presence of biofilms. This was highlighted in patients with chronic *P. aeruginosa* infection who are unable to clear bacteria from the lungs despite high antibody responses to *P. aeruginosa* suggesting that the adaptive response in these patients is ineffective (Winnie and Cowan, 1991).

1.12. Glucose homeostasis and the role of glucose in immune responses

Glucose is an essential requirement for the function of most mammalian cells including brain and blood cells as well as for the activation of neutrophils which is relevant in the case of infection and hyperglycaemia (Tan et al., 1998). Under normal conditions, glucose levels are tightly regulated but during the acute stages of bacterial infection, glucose levels in the plasma rise (Baker et al., 2006), leading to detrimental effects.

In some instances, the presence of glucose can dampen immune function. Previous *in vitro* studies have demonstrated that glucose levels in the ASL are increased by pro inflammatory mediators suggesting that when tight junction integrity is compromised there is more glucose in the ASL. In the *in vitro* study used to determine this, tight junction damage caused more paracellular glucose transport and an increase in apical glucose uptake by cells which was not sufficient to counteract the increase in glucose (Garnett et al., 2012). This suggests that it is not susceptibility alone that contributes to infection but uncontrolled glucose levels in the ASL.

During acute infection there is an influx of neutrophils to the site of infection and previous studies have demonstrated that glucose is important in the activation of neutrophils (Kiyotaki et al., 1984, Naftalin and Rist, 1993). However when glucose levels are consistently high in the blood and airways, neutrophils are constantly exposed to glucose and therefore become inactivated in response to infection. This was demonstrated by Kummer et al. (2007) where the link between diabetes and an aberrant neutrophil response was studied using a computational approach to determine the role of glucose and demonstrated that elevated glucose levels *in vitro,* similar to glucose levels in the blood of uncontrolled diabetes, increased the production of reactive oxygen metabolites by neutrophils. It has therefore been suggested that excess glucose causes tissue damage by activating neutrophils and increasing susceptibility by compromising neutrophil responses. Jakelic et al. (1995) demonstrated that people with have elevated blood glucose levels greater than 12 mmol/l had significantly lower phagocytic activity compared to people with lower glucose levels further emphasising the damaging effect uncontrolled glucose has on neutrophil responses.

In addition to damaging cells involved in the innate immune response, there is evidence demonstrating the pro-inflammatory actions of glucose (Mohanty et al., 2000, Tripathy et al., 2003) which is supported by an increase in the activity of NK- κB and TNF-α mRNA after glucose administration in non-diabetic people (Aljada et al., 2006, Dhindsa et al., 2004). Also an increase in levels of pro-inflammatory cytokines IL-6, TNF-α and IL-8 was also observed in non-diabetic people given an insulin release inhibitor octreotide suggesting hyperglycaemia could lead to inflammation (Esposito et al., 2002).

It is known that there is a host requirement for glucose, for example activated macrophages readily consume glucose {Newsholme, 1986 #751} and there is evidence of enhanced glycolysis, the conversion of glucose to pyruvate, in macrophages and DCs activated by LPS {Rodriguez-Prados, 2010 #752}. The uptake of glucose and the conversion to pyruvic acid leads to the generation of ATP for cell function such as phagocytosis and cytokine release by macrophages. This raises the suggestion that host immune cells require glucose once activated and that increased glycolysis is a signature of immune cell activation in response to pathogens. Therefore there is a balance between host requirement for glucose and an effective response to pathogens versus an aberrant immune response driven by immune response and a potential increase in bacterial burden.

1.12.1. Glucose and Bacterial Infection Previous studies

In support of the risk factors associated with uncontrolled hyperglycaemia, patients with hyperglycaemia, defined by the WHO as non-fasting blood glucose levels in

excess of 11mmol/l and fasting levels above 6mmol/l, admitted to intensive care units have improved mortality rates when insulin is administered to control glucose levels (van den Berghe et al., 2001).

Despite evidence that diabetes is associated with an increased risk of infection, there are conflicting studies on the role of glucose during infection as glucose is required for the host as well as potentially promoting bacterial growth. As bacteria are able to utilise glucose, in a hyperglycaemic environment it would seem logical that the excess glucose would contribute to bacterial infection. However, there is also the possibility that the reason for increased bacterial infection in people with diabetes where glucose levels are hard to control is due to defective immune responses due to excess glucose.

The importance of glucose in *P. aeruginosa* growth *in vitro* has been highlighted by Pezzulo et al. (2011) who demonstrated that low glucose levels in the ASL impaired bacterial growth. The same group also found that the ASL alone did not support *P. aeruginosa* and low glucose levels in the lung could be an important mechanism for prevention of infection, suggesting the potential problematic implications of a rise in glucose in the ASL. The same study also showed that expression of glucose transporters GLUT-1 and GLUT-10 increases in response to glucose levels in an attempt to lower ASL glucose levels further supporting the importance of lower glucose in the airways compared to circulating levels.

The link between glucose levels in the ASL and paracellular permeability was assessed by Garnett et al. (2013), who developed polarised monolayers with apical and basolateral surfaces and confirmed that ASL glucose concentration increased

in response to exposure to pro-inflammatory cytokines which disrupt tight junctions between cells and increase paracellular permeability. Although it is known that patients with chronic inflammation are more susceptible to *P. aeruginosa* infection, how a hyperglycaemic environment contributes to this is and if it is hyperglycaemia in the lungs alone is not clear.

The evidence supports a theory which links inflammation, infection and available nutrients, due to hyperglycaemia leading to favourable conditions for respiratory bacterial infections. As hyperglycaemia but not diabetes alone is a risk factor for *P. aeruginosa* infection, other conditions with an abundance of glucose, such as obesity could be implicated in infection and chronically inflamed lungs and elevated levels of glucose could promote the establishment of *P. aeruginosa* infection. This is also supported by a study in which individuals with elevated levels of blood glucose have higher levels of glucose detected in endotracheal secretions suggesting that blood glucose is a good indicator of increased levels of glucose in the ASL (Philips et al., 2003).

Glucose and the increased risk of bacterial infection have been alluded to in recent publications for example, glucose in bronchial aspirates increases the risk of Methicillin Resistant *Staphylococcus aureus* infections and patients who were infected with MRSA had an increased duration of stay in intensive care (Philips et al., 2005) which adds to the burden on the healthcare systems further increasing the importance of developing novel treatment strategies against multidrug resistant bacteria. However, a conclusive link between glucose levels in the airways and increased bacterial burden without complicating factors is yet to be established. Diabetes and Impaired Immune Responses

The pathology associated with diabetes could contribute to the increase in bacterial infection due to a dysregulated immune response as well as an increase in available glucose, however this remains unclear. In people it has been observed that in diet induced diabetes neutrophils are recruited to the adipose tissue leading to inflammation (Rensen et al., 2009) and it is known that obesity increases the risk of developing type 2 diabetes.

In obese individuals, a state of chronic inflammation results in increases in chemokines and pro-inflammatory cytokines (Heilbronn and Campbell, 2008). Previous studies have demonstrated that glucose increases superoxide production and decreases motility of neutrophils (Oldenborg and Sehlin, 1997, Lin et al., 1993) contributing to the immune deficiencies observed in people with diabetes. The neutrophil deficiency associated with diabetes has been also linked to changes in chemotaxis and phagocytosis (Ortmeyer and Mohsenin, 1993, Shah et al., 1983).

In addition to decreased motility, neutrophils isolated from people with diabetes have an ineffective respiratory burst (Nielson and Hindson, 1989) which suggests that neutrophils constantly exposed to glucose become defective and damaged as they have previously been over activated which causes non-specific tissue damage.

If hyperglycaemia leads to defective immune responses and if a clear link between non-diabetes associated hyperglycaemia and respiratory bacterial infection can be made, the presence of glucose in the lungs could potentially be identified as a key risk factor in *P. aeruginosa* infections.

1.13. Hypothesis and Aims

1.13.1. Hypothesis

The increased risk of bacterial infection in the lungs of patients with co-morbidities such as hyperglycaemia is due to elevated airway glucose levels. Therefore in conditions such as hyperglycaemia in obesity and diagnosed and undiagnosed diabetes, *P. aeruginosa* infection is likely.

1.13.2. Aims

There were 2 main aims of this thesis

1. **Link glucose to disease**

   By generating mutants with reduced capability of glucose utilisation the role of glucose can be determined to show that glucose does promote bacterial growth and mutants generated are unable to use excess glucose.

   Secondly, *in vitro* and *in vivo* infection models were developed to test both wild type and mutant *P. aeruginosa* to test the hypothesis.

2. **Exploit glucose control to treat infection**

   Using the *in vitro* and *in vivo* infection models to link glucose with increased bacterial burden then targeting glucose levels to reduce bacterial infection in the lungs offers a novel alternative to antibiotics to reduce bacterial burden.

   This part of the project involved using the anti-diabetic drugs metformin and insulin.

2. Materials and Methods

2.1. Reagents List

0.45 µm Syringe filter	Appleton Woods	FC122
1.5 ml Eppendorf	SSI	1220-00
100 µm cell strainer	Becton Dickenson	340 638
10x Taq Buffer	New England Biolabs	M0273S
24 well transwell	Sigma Aldrich	CLS3396-2EA
5 M Betaine	Sigma Aldrich	B0300
50 ml falcon	Corning	734 0453
5x PCR buffer	New England Biolabs	M0285L
60 mm Petri Dish	Jencons	391---1917
96 well tissue culture plate	Corning	353072
ACK lysis buffer	Lonza	10---548E
Agarose	Sigma Aldrich	A9539
Ampicillin	Sigma Aldrich	A0166
Amplex Red Glucose Kit	Thermo Fisher	A22189
Anhydrous Citric Acid	Sigma Aldrich	251275
BSA	Sigma Aldrich	A7906
75 cm Cell Culture Flasks	Sigma Aldrich	CLS3799
Crystal Violet	Sigma Aldrich	HT9018FOZ
CXCL1 KC ELISA Kit	R&D Systems	PMKC00B
D-Glucose	Sigma Aldrich	158968
Dipotassium phosphate	Sigma Aldrich	P3786
DMEM	Sigma Aldrich	D6546

DMSO	Sigma Aldrich	D8418
DNA ladder	Invitrogen	10488---058
Elisa plates	Thermo Scientific	430341
Eosin	Sigma Aldrich	HT110116
Ethanol	Sigma Aldrich	E7023
Fructose	Sigma Aldrich	F0127
Gel extraction Kit	QIAgen	28704
Gel Pilot Loading Dye	QIAgen	239901
Genomic Extraction Kit	Invitrogen	K1820-01
Gentamycin	Gibco	15710---049
Glyerol	Sigma Aldrich	G5516
Haematoxylin	Sigma Aldrich	HHS15
Hifi^{plus} polymerase	Thermo Fisher	11304-011
Histomount	National Diagnostics	HS-103
IL-6 ELISA KIT	R&D Systems	PM6000B
Insulin needles,	Terumo	BS30M2913
Isoflurane	Merial	Imperial CBS
KH_2PO_4	Sigma Aldrich	1551139
L spreaders	Sigma Aldrich	Z376779
LB	Sigma Aldrich	L3022
LB agar	Sigma Aldrich	L2897
Methanol	Sigma Aldrich	42105
$MgSO_4$:7H2O	Sigma Aldrich	1374361
Miniprep kit	QIAgen	L3022

NH_4Cl	Sigma Aldrich	A9434
pCR2.1	Invitrogen	45-0046
Pen/strep	Gibco	15140---122
Phosphate Buffered Saline	Gibco	14190-169
PIA	Sigma	17208
Primers	Eurofins	N/A
RPMI	Sigma Aldrich	R0883
S.O.C.	Sigma Aldrich	120883
Sodium Ammonium Phosphate	Sigma Aldrich	S4172
Sodium Chloride	Sigma Aldrich	S7653
Sodium Phosphate Dibasic	Sigma Aldrich	S3264
Sterile loops	Fisher Scientific	22-363-595
Streptomycin	Sigma Aldrich	S6501
Succinate	Sigma Aldrich	W327700
Sucrose	Sigma Aldrich	S7903
Sybr Safe	Invitrogen	S33102
Syringes	BD	300185
T4 DNA Ligase	New England Biolabs	M02025
Taq polymerase	Invitrogen	18038-042
TOP10 *E. coli*	Thermo Fisher	C4040-03
U bottom 96 well	Sigma Aldrich	CLS3799
Foetal Bovine Serum	Gibco	10500

2.2. Microbiology Techniques

Standard sterile techniques were used throughout the preparation of bacterial cultures, mutant generation and characterisation, all of which are detailed below.

2.2.1. Bacterial Strains

All strains of *P. aeruginosa* were supplied by Professor Alain Filloux's laboratory at Imperial College London – *P. aeruginosa* PAO1, PAK and PA14. For most studies PAO1 was used and was the background strain used to generate mutants. *Escherichia coli* strains were used for mutant generation and all strains including

origin are summarised below.

Strain	Origin
PAO1	Prof Alain Filloux
PAK	Prof Alain Filloux
PA14	Prof Alain Filloux
E. coli TOP10	Thermo Fisher
E. coli CC118pir	Prof Alain Filloux
E. coli 1047	Prof Alain Filloux
PAO1 Δ*glk*	Constructed in House
PAO1 Δ*gltK*	Constructed in House
PAO1 Δ*gtrS*	Constructed in House
PAO1 Δ*oprB*	Constructed in House

2.2.2. Glycerol Stocks

For wild type and mutant bacteria stocks, 700 µl of overnight culture was added to 700 µl of sterile 80 % glycerol to give a final 40 % volume/volume glycerol stock stored at -80 °C. Thawing of glycerol stocks was minimised by keeping stocks on ice for plating and inoculation and each stock was made in triplicate from the same colony and overnight culture.

2.2.3. Cultures

Overnight cultures were prepared under sterile conditions from single colonies isolated from LB agar plates used to inoculate LB broth. Cultures were incubated overnight at 37 °C, 200 RPM. All strains were grown in LB for infectious dose preparation and growth characterisation.

To quantify bacterial growth, overnight cultures were serially diluted and plated on LB plates and incubated at 37 °C to determine CFU/ml. Fresh cultures were prepared by taking a sample from a colony on an LB plate prepared from glycerol stocks and inoculating 5 ml LB broth and growing at 37 °C overnight. Once optimisation of overnight culture CFU/ml generated consistent results, culture CFU/ml was determined by OD readings.

2.2.4. Colony Forming Units/ml Calculations

For plating 100 µl or 50 µl was spread on LB plates using sterile L spreaders. Another technique was used for plating 1:2 serial dilutions in parallel to each other on a plate. 20 µl of each dilution was applied to an agar plate using a multichannel pipette and the plate was held at a 45 ° angle for 20 µl to run along the plate. Plates were incubated overnight at 37 °C resulting in 6 streaks from which CFU/ml could be calculated. The same procedure was used for *in vivo* CFU/ml calculations.

2.2.5. Growth and Dose Preparation

Bacteria were streaked on LB agar plates from glycerol stocks and single colonies were used to prepare overnight cultures of 5 ml LB in 50 ml Falcon tubes at 37 °C, 200 RPM. Overnight cultures were used to inoculate fresh LB at a ratio of 1:100 and grown at 37 °C, 200 RPM until an OD_{600} of 0.4 – 0.5 was reached. The culture was centrifuged for 5 minutes at 4000 RPM and the supernatant was removed. The pellet was resuspended in 1 ml of sterile PBS in a 1.5 ml Eppendorf and centrifuged at 12, 000 RPM for 3 minutes. The supernatant was removed and the pellet was resuspended in 400 µl of PBS for *in vivo* infections or antibiotic free cell culture medium for *in vitro* infections. This culture was the 10^{10} CFU/ml stock and was serially diluted to yield desired concentrations. Cultures were serially diluted and plated to confirm CFU/ml.

2.2.6. Characterisation of Bacterial Strains in Different Growth Media

For characterisation, all strains were grown in LB in a shaking incubator set at 37 °C, 200 rpm. Overnight cultures were used to inoculate sterile LB broth at a dilution of 1:100. Optical density (OD) was used as a measure of bacterial growth in addition to CFU/ml calculations, 1 ml was taken from individual cultures and OD_{600} was determined using a spectrophotometer (Ultrospec 10 Cell Density Meter, Amersham Biosciences).

For characterisation of mutants, growth was compared in M9 minimal medium supplemented with glucose or succinate was added as the sole carbon source.

Overnight cultures were washed, resuspended and diluted in PBS and added to a 5 ml culture at a ratio of 1:100.

Minimal Medium M9 Salts were prepared as detailed below and kept sterile.

H_2O 800 ml

Na_2HPO_4 64 g

KH_2PO_4 15 g

NaCl 2.5 g

NH_4Cl 5 g

The salts solution was mixed thoroughly and adjusted to 1 litre volume and autoclaved. M9 salts solution was used to make growth medium no more than a week in advance by preparing the mixture below under sterile conditions and filtered using a 0.40 µm filter.

H_2O 700 ml

M9 Salts 200 ml

1 M $MgSO_4$ 2 ml

1 M $CaCl_2$ 100 µl

When adjusted to 1 l, the solution formed the carbon free M9 medium to which carbon sources were added immediately prior to use and at the desired concentration.

2.2.7. High Throughput Growth of Bacteria

A 96 well growth assay was developed to screen a high number of colonies simultaneously under different medium and glucose conditions using a smaller volume of growth medium. Optimisation of OD using a plate reader was performed to sample 100 – 200 µl of culture allowing for greater sampling and growth in 96 well

culture plates. To ensure there was adequate gas exchange, plates were covered using 96 well microplate covers (Thermo Scientific). Overnight cultures were diluted in fresh LB and incubated at 37 °C, 200 RPM, OD readings were taken every 30 minutes and samples were taken to determine CFU/ml by serial dilution and plating.

2.2.8. Mutant generation

Genes were selected based on literature reviews on *P. aeruginosa*. Four genes were selected, gene sequences taken from www.pseudomonas.com, accession numbers given after each gene - *gltK* (PA3187), *glk* (PA3193), *gtrS* (PA3191) and *oprB* (PA3186). Primers were designed by sequence retrieval from the online *Pseudomonas* database (Winsor et al., 2011) and standard primer design protocol was followed optimising GC content and annealing temperature using NEB Tm calculator and PCR selection tool (New England Biolabs). For each gene 6 sets of primers were designed to generate fragments and confirmation of deletion as detailed in Figure 2.1.

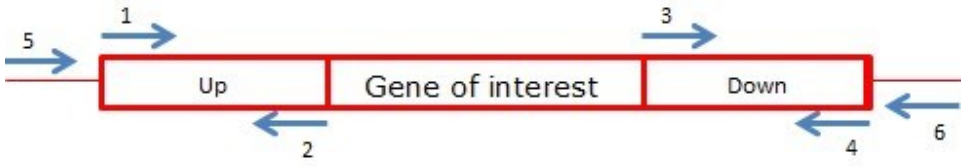


Figure 2.1 Location of primers for gene deletion

For each gene, 500 bp sequences either side of the gene to be deleted were amplified using primers (1+2) and (3+4). Primers 2 and 3 had overlapping sequences so the up and down fragments could be joined using overlapping PCR. For final confirmation of gene deletion, primers (5+6) were used which were external to the gene of interest.

2.2.9. Mutagenesis Primers

Primers for each of the 4 genes are listed below and all sequences are written in 5'→3'. Primers 2, 4 and 6 for each gene were designed by using 'Reverse Complement' to reverse the sequence from the 3' to 5' direction.

2.2.9.1. gltK Primers

gltK 1 GTCGAAGTACTGCTGCTTGA

gltK 2 ATGGCAACCAACTCCTGAATCCTTTCGT
gltK 3 TCAGGAGTTGGTTGCCATGTTTCGTTC

gltK 4 GAGGATCACCTGGAACGGCA

gltK 5 GCTGCCGCTCATCTTGAAGCCG

gltK 6 GCTGGTCAGCAGCATCACCAGC

2.2.9.2. oprB Primers

oprB 1 CGCTGCCGTTCGGATGGCT

oprB 2 ATGTACAAGCGGTGTTCTGATCGTCGCGTT

oprB 3 TCAGAACACCGCTTGTACATTTCCAG

oprB 4 GTTCGTCGCCAGCTTCATCG

oprB 5 GCGAGCCGACTCCAGCTCCATG

oprB 6 AGGTCTACCTGTTCGACGAGCCG

2.2.9.3. gtrS Primers

gtrS 1 TTCGGAGCAAGTCTGCGCTTGCAATGC

gtrS 2 ATGCTGCTCCTGCTGGAGTGATGGGCTGG

gtrS 3 TCACTCCAGCAGGAGCAGCATGCG

gtrS 4 GCCGACCGGGTGATCGG

gtrS 5 CGGGTCTTCAGCACGGTCATCG

gtrS 6 GCGAACGGACGATCGATCCT

2.2.9.4. glk Primers

glk 1 TCGCCGTCCTCGTGGAACAGCC

glk 2 ATGAATAACGAGGGCTGAGTCGGCGTCG

glk 3 TCAGCCCTCGTTATTCATCGTCGT

glk 4 TGGTCGCCGTGGTGCGTTTC

glk 5 GCCGCCAGCAGGTAGCCACTGCCG

glk 6 TCTGGCGCGAAGGGCCGGAAC

2.2.10. Plasmid Primers

To confirm ligation into pcr2.1 and pKNG101 the primers listed below were used.

M13 F GACCGGCAGCAAAAT

M13 R GAGGAAACAGCTATCAC UpKN

CCCTGGATTTCACTGATGAG RpKN

CATATCACAACGTGCGTGGA

2.2.11. Genomic DNA extraction

Genomic DNA was isolated using Pure Link Genomic DNA Kit (Invitrogen) following the manufacturer's guidelines. 1 ml of overnight PAO1 culture was pelleted in a 1.5 ml Eppendorf tube by centrifugation for 3 minutes at 12 000 RPM. The pellet was suspended in 180 µl of Genomic Digestion Buffer and 20 µl Proteinase K supplied with the kit and briefly vortexed. The Eppendorf tube was incubated at 55 °C for 3 hours, with vortexing every 30 minutes, after which 20 µl of RNase A was added and the Eppendorf tube was incubated at room temperature for 2 minutes. 200 µl of Genomic Lysis buffer was added to the solution and mixed well and 200 µl of 100 % ethanol was subsequently added to the Eppendorf tube. The solution was then

pipetted into a spin column supplied with the extraction kit. The column was centrifuged at 14 000 RPM for 1 minute and placed into a clean collection tube. 500 µl of wash buffer supplied with the kit was added and centrifuged again and collection tube was discarded. The tube was then placed in a clean collection tube and 500 µl of wash buffer 2 was added to the tube and centrifuged at 14 000 RPM for 3 minutes and the collection tube was discarded. The spin column was then placed into a sterile 1.5 ml Eppendorf tubeand 30 µl of molecular biology water was added to the column and incubated at room temperature for 1 minute. The Eppendorf tube and column were centrifuged for 1 minute at 14 000 RPM. 2 µl of the sample was run of 1 % agarose gel to confirm genomic DNA isolation.

2.2.12. Agarose Gel Electrophoresis

Genomic DNA and PCR products were separated on 1 % agarose gel and run for 45 minutes at 100 V. A 1 kb ladder (New England Biolabs) was used to compare product sizes to corresponding bands. This was done after each PCR round to confirm product size.

2.2.13. Polymerase Chain Reaction

For mutant generation, two PCR protocols were used – cloning for amplification of DNA fragments and colony PCR for confirmation of transformation and gene deletion.

2.2.14. PCR for Cloning

Cloning of fragments was achieved using High fidelity plus PCR system (Roche) with the mixture of reagents detailed below.

Genomic DNA (50-100 ng/µl)	3 µl
Forward primer	5 µl
Reverse primer	5 µl
dNTPs (20 mM stock)	1 µl
5x PCR buffer	10 µl
5M Betaine	10 µl
Hifiplus polymerase	0.5 µl
Molecular biology water	15.5 µl

Conditions for PCR were generally followed as detailed below with PCR running for 20 cycles.

Initialisation step	95 °C for 5 minutes
Denaturation step	95 °C for 30 seconds
Annealing step	55 °C for 30 seconds
Extension step	72 °C 1 minute/kb Final
extension step	72 °C 10 minutes

After each PCR amplification, in fragment generation was confirmed by gel electrophoresis. The fragments were PCR purified using PCR purification kit (Qiagen) and the eluted product was diluted 1:10 and 1.5 µl of each fragment was used in PCR following the conditions used for PCR cloning.

The fragment was then ligated into the high copy number vector pCR2.1 (Invitrogen) which carries ampicillin and kanamycin resistance genes as well as a lacZ marker in a 1.5 ml Eppendorf tube.

pCR2.1 vector	2 µl
Ligase	1 µl
Buffer	1µl
Insert	1 µl
Molecular Biology water	5 µl

The ligation mixture was then placed in a water bath set at 16 °C and left overnight. The ligation product was then transformed into competent TOP10 *E. coli* cells.

2.2.15. Transformation

Transformation was carried out by heat shock. The 10 µl ligation mixture was added to a vial of TOP10 *E. coli* (Thermo Fisher) cells on ice and left for 30 minutes. The vial was transferred to a heat block set at 42 °C for 30 seconds and returned to ice for 1 minute. 500 µl of Super Optimal Broth (S.O.C.) medium was added and incubated for 1 hour at 37 °C and 100 µl was plated on selective agar plates. The remainder was centrifuged at 13 000 RPM for 1 minute and the pellet was resuspended in 200 µl S.O.C. and plated on a selective agar plate. Selective plates used were LB agar with X-gal**,** ampicillin and kanamycin (50 µg/ml) and incubated overnight to select for colonies with successful transformations. Colonies able to grow in the presence of ampicillin and kanamycin were screened by PCR using primers for sequences in the pCR2.1 plasmid flanking the insert (Figure **2.2**) and successful clones identified by colony PCR were grown overnight for DNA extraction and sequencing.

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase.

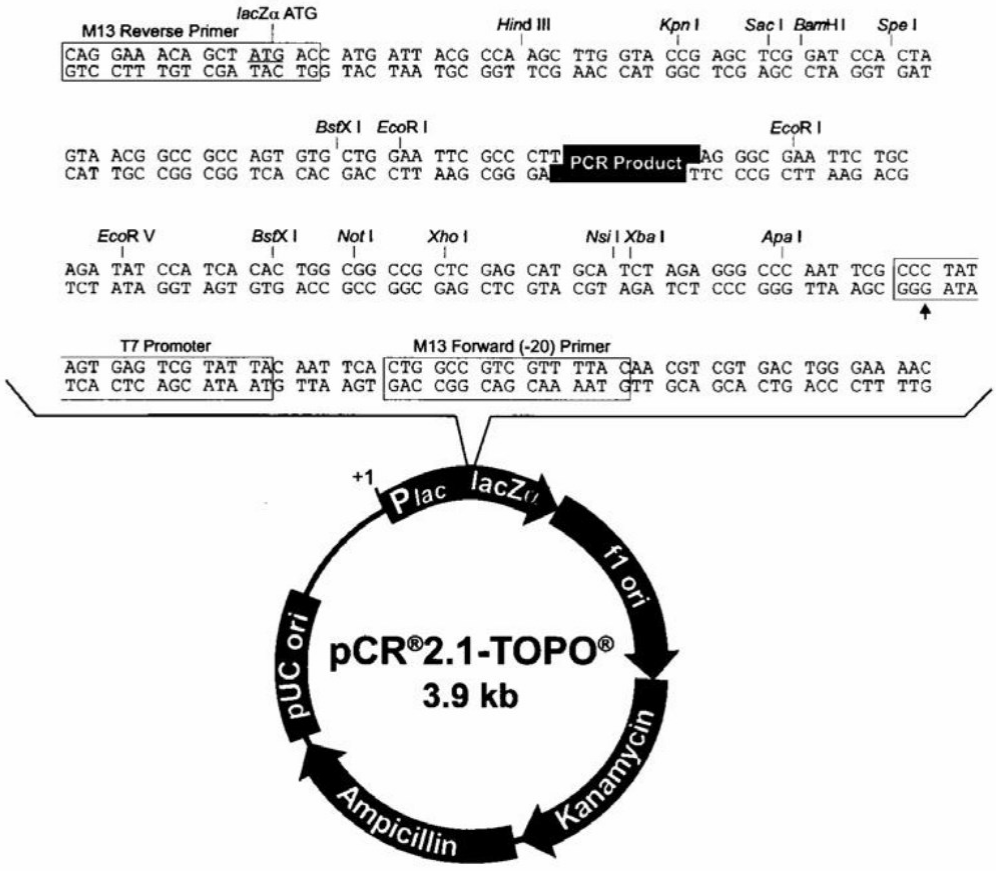


Figure 2.2 pCR2.1 plasmid with location of insert

The pCR2.1 plasmid was used as a high copy number vector to generate a high concentration of the upstream and downstream fragments joined by PCR flanking the gene of interest. The fragment was ligated into pCR2.1 overnight with T4 ligase and buffer for subsequent transformation into TOP10 *E. coli* cells. Following transformation, M13 primers which flank either side of the insert were used for PCR to identify clones which carry the plasmid with insert. Adapted from Thermo Fisher website

2.2.16. Colony PCR

PCR samples were prepared by suspending single colonies in 30 µl sterile molecular biology water. The standard PCR mix below was used throughout colony PCR.

DNA (colony suspended in MBW)	5 µl
10x Taq Buffer	3 µl
5' primer (10 µM stock)	2 µl
3' primer stock (10 µM stock)	2 µl
dNTPs (20 µM stock)	2 µl
Molecular Biology Water	14.3 µl
Taq polymerase	0.7 µl
DMSO	1 µl

2.2.17. Plasmid Isolation for pCR2.1 and pKNG101

Overnight cultures obtained from single colonies were used to inoculate LB and incubated at 37 °C, 200 RPM and incubated overnight for plasmid isolation. 1 ml of overnight culture was centrifuged at maximum speed for 3 minutes and the supernatant removed. Pellets were resuspended in 250 µl of buffer P1 and 250 µl of buffer P2 was added the Eppendorf tube and mixed by inversion until slightly clear. 350 µl of buffer N3 was added to the Eppendorf tube and mixed by inversion. Samples were centrifuged for 10 minutes at 13 000 RPM. Supernatants were applied to a QIAprep spin column plated into an Eppendorf tube and centrifuged at maximum speed for 60 seconds and flow through was discarded. The spin column was washed by applying 0.5 ml of buffer PB and centrifuging at maximum speed for 60 seconds and discarding flow through. The spin column was washed again using 0.75 ml of buffer PE and centrifuged for 6o seconds and flow through was discarded. Residual

PE buffer was removed by centrifuging again and the spin column was placed in a clean Eppendorf tube for elution. 30 µl sterile H_2O was added to the spin column and left to stand for 60 seconds at room temperature and centrifuged at maximum speed for 1 minute.

Buffer P1	50 M Tris-HCl pH 8.0
	10 mM EDTA
	100 µg/ml RNaseA
Buffer P2	200 mM NaOH
	1% SDS
Buffer N3	4.2 M Gu-HCl
	0.9 M potassium acetate
Buffer PE	10 mM Tris-HCl
	80% ethanol

2.2.18. Restriction Digest of Ligated Fragments

Double digests were used to isolate fragments using either *BamH*1 and *Apa*1 or *Spe*1 and *Sma*l using the following mixture which was incubated at 37 °C for 1.5 hours.

5 µl DNA

0.5 µl enzyme 1

0.5 µl enzyme 2

2 µl 10x buffer

12 µl water

The digested sample was then run on 1% agarose gel and gel extraction of the correct product size was performed using a Qiagen gel extraction kit. The concentration of the fragment was calculated using a NanoDrop and the product was ligated into the suicide vector pKNG101 overnight at 16 °C at a ratio of 1:3 with 10 x ligation buffer and T4 ligase.

To confirm ligation, PCR was performed using colony PCR and primers for sequences in the suicide vector flanking the insert.

The ligation was transformed into CC118pir *E. coli* cells and plated on LB plates with streptomycin and incubated overnight at 37 °C. Colonies were screened again using plasmid primers to identify a clone to use in three partner conjugation.

2.2.19. Tri Parental Mating

For conjugation, three strains were prepared overnight in 5 ml LB at 37 °C, 200 RPM. The donor strain, CC118pir *E. coli*, carries the suicide vector, the receiver strain, WT PAO1 and the helper strain, *E. coli* 1047 carries the pRK2013 plasmid. In parallel a 20 µl drop of the helper strain was added on top of 20 µl of the donor strain and also separately as controls (Figure 2.3) onto LB and *Pseudomonas* Isolation Agar (PIA) plates. Plates was then incubated for 2 hours at 37 °C after which 40 µl of the receiver strain was added to the helper and donor strains and also separately to act as a negative control (Figure 2.3) and incubated for a further 4 hours. The patches were then collected using sterile loops and resuspended in 500 µl of LB and 200 µl of each patch was plated on PIA with donor, helper and receiver patches alone acting as negative controls. The sample from the patch comprising of all 3 strains was also plated on PIA and the remaining 400 µl was pelleted using a microcentrifuge and resuspended in 200 µl of fresh LB and plated on PIA and plates were incubated overnight at 37 °C.

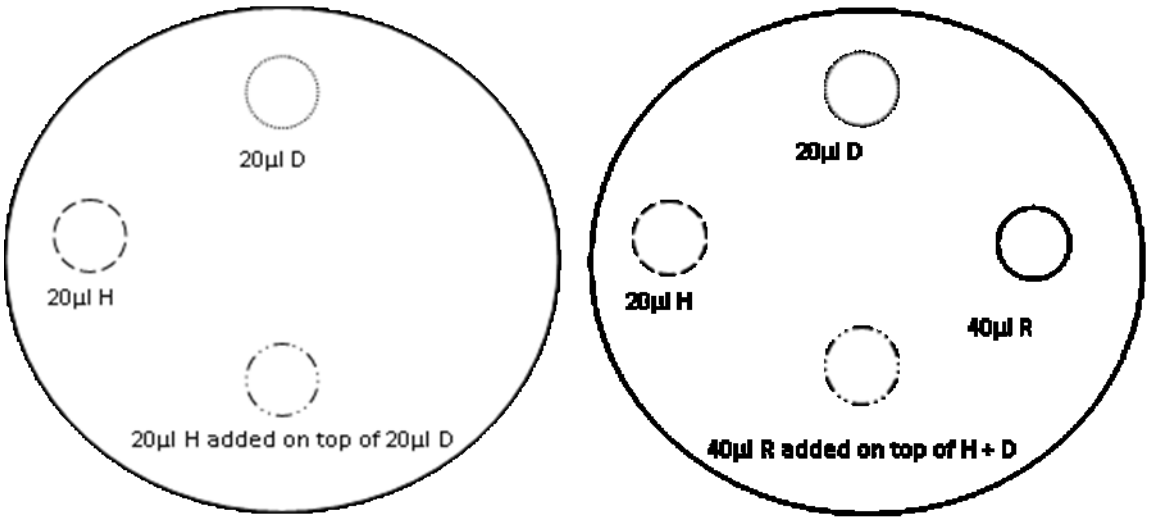
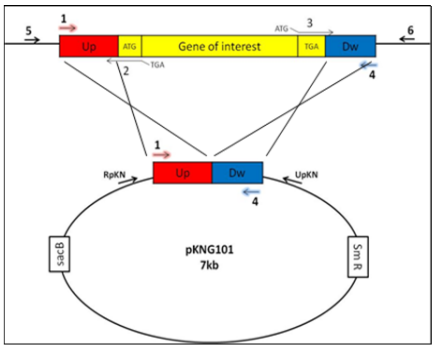


Figure 2.3 Tri Parental Mating.
The three strains, H- helper strain *E. coli* 1047, D- donor strain CC118pir *E. coli* and R- receiver strain WT PAO1 were plated using the above layout. H, D and R were plated individually to act as controls and plates were incubated at 37 ° C for a total of 6 hours.

The first recombination event was confirmed by PCR using primers external to the upstream and downstream fragments with primers specific to the suicide vector respectively to identify if the plasmid integration occurred upstream or downstream the gene of interest. From the successful clone 2 upstream clones and 2 downstream clones were restreaked on LB plates with streptomycin and one colony from either plate was selected for sucrose selection. This colony was streaked on LB plates containing 5 % sucrose and grown at room temperature for 48 hours to allow for the ejection of pKNG101. Single colonies were then selected and plated using the same sterile loop on LB plates containing streptomycin (50 µg/ml), pseudomonas isolation agar plates and LB plates in that order. This confirmed that the pKNG101 plasmid has been expelled from the cell by observing no growth on

the LB plates + streptomycin. Recombination events are detailed in Figure 2.4 where the first recombination event results in inserts either upstream or downstream of the gene to be deleted. The second recombination event occurs as a result of the cutting out of the toxic *sacB* gene as well as the gene of interest.



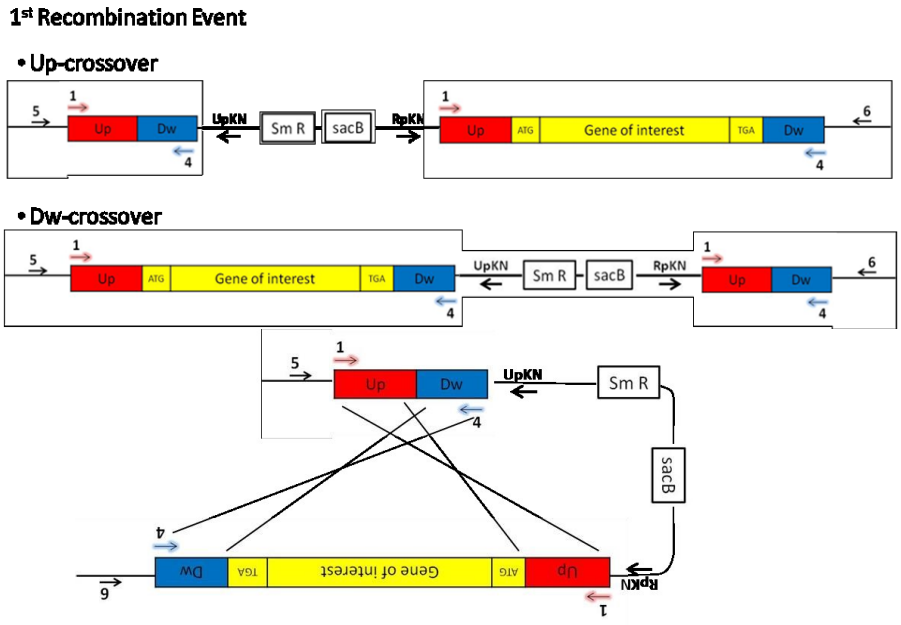


Figure 2.4 Summary of recombination events during conjugation steps of mutant generation. Ligation of the mutator fragment, consisting of the upstream and downstream fragments of the gene to be deleted, into the suicide vector plasmid – pKNG101. The pKNG101 plasmid with the upstream and downstream fragments was transformed into competent cells for use in conjugation and through a series of recombination events, the gene of interest was deleted.

Colonies on the PIA and LB plates were checked by PCR using the primer pairs (1 + 4) and (5 + 6) to confirm gene deletion. Any clones with successful deletions were restreaked to generate single colonies. This is to ensure the glycerol stocks are homogenous as clones isolated may not be pure. Single colonies were checked by PCR using the external primers and overnight cultures were prepared for glycerol stocks.

2.2.20. Transposon Library

Single transposon knockouts were kindly provided by Prof Alain Filloux and were in the *P. aeruginosa* PA14 background (Liberati, *et al.*, 2006). Samples were taken from 96 well glycerol stocks and incubated at 37 °C with shaking until cultures reached a high OD to create glycerol stocks.

2.2.21. Clinical Isolates

Clinical isolates from CF patients infected with *P. aeruginosa* were obtained from Professor Jane Davies at the Royal Brompton Hospital, Imperial College London. Samples were stored in 96 well glycerol stocks and duplicate plates were prepared by inoculating fresh LB using multichannel pipettes.

2.2.22. Biofilm Assay

To quantify biofilm formation, *P. aeruginosa* strains were grown in 96 well culture plates at 37 °C, 1:100 dilution in LB broth in a stationary incubator. After 24 hours of growth, LB medium was gently removed and 0.01 % crystal violet was added to each well and incubated at room temperature for 15 minutes.

Plates were then washed and dried and crystal violet was solubilised by adding 30 % acetic acid. OD_{550} readings were taken to compare biofilm formation.

2.2.23. Statistical Analysis

All statistical analysis and data presentation was performed using Graph Pad Prism 6.0.

2.3. In vitro Methods

To characterise mutants, *in vitro* tissue culture models were established using co- culture monolayers and transwell co-cultures of airway epithelial cells.

2.3.1. Cell Culture

Co-culture models were developed using monolayers and transwell inserts to determine what effect cells have on bacterial growth with and without glucose. Airway epithelial cell strains H441 and A549 were used for optimisation and A549 cells were used in all transwell, monolayer and co-culture experiments. H441 cells were kindly supplied by Prof Deborah Baines, St George's University London and A549 cells were available from Dr Tregoning. Cells were stored in liquid nitrogen and were recovered and seeded into T75 flasks with either RPMI (A549 cells) or DMEM (H441 cells) medium supplemented with penicillin/streptomycin and L-glutamine which was changed every 3 days and grown until confluent, after which Trypsin EDTA was used to detach cells for seeding and cell passaging.

2.3.2. Monolayer Co-culture

A monolayer co-culture was developed by seeding 10^5 cells/ml in 6 well culture plates until confluent. Prior to infection, cells were washed twice with PBS and bacteria were suspended in antibiotic free growth medium. Monolayer co-

cultures were incubated with bacteria for 4 hours at 37 °C, 5% CO_2. To measure bacterial growth the cells and bacteria were homogenised, serially diluted and plated on LB plates for overnight incubation at 37 °C. Both cell lines were used for monolayer co- cultures in 6 well cultures.

2.3.3. Air Liquid Interface Co-culture

H441 cells were used in transwell co-cultures for optimisation of dose and incubation time. For wild type and mutant growth co-cultures with A549 cells were used. Cells were seeded onto 24 well transwell (Corning, Sigma-Aldrich) with glucose free RPMI culture medium in the basolateral compartment and incubated at 37 °C, 5% CO_2. One day after seeding, culture medium was changed in the basolateral compartment and cells were left for 5 days to form a confluent layer of cells and medium on the apical side was removed. Transwells were subsequently incubated for 7 – 10 days and transwell epithelial resistance measurements were taken with an Ohmmeter attached to electrodes to determine when the transwells were ready to use which was when resistance was in excess of 300 Ωcm^2. 10 µl of 10^5 CFU/ml of wild type or mutant bacteria in log phase growth was applied to the apical side and medium was supplemented with glucose or succinate applied to the basolateral side. For control experiments, glucose and succinate was applied to the apical side with bacteria.

2.4. In vivo Methods

Acute murine *in vivo* infections were developed with PAO1 as well as a hyperglycaemic model to test the role of glucose on infection.

2.4.1. Ethics statement

All animal experiments were carried out in accordance with the UK animals scientific procedures act under project licence number PPL 70/7756 and personal licence number 70/24601. Animals were housed in the Central Biomedical Services facility at St Mary's Campus, Imperial College London.

Mice used in all *in vivo* models were C57BL/6, unless otherwise stated, and were 6-8 weeks old when ordered from Harlan or Charles River. Mice were weighed prior to infection and were anaesthetised using isoflurane set at 2.5 % and O_2 set at 0.8 l/minute. Once unconscious, 100 µl of PAO1 10^7 CFU/ml was delivered intranasally (i.n.) resulting in 10^6 CFU/mouse. Mice were monitored after infection in accordance with severity limits on the animal licence. 24 hours post infection mice were sacrificed by intraperitoneal (IP) injection of 100 µl pentobarbital.

2.4.2. Induced Hyperglycaemia

To induce hyperglycaemia, a type one diabetes model was developed using the drug streptozocin (Sigma Aldrich) which kills the insulin producing β cells of the pancreas. Previous studies on murine models of hyperglycaemia used single high doses for a type one phenotype and multiple low doses to induce a type 2 phenotype (Sakata et al., 2012). For this study C57BL/6 mice were injected IP with 5 mg, 0.25 mg/g of streptozocin in 100 µl PBS. Blood glucose was checked using a commercial glucose testing kit (One Touch, Ultra Mini) and urine glucose levels were checked every day using urine glucose testing sticks (Bayer Diastix). One week after the first injection, mice were injected with a second 5 mg dose of streptozocin and water was replaced with 10 % sucrose water overnight to avoid

hypoglycaemia caused by elevated and sudden insulin release by dead and dying β cells. 14 days after the first streptozocin injection, hyperglycaemia was confirmed by blood glucose levels.

Metformin was prepared at a concentration of 4 mg/ml and 200 µl was delivered IP. Insulin was delivered IP at a concentration of 0.5 units/kg (~10ul/unit).

2.4.3. Calculation of Colony forming units

CFU calculations were determined as described in '2.1.4 Colony Forming Units/ml Calculations'

2.4.4. Bronchoalveolar Lavage

BAL fluid was collected using sterile syringes and 1 ml sterile PBS was used to inflate the lungs 2-3 times by exposing the trachea and inserting a tube into the lungs. Samples were stored on ice and 100 µl was taken for serial dilution and plating. The remainder was centrifuged for 1 minute and maximum speed and the supernatant was collected and aliquoted for cytokine analysis. To lyse red blood cells, 200 µl Ammonium-Chloride-Potassium Lysing Buffer (ACK) was used for the lysis of red blood cells and was added to the cell pellet and resuspended and incubated at room temperature for 3 minutes after which 200 µl of complete cell growth medium, DMEM/RPMI was added. The samples were centrifuged again for 1 minute and supernatant was removed. The pellet was suspended in 200 µl of cell growth medium and the sample was used for cell counts.

2.4.5. Airway Cell counts

Airway cells counts were performed by Trypan blue exclusion, 10 µl of cell suspension was added to 10 µl of Trypan blue and counted by light microscopy. The remainder of the cell suspension was used for differential cell counts by staining.

2.4.6. Differential Cell Counts

For differential cell counts a cytocentrifuge was used and 100 µl of airway cells were spun at 500 RPM for 5 minutes. Slides were left to air dry and fixed in methanol for 5 minutes. Once dry cells were stained with haematoxylin for 5 minutes and washed with water. Slides were then washed in eosin 10 times at 5 second intervals and briefly washed in water. Once dry, coverslips were applied using histomount and percentage cells were determined. From this, total neutrophil counts were calculated.

2.4.7. Lung Processing

Lungs were removed after airway lavage and stored in 1 ml PBS in bijou tubes on ice until ready for processing. Lungs were homogenised using a plunger from sterile 2 ml syringes passed through a 70 µl cell strainer and flushed with PBS. Samples were then serially diluted for CFU/ml calculations by plating.

2.4.8. Cytokine analysis

To compare cytokine and chemokine responses to PAO1 infection under normal and hyperglycaemic conditions ELISAs were performed following the manufacturer's guidelines. 100 µl of diluted capture antibody (2 µg/ml) in PBS

was added to each well of a 96-well microplate and sealed and incubated at 4 °C overnight. Following incubation, capture antibody was removed by decanting and wells were washed 3 times with wash buffer (PSB, 0.05 % Tween). 300 µl of 1 % bovine serum albumin (BSA) in PBS was added to each well and plates were covered and incubated for 1 hour at 37 °C. Standard curves were prepared using supplied standards and an eight point standard curve using 2 fold serial dilutions in PBS 1 % BSA was prepared with a high standard of 1000 pg/ml. 100 µl of each sample or standard was added to wells and incubated for 2 hours at 37 °C after which plates were washed with wash buffer. Detection antibody diluted to form a working concentration of 200 ng/ml in 1 % BSA and 100 µl of detection antibody was added to each well and plates were incubated for 2 hours at 37 °C. Wells were subsequently washed and 100 µl of Streptavidin-HRP was added to each well and incubated for 30 minutes. Plates were washed and 100 µl of 1:1 mixture of H_2O_2 and Tetramethylbenzidine was added to each well and developed in the dark for 30 minutes. After incubation, 50 µl of H_2SO_4 was added and absorbance was read using a microplate reader. This was performed for interleukin 6 and CXCL1 /KC (R&D systems). Samples were tested in duplicate from aliquots stored at – 80 °C.

2.4.9. Glucose Levels

Glucose levels in the lung were assessed by using an Amplex Red Glucose Kit (ThermoFisher) which uses fluorescence to quantify glucose in samples. BALF was stored in aliquots at -80 °C for and the manufacturer's protocol was followed. Amplex red supplied with the kit was dissolved in 60 µl DMSO. Reaction buffer was prepared by diluting 4 ml of 5x Buffer in 16 ml H_2O and

Horseradish Peroxidase (HRP) was prepared by dissolving the supplied vial in 1 ml of reaction buffer. Glucose oxidase was prepared by dissolving supplied glucose oxidase in 1 ml of reaction buffer and glucose stock solution was prepared by dissolving 72 mg in 1 ml of reaction buffer to yield a final concentration of 400 mM, Standard curves were prepared by diluting glucose stock solution in reaction buffer to produce glucose concentrations of 0 to 200 µM. For the assay, 50 µl of samples and glucose standards were aliquoted into each well. To prepare the working solution each of the above prepared solutions were mixed using the following volumes and 50 µl was added to each well loaded with samples or standards.

50 µl	Amplex Red
100 µl	HRP
100 µl	Glucose Oxidase
4.75 ml	Reaction Buffer

In collaboration with Dr James Garnett (Newcastle University) at St George's University London the Glucose Oxidase Analyser (Analox Instruments, UK) was used for glucose measurements.

Chapter 3 Results

3.1. Introduction

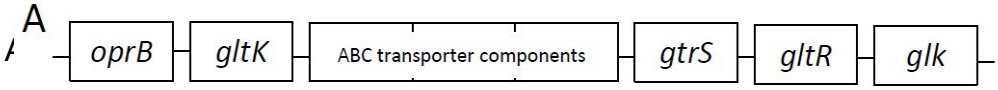
Based on supporting data in previous publications, this project aims to link elevated glucose levels with susceptibility to bacterial lung infections by promoting bacterial growth. Confirming glucose as a driver of respiratory bacterial growth may enable the development of alternative strategies to treat *P. aeruginosa* infections. *Pseudomonas aeruginosa* was used in the study as it has been identified as an opportunistic pathogen resistant to common antibiotics as well as being easily manipulated for generation of mutants to obtain a greater understanding of the effect of glucose on *P. aeruginosa* growth.

The hypothesis of this project was tested through the generation of glucose uptake and utilisation mutants which were used in *in vitro* and *in vivo* infection models developed during this project.

Some of the data presented in this chapter has been included in published studies (Garnett, 2013) and Gill et al. (2016).

3.2. Gene selection

Genes were selected based on literature reviews of *P. aeruginosa* mutant generation and glucose utilisation. Deletion of *gltK* was selected first for deletion as it has previously been characterised (Adewoye and Worobec, 2000), but the consequence for bacterial growth *in vitro* and *in vivo* in the presence of glucose was not explored. Glucose uptake and utilisation genes were located in a cluster of genes (Winsor et al., 2011) and three additional genes were selected for deletion. GltK was previously identified as a high affinity glucose transporter and resulted in a reduction in glucose transport (Adewoye and Worobec, 2000). OprB was selected as a carbohydrate specific porin which contributes to glucose transport, located in the outer membrane (Wylie and Worobec, 1994, Adewoye and Worobec, 1999). GtrS is thought to act as a sensor located downstream of GltK in the glucose pathway facilitating the final stage of glucose uptake (Daddaoua et al., 2014). Glk is downstream of GtrS acts as a kinase involved in carbon utilisation and entry into the pentose phosphate pathway (Winsor et al., 2011, Mao et al., 2009). Details of genomic and cellular location of each gene selected are detailed in Figure 3.1.



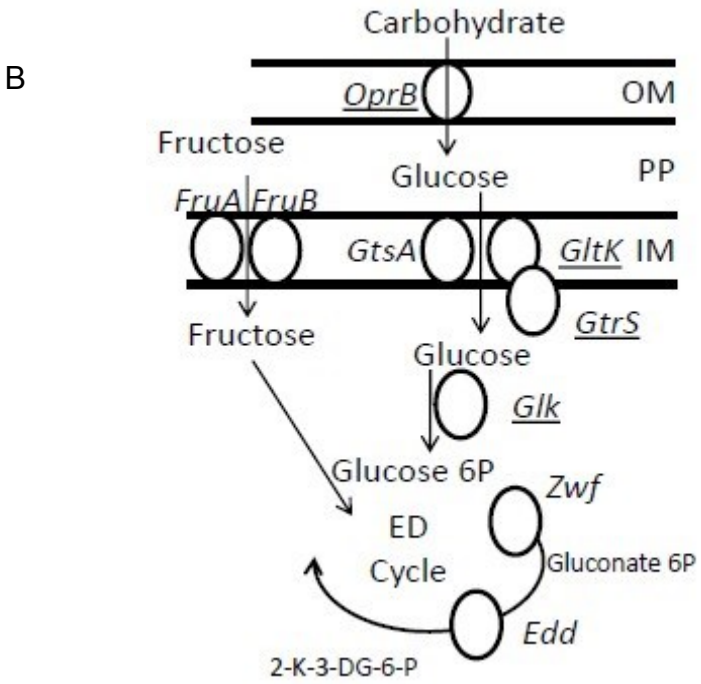


Figure 3.1 Location of Deleted Genes

Each of the genes were selected based on location and function using the *Pseudomonas* online database (Winsor et al., 2011) and genomic location of each gene is shown in sequence (A). The cellular location of each gene product was determined using the *pseudomonas* database and references for each gene function (B). Underlined genes were selected for deletion

Adapted from Gill et al. (2016)

3.3. Mutant Generation

Each mutant was generated following a protocol adapted from A. Hachani (unpublished). *The results shown here are for the generation of gltK and the same steps were followed for the other 3 genes and are shown in the appendix.*

The fragment was generated by PCR amplification of 500 bp fragments either side of the gene to be deleted using primers specific for the gene Sequences were retrieved from the online *Pseudomonas* database as detailed in Figure 2.1. Each fragment size was confirmed by PCR (Figure 3.2A) with overlapping PCR used to join the fragments (Figure 3.2B). The fragment was then ligated into pCR2.1 and transformed into TOP10 competent *E. coli* cells. Colonies were screened by PCR to identify a clone carrying the plasmid based on PCR product size using plasmid primers (Figure 3.2C).

Clones with the correct band size were digested using *BamH*1 *and Apa*1 restriction enzymes to isolate the fragment and the linear plasmid was extracted from the agarose gel (Figure 3.2D). The fragment was ligated overnight at a 3:1 ratio into the suicide vector pKNG101. The plasmid carrying the fragment was then transformed into the cc118λpir *E. coli* competent cells, to identify a clone to use for tri-parental conjugation which was confirmed by PCR and based on product size using primers specific to the suicide vector which flanked the insert (Figure 3.2E).

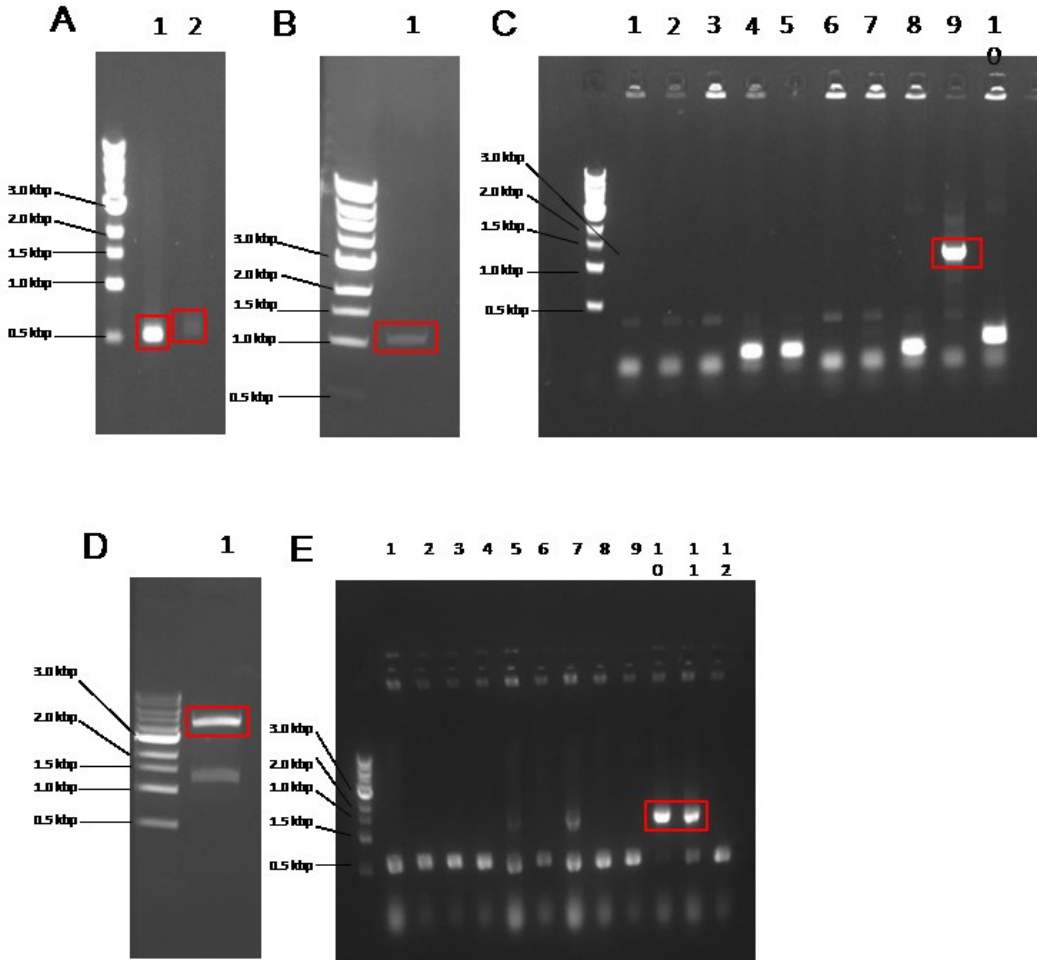


Figure 3.2 Construction of mutator fragments.
Using primers designed upstream and downstream of *gltK* flanking sequences were generated by PCR and gel electrophoresis on 1 % agarose using a 1 kb ladder with a product size of approximately 500 bp (A) and overlapping PCR to join the 2 overlapping fragments using the forward and reverse primers for the upstream and downstream fragments respectively with 1 clone generating the correct product size of 1000 bp (B). Ligation of the fragment into the high copy number vector pCR2.1 was performed for transformation into TOP10 *E. coli* cells which was confirmed by PCR using plasmid specific primers (C). Overnight cultures were set up for plasmid extraction and isolation by restriction digest, gel electrophoresis and gel extraction (D). The final ligation into the suicide vector pKNG101 and transformation into cc118λpir *E. coli* was confirmed by PCR using primers FpKN and RpKN, primers for pKNG101 (E). Basepair (bp) indicates approximate fragment size for correct PCR product determined using a 1 kb ladder. Lane number represents individual clones tested.

Successful recombination was confirmed by PCR (Figure 3.3A) and positive clones were plated on sucrose selection plates. PCR confirmation of colonies identified clones with a gene deletion (Figure 3.3B) to use for patching to generate homogenous colonies.

Colonies were checked again using external primers for the gene of interest, where successful deletion would yield a product size of 1000 bp using external primers and glycerol stocks were prepared.

The generation of the mutator fragment to ligate into the high copy number vector, extraction and then identification of a clone to use to ligate into the suicide vector is shown in full for the gltk mutant (Δ*gltk*). The same procedure was followed for generation Δ*oprB*, Δ*glk and* Δ*gtrS* and is summarised in table 3.1 and the final products shown in Figure 3.4.

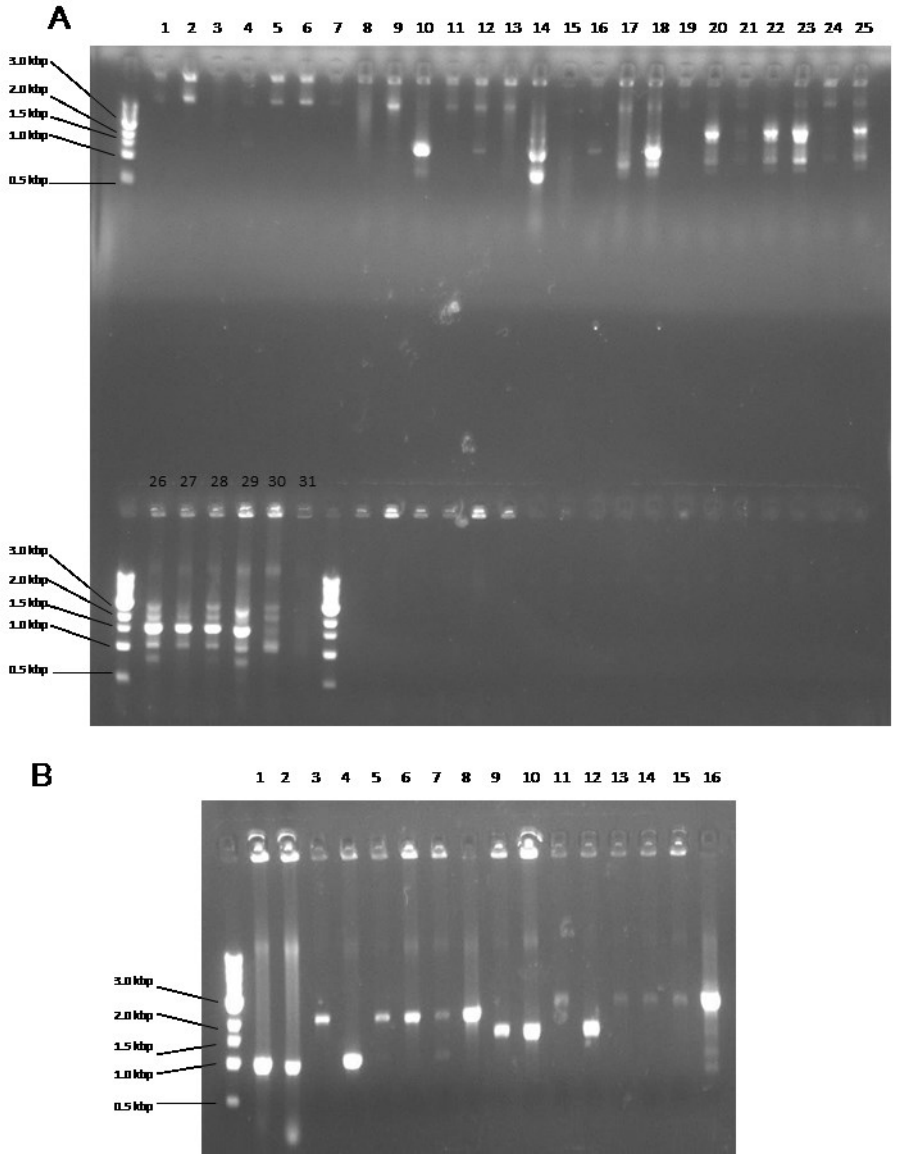


Figure 3.3 Recombination and Conjugation Confirmation

Successful recombination was confirmed by PCR and gel electrophoresis using primers specific for PKNG101 either side of the insert (A) and positive clones were plated on sucrose selection plates to force expulsion of the suicide vector and PCR confirmation of colonies identified clones with a gene deletion (B). 1 kb ladder and 0.5 % agarose gel. Basepair (bp) indicates approximate fragment size for correct PCR product determined using a 1 kb ladder. Lane number represents individual clones tested

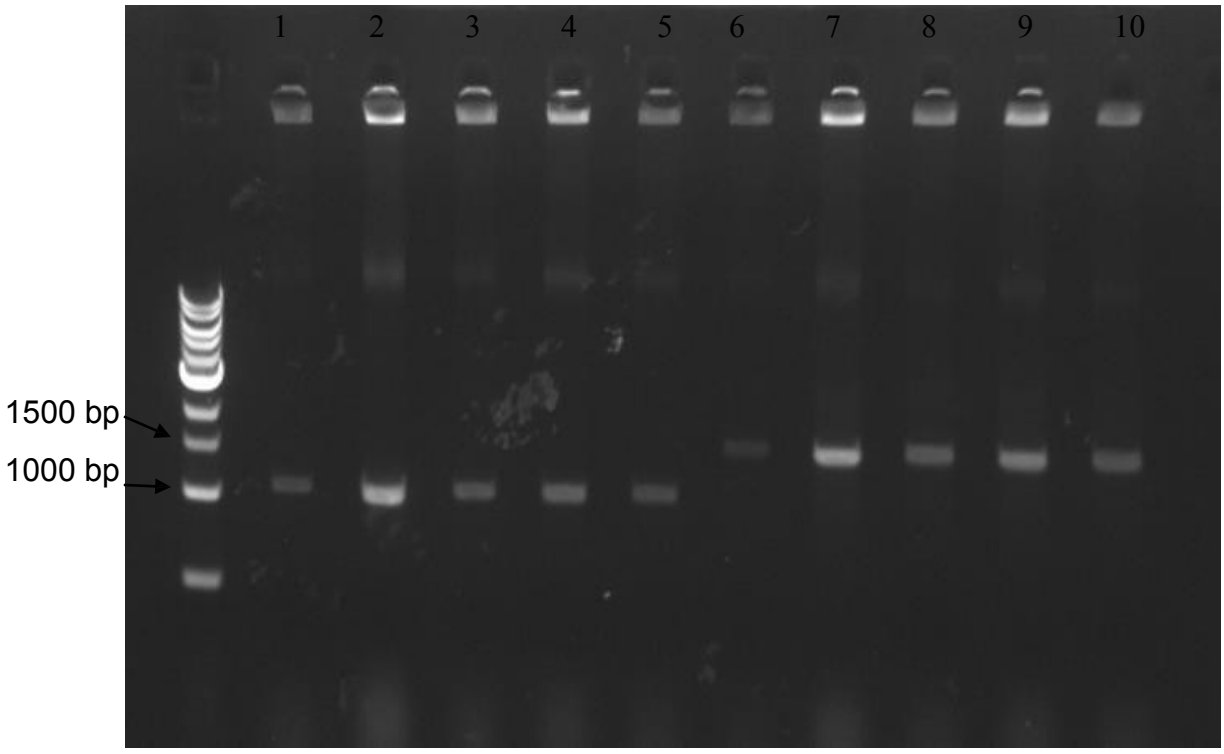


Figure 3.4. Confirmation of Gene Deletion

Final gene deletion for all mutants was confirmed by PCR using the external primers (5+6) Lanes 1- 5 showing that the genes *glk* (in duplicate)*, gltK, gtrS, opr* are no longer present – expected band size 900-1100 bp. When primers for each gene were used on wild type PAO1 the product size would be larger due to presence of the gene (lanes 6-10). 1 kb ladder and 0.5 % agarose gel.

	Monday	Tuesday	Wednesday	Thursday	Friday
Week 1	• PCR amplification of upstream and down stream fragments • Confirmation by gel electrophoresis • PCR product purification • Overlapping PCR • Confirmation by gel electrophoresesis • Overnight ligation at 16°C into pCR2.1	• Transformation into TOP10 cells • Plate cultures on LB plates + ampicillin and kanamycin • Incubate at 37 °C overnight	• Patch white colonies on LB plates + ampicillin and kanamycin • Incubate at 37 °C overnight	• Screen colonies by colony PCR using pCR2.1 primers • Set up overnight cultures of successful clones in LB +ampicillin and kanamycin	• Miniprep samples to isolate plasmid
Week 2	• Isolate mutator fragment by double digest • Gel electrophoresis to check size and gel extraction • Ligate overnight at 16°C into pKNG101	• Colony PCR to confirm ligation • Transformation into CC118λ*pir* *E. coli* • Plate cultures on LB plates+ streptomycin • Incubate overnight at 37°C	• Patch colonies on LB plates + streptomycin • Incubate at 37 °C overnight	• Screen colonies using plasmid primers • Set up overnight cultures of successful clone, the helper strain and receiver strain in LB at 37°C	• Conjugation
Week 3	• Isolate conjugants on PIA + streptomycin • Incubate plates overnight at 37 °C	• Patch colonies on LB plates + streptomycin • Incubate plates overnight at 37 °C	• Colony PCR to confirm recombination using plasmid primers and external primers • Streak single colonies on LB plates + streptomycin • Incubate plates overnight at 37 °C	• Select single conlony to streak on LB plate + 5% sucrose • Incubate plates for 48 hours at room temperature	
Week 4	• Patch colonieso LB plates + streptomycin, PIA and LB • Incubate plates overnight at 37 °C	• Colony PCR clones sensitive to streptomycin and able to grow on PIA and LB using mutant primers to confirm deletion • Streak successful clones on LB • Incubate plates overnight at 37 °C	• Patch single colonies on LB • Incubate plates overnight at 37 °C	• Colony PCR to confirm gene deletion • Set up overnight culture of successful mutants	• Set up glycerol stocks of mutants for storage at -80°C

Table 3.1 Summary of mutant generation

The table shows a week by week schedule of mutant generation which can be completed in 3-4 weeks if every step is successful.

3.4. Glucose uptake and utilisation mutants display reduced growth in M9 minimal medium supplemented with glucose

To test the role of glucose on bacterial growth, strains carrying deletions in genes involved in glucose uptake and utilisation, *gltK, glk, gtrS* and *oprB,* were grown in different nutrient conditions. Firstly, to assess the effect gene deletion has on growth in rich medium, all strains were grown in LB and OD was taken of over a period of 24 hours at intervals of 1,2,4,8 and 24 hours. In complete medium, gene deletion had no significant effect on the growth of mutant strains compared to the WT over 24 hours (Figure 3.5A). In rich medium, WT and KO strains plateaued at 24 hours as the exponential growth phase ends due to nutrients being depleted and the lag growth phase is entered.

Overnight cultures of WT and mutant strains grown in LB were cultured in M9 minimal medium and OD was taken over a period of 24 hours. In M9 minimal medium supplemented with succinate the growth plateaued after 8 hours for all strains. With the addition of succinate as the sole carbon source, the mutant strains displayed similar growth to the WT over 24 hours (Figure 3.5B). Although there was a reduction in OD after 24 hours of growth with mutant strains this difference is not significant.

When overnight cultures of mutant strains were grown in M9 minimal medium with glucose as the sole carbon source there was a significant reduction in growth as determined by OD over 24 hours compared to the WT strain (Figure 3.5C). A further control using fructose as the sole carbon source also demonstrated that there was no difference in growth between wild type and mutant strains when alternative carbon sources were available (Figure 3.5D).

This demonstrates that deleting genes involved in glucose uptake and utilisation does not significantly affect growth in rich medium, as other nutrients required for growth are available. However when only glucose is available as the sole carbon source all strains carrying gene deletions display attenuated growth compared to the WT after 8 hours, which is not observed when an alternative carbon source is added. This is explained by the genes selected for deletion which were involved in glucose uptake and utilisation. The fact that deletion of genes *glk, gltK, gtrS* and *oprB* does not result in reduced growth with alternative carbon sources compared to wild type PAO1 suggest that they can be used to determine the impact of glucose on growth in *in vitro* and *in vivo* models.

Mutants grow to different levels under different carbon sources and this is due to the structure of each carbon source.

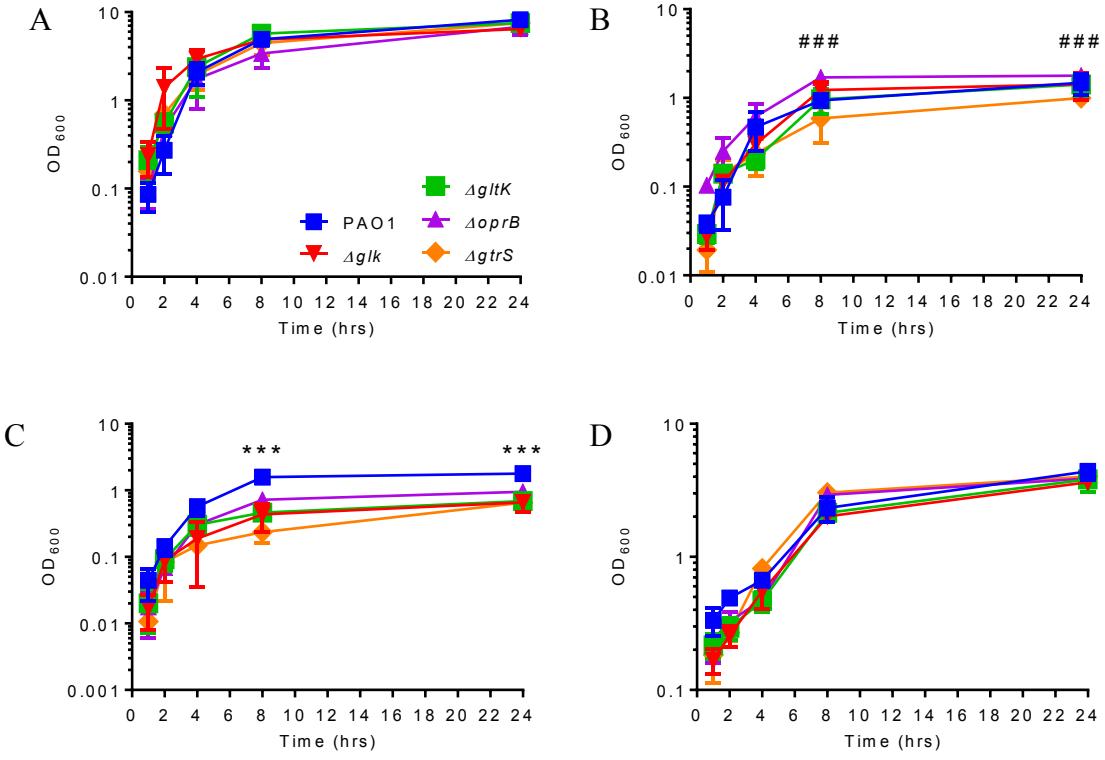


Figure 3.5. Growth of *P. aeruginosa* mutants compared to WT in rich or minimal medium supplemented with carbon sources other than glucose

Wild type and strains carrying gene deletions were grown overnight in LB at 37 °C, 200 rpm and used to inoculate fresh LB medium or M9 medium at a ratio of 1:100. Strains were grown in LB (A), M9 minimal medium supplemented with 50 mM succinate (B), M9 minimal medium supplemented with 50 mM glucose (C) or M9 minimal medium supplemented with 50 mM fructose (D). OD_{600} measurements were taken over a period of 24 hours. Mean SD n=3. *** p≤0.001 between WT and mutants in M9 medium + glucose and ### p=<0.001 between Δ*oprB* and Δ*gtrS* in succinate where relevant. P values determined by 2 way ANOVA.

3.5. Basolateral glucose supports apical bacterial growth in a dose dependent manner in an airway epithelium co-culture

The effect of glucose on growth in the presence of epithelial cells could differ from growth in LB alone or in minimal medium so bacterial growth was assessed using monolayer and transwell co-cultures of H441 and A549 epithelial cells. The first optimisation involved using serial dilutions of overnight cultures, which typically have a concentration of 10^9 CFU/ml, and increasing levels of glucose in the basolateral compartment.

Overnight cultures of WT PAO1 were diluted in cell growth medium RPMI and applied to the apical side of fully differentiated H441 cells grown on transwell inserts (Figure 3.6A) with increasing concentrations of glucose on the basolateral side. As expected there was a dose dependent increase in recovered CFU from the apical side with increasing infectious doses of *P. aeruginosa* and glucose concentration (Figure 3.6B).

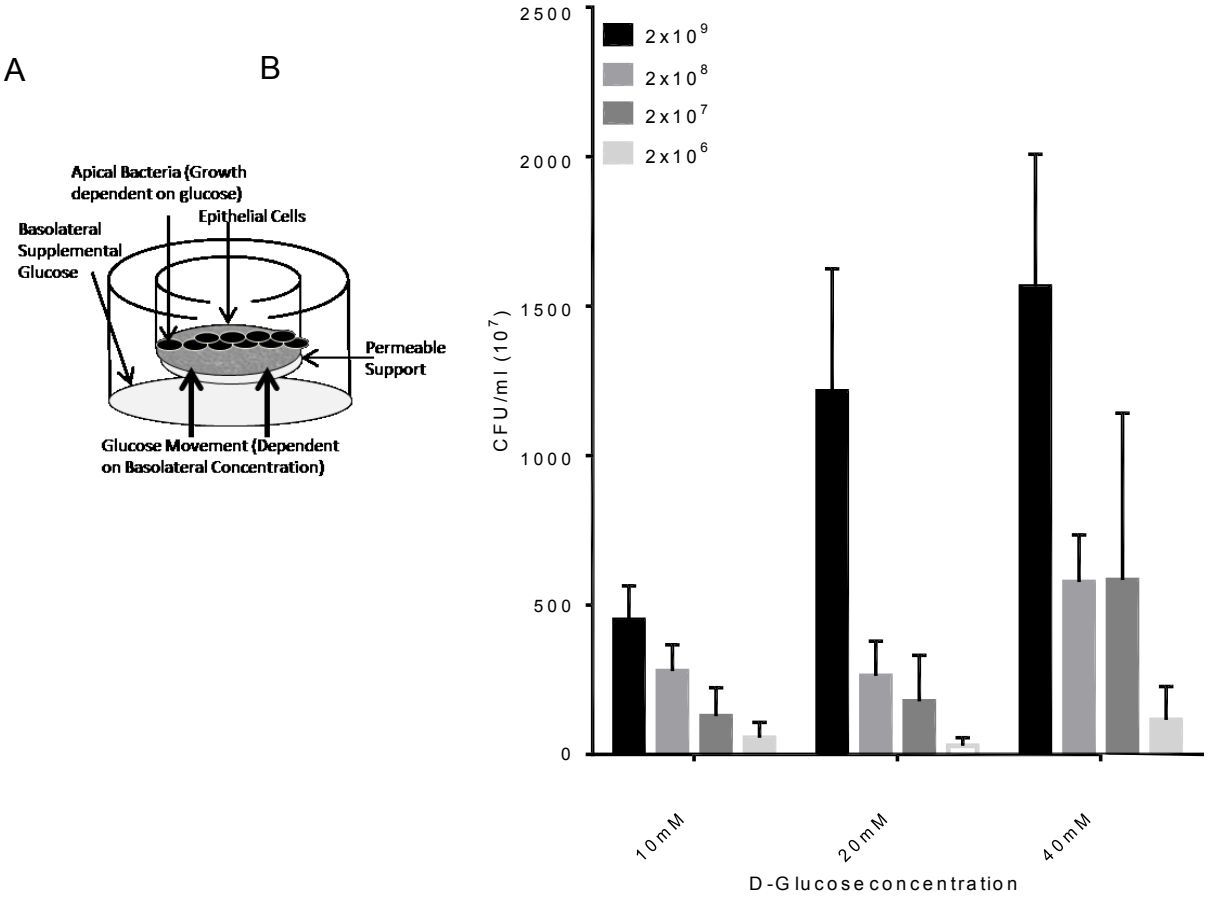


Figure 3.6 Basolateral glucose promotes bacterial growth on the apical side in air-liquid interface co-culture.
Experimental set up (A) and after 7-10 days air liquid interface cell cultures were infected apically with 50 µl of 4 different CFU/ml of *P. aeruginosa* overnight cultures. Basolateral compartments filled with glucose free RPMI cell culture medium were supplemented with glucose and co-cultures incubated for 5 hours at 37 °C, 5% CO_2. CFU/ml were calculated by serial dilution and homogenisation from co-cultures onto LB plates and incubated overnight at 37 °C (B). N=3.

3.6. Monolayer and bacteria co-cultures show dose dependent increase in recovered CFU/ml of P. aeruginosa with glucose.

Monolayers of A549 cells were also used in co-cultures and increasing glucose in the culture medium increased the recovered CFU/ml of WT PAO1. As the growth curves showed that in the presence of glucose only, mutants were unable to grow to the same levels as WT PAO1, determined by OD, the same was tested in co-culture models with A549 epithelial cells. In a monolayer co-culture, WT and mutant strains in log phase growth were grown in medium with increasing levels of glucose. Wild type PAO1 was able to grow with increasing levels of glucose, determined by CFU/ml (Figure 3.7A) and with all mutants there was a decrease in recovered CFU/ml compared to WT PAO1 (Figure 3.7B-e), despite increased glucose concentrations.

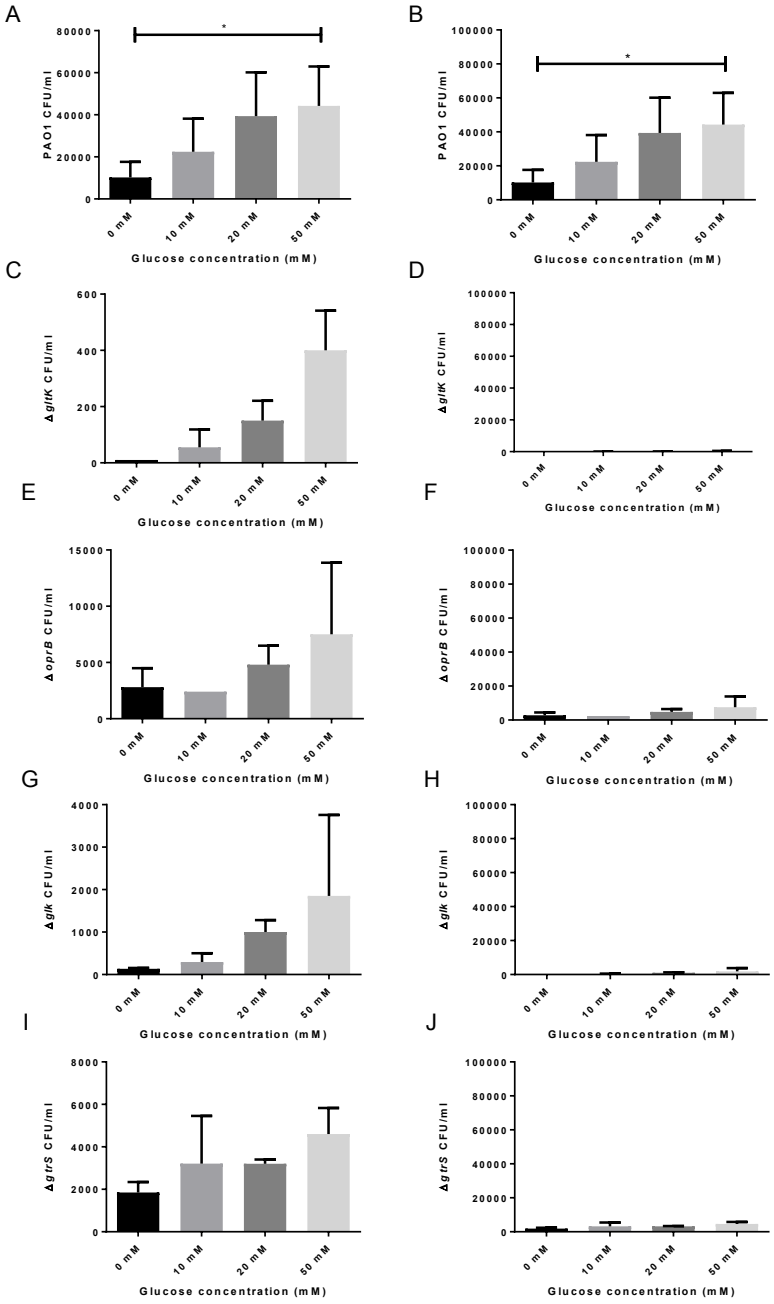


Figure 3.7 Glucose promotes *P. aeruginosa* WT growth in dose dependent manner but not mutants.

Monolayers of A549 cells were infected with WT (A-B) or mutant PAO1 (C-J) grown to log phase and diluted to 10^5 CFU/ml. Bacterial cell cultures were washed and diluted in antibiotic and glucose free RPMI medium with increasing concentrations of glucose and applied to the apical compartment. Co-cultures were incubated for 5 hours at 37 °C, 5% CO_2. Cultures were homogenised a serially diluted for CFU/ml calculations. n=3. Values also plotted on same axis for each strain.

3.7. Mutant and WT air liquid interface Co-culture

To further test the role of glucose in a co-culture model, a transwell co-culture with A549 cells was developed. Apical bacterial growth was compared between WT and mutant strains with different glucose concentrations in the basolateral compartment after 4 hours of growth. As expected, after infection with 10^5 CFU/ml there was an increase in CFU/ml recovered from the apical side with higher concentrations of glucose in the basolateral medium for WT PAO1 (Figure 3.8A). When mutant bacteria were applied to the apical side, there was a significant reduction in recovered CFU at all glucose concentrations but there was still an increase in CFU with increasing glucose levels for Δ*glk,* Δ*gltK* and Δ*oprB* mutants (Figure 3.8B,C,E) whereas the Δ*gtrS* mutant displayed similar growth with all concentrations of glucose (Figure 3.8D). Despite a dose dependent increase in CFU/ml for mutant strains with increasing glucose levels, the CFU/ml values were significantly lower compared to WT PAO1.

This suggests that *in vitro*, mutants are unable to utilise the excess nutrients in the form of glucose, however as only one carbon source was focused on more experiments need to be undertaken to confirm this.

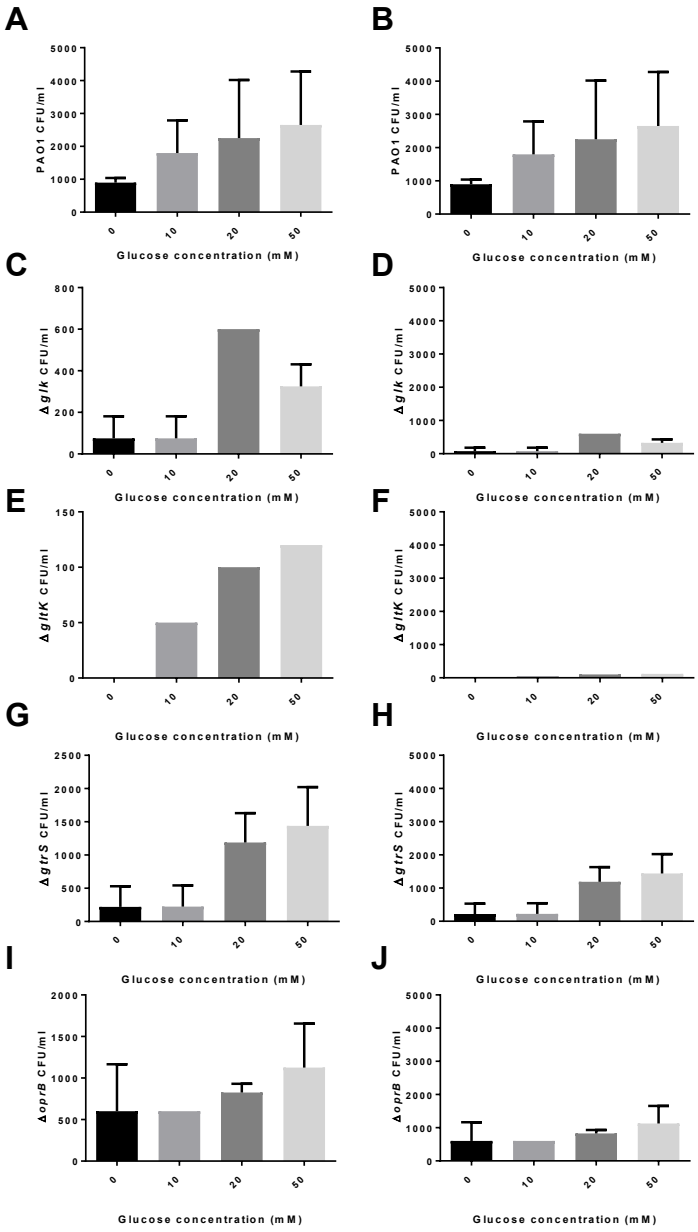


Figure 3.8 Basolateral Glucose promotes apical growth of WT PAO1.

A549 cells seeded on transwells were grown until confluent and glucose free RPMI medium in the basolateral compartment was supplemented with increasing levels of glucose. Log phase WT (A-B) or mutant strains (C-J) were applied to the apical side and co-cultures were incubated for 5 hours at 37 °C and 5 % CO_2. After this time transwell epithelial resistance measurements were taken to confirm epithelial layer integrity and co-cultures were homogenised for serial dilution and CFU/ml calculations by plating. n=3. * p≤ 0.05. Data plotted on same axis (B,D,F,H,J).

To confirm that the reduction in recovered CFU in transwells was due to the inability of *P. aeruginosa* mutants to utilise glucose in the basolateral compartment and not due to diffusion or active transport or glucose, glucose was applied to the apical side with WT or mutant PAO1. As observed before there was an increase in recovered CFU with WT PAO1 from 0 mM glucose to 20 mM glucose (Figure 3.9A) but mutant strains still displayed a reduction in growth at both concentrations of glucose (Figure 3.9). A further control study was used in an attempt to restore KO growth to the same levels as WT by adding succinate to the apical side with log phase bacteria. This resulted in similar levels of recovered CFU across all strains (Figure 3.9B). An acute respiratory infection is achieved at a P. aeruginosa PAO1 dose of 10^6 CFU/mouse with peak infection 24 hours post inoculation.

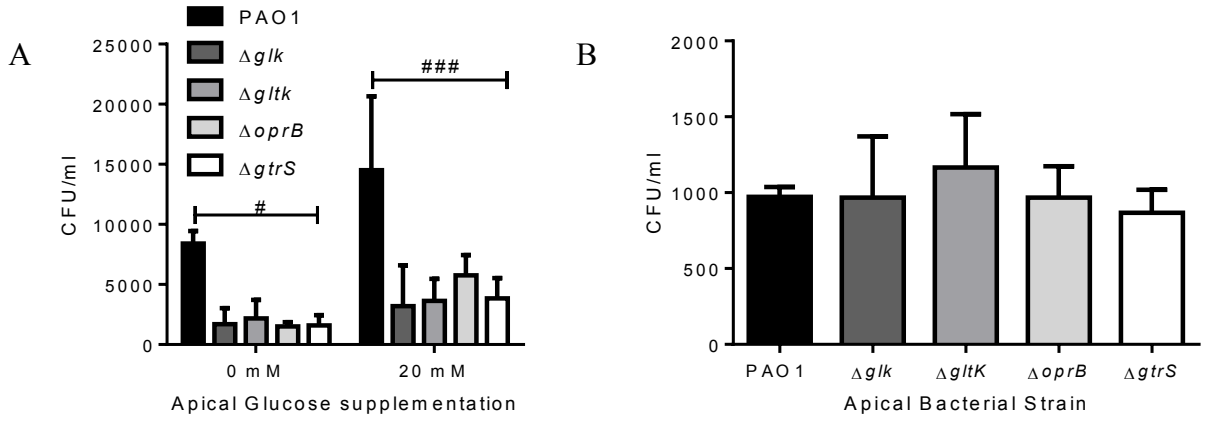


Figure 3.9 Transwell Co-cultures with Apical Succinate or Apical Glucose with Wild type and *P. aeruginosa* mutant strains.

Transwell co-cultures of A549 epithelial cells and 100 µl of log phase 10^5 CFU/ml wild type or knockout mutant strains were incubated for 4 hours at 37 °C, 5 % CO_2 and homogenised for CFU/ml calculations by serial dilution and plating. Instead of basolateral addition of carbon sources, 20 mM glucose (A) and succinate (B) was applied to the apical side with each mutant. N=3. # annotates significant difference between WT and all *P. aeruginosa* mutants by 2 way ANOVA and post-test.

As the role of glucose in vitro was shown to be important in promoting bacterial growth, an in vivo infection model was developed. Firstly, infectious dose titrations were performed using 3 100 µl doses of log phase PAO1, 10^8, 10^7 and10^6 CFU/mouse diluted in sterile PBS but grown in LB broth and confirmed by plating (Figure 3.10). Mice were infected by intranasally using a 200 µl pipette and dropping 100 µl of the infectious inoculum onto the tip of the nose of the mouse. At 10^8 CFU, mice were beyond severity limits as determined by the Home Office license and were culled after 5 hours. After 24 hours, 2 mice of the 10^7 group survived, but were reaching severity limits and one was found dead indicating that this dose was also too high. All mice from the 10^6 group survived and there was a significant difference in weight 24 hours post infection (Figure 3.10A) as well as an increase in CFU recovered from BALF and homogenised lungs (Figure 3.10B, C). There was also an increase in airway cells (Figure 3.10D) and neutrophils (Figure 3.10E, F). As three mice were used per group due to the 3 Rs – reduction, refinement and replacement and as this was a pilot study statistical analysis was not performed.

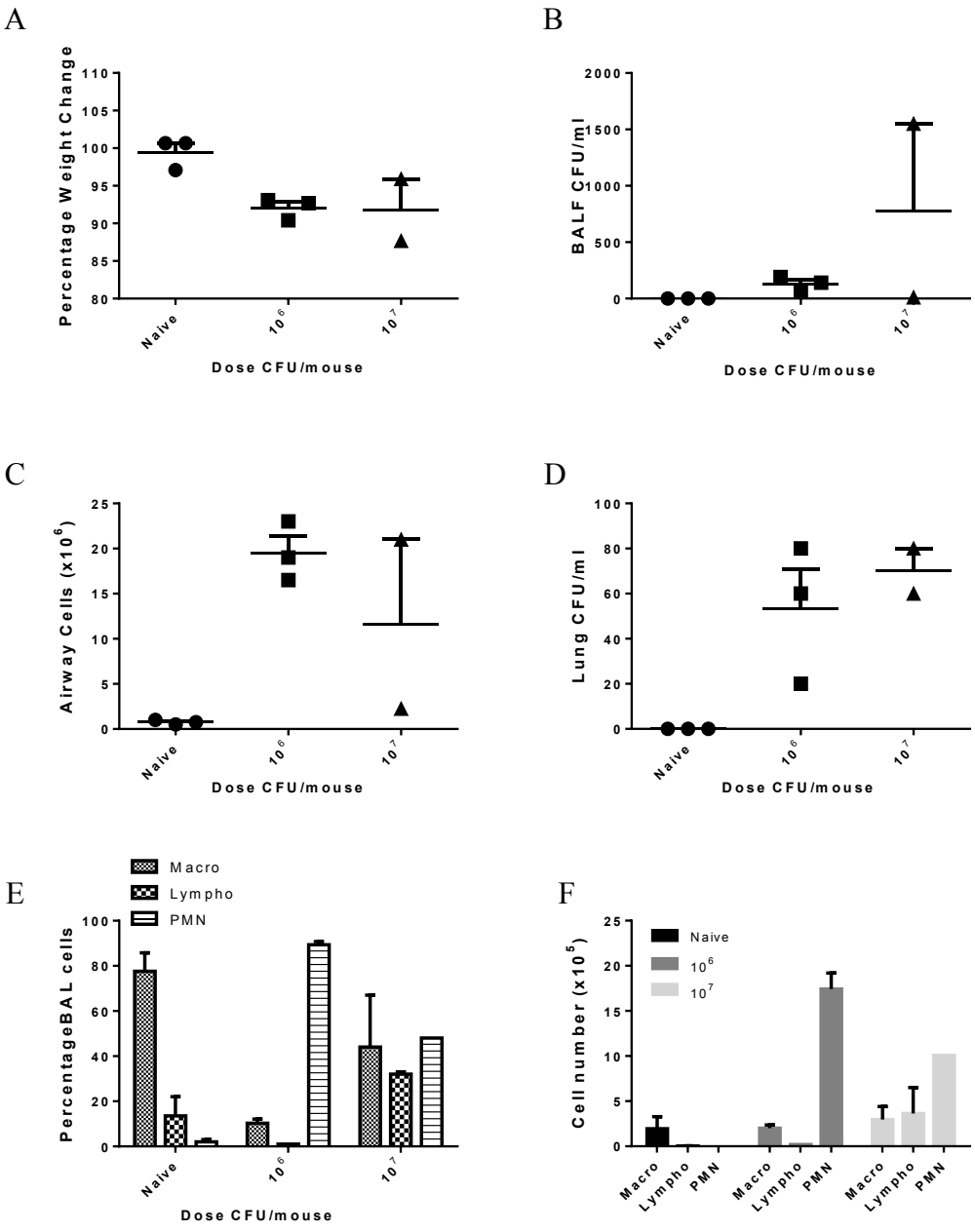


Figure 3.10 PAO1 dose titration

C56BL/6 mice were intranasally infected with 100 µl of PAO1 in log phase at 3 different CFU/ml determined by OD600 and confirmed by plating and overnight incubation at 37 °C. Weight loss was compared to starting weight and 24 hours post infection (A). BALF was used to determine CFU/ml in the lung (B) and airway cells (C). Homogenised lungs were used to determine lung CFU/ml (D). Percentage cell counts were performed by cytospins and H&E staining (E) and used to determine total cell population numbers (F). N=3.

3.8. Effect of bacterial strain on acute infection

As three *P. aeruginosa* strains, PAO1, PAK and PA14 were available for this study, their virulence was compared *in vivo* using the infection model developed and all 3 strains were similar in virulence.

Airway CFU/ml was compared between strains and PAO1 produced plated with viable cell counts (Figure 3.11A). Weight loss was significantly greater with PAO1 compared to PA14 and PAK (Figure 3.11B). All strains had significantly higher airway cells in infected mice compared to naïve mice (Figure 3.11C) and PAK caused a greater influx of neutrophils in the airways compared to other strains (Figure 3.11D). For this experiment, a final concentration of 10^5 CFU/mouse was used to reduce the risk of mortality.

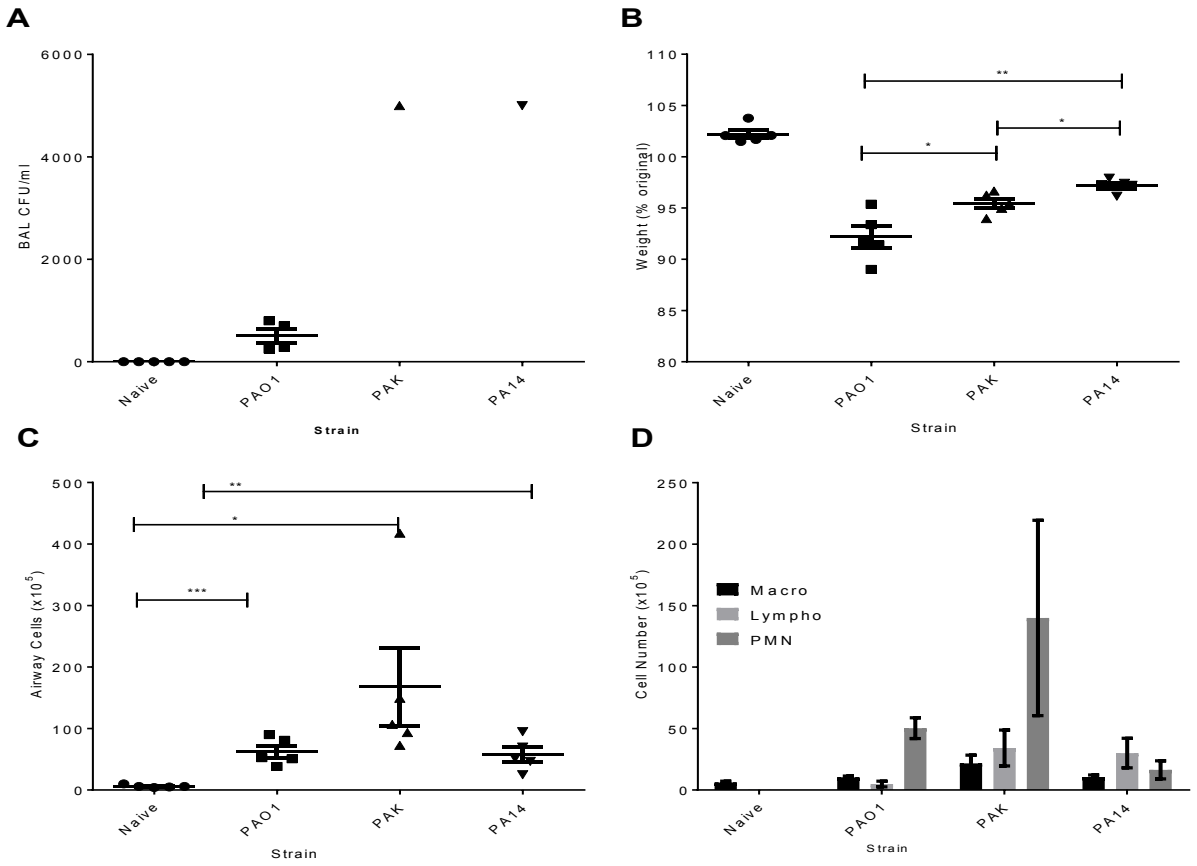


Figure 3.11 *P. aeruginosa* Strain Comparison

C57BL/6 mice were intranasally infected with 100 µl of 3 strains at a final concentration of 10^5 CFU/mouse. 24 hours post infection, mice were sacrificed by injecting 100 µl pentobarbital. BAL CFU/ml was compared between groups (A) and weight loss over 24 hours (B). BALF was used to calculate airway cells (C) and cell population by H&E staining (D). * p≤ 0.05, ** p≤0.01 and *** p≤0.001 by t-test.

Mouse strains were also compared and in line with previous *in vivo P. aeruginosa* infections, C57BL/6 mice were more susceptible than BALB/c mice in an acute infection model based on percentage weight loss, CFU recovery from BALF (Figure 3.12B) and airway cells (Figure 3.12C). Differential cells counts were also compared and showed a reduced influx of neutrophils (Figure 3.12D) compared to PAO1 infected C57BL/6 mice. Heat killed (HK) PAO1 was used as a control to see the effect of delivery as well as to determine if extracellular debris had an effect on immune responses in the acute infection model.

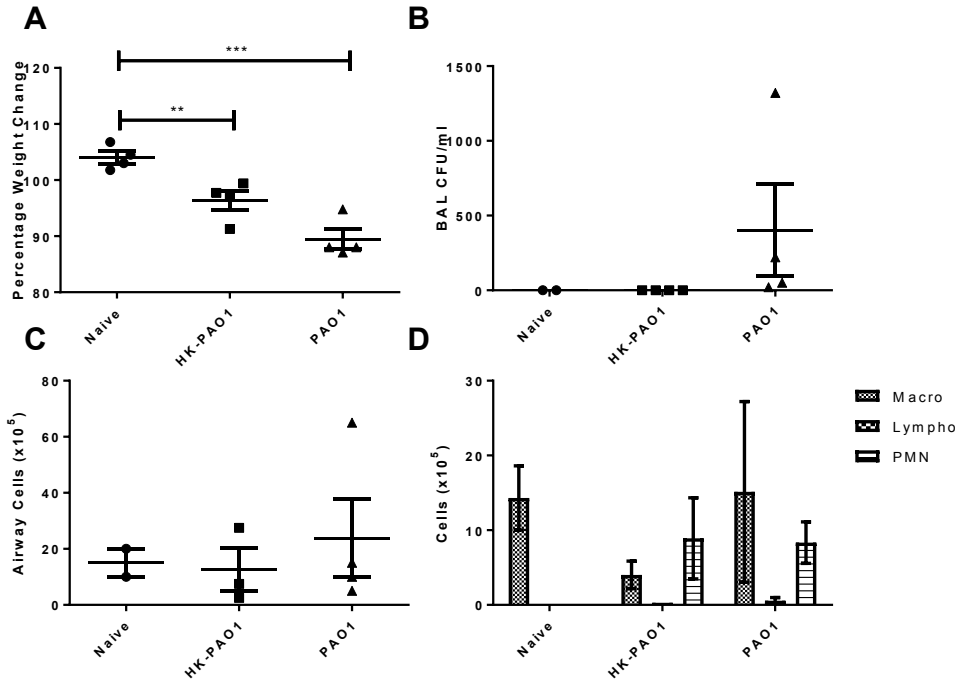


Figure 3.12 *P. aeruginosa* infection in BALB/c mice

BALB/c mice were intranasally infected with 10^6 CFU/mouse of heat killed (HK) PAO1 or live PAO1. 24 hours post infection, mice were sacrificed and percentage weight loss was compared between groups (A), BAL CFU/ml (B). BALF was used to calculate airway cells (C) and cell population by H&E staining (D). * p≤0.05 and *** p≤0.001 determined by t-test. N=5.

3.9. Time Course

In order to determine the kinetics of acute PAO1 infection in mice a time course study was performed over a period of 72 hours. The peak bacterial recovery was 24 hours after infection (Figure 3.13A), and mice were able to clear bacteria 72 hours after infection (Figure 3.13A). Bacterial burden was also compared after 6 hours with 24 hours to determine if 6 hours would be a suitable time point to keep in line with animal severity limits (Figure 3.13B) however, airway cells (Figure 3.12C) and neutrophil responses (Figure 3.13D) were higher after 24 hours compared to 6 hours and mice were able to recover from infection as neutrophil counts were lowest 72 hours post infection (Figure 3.13D).

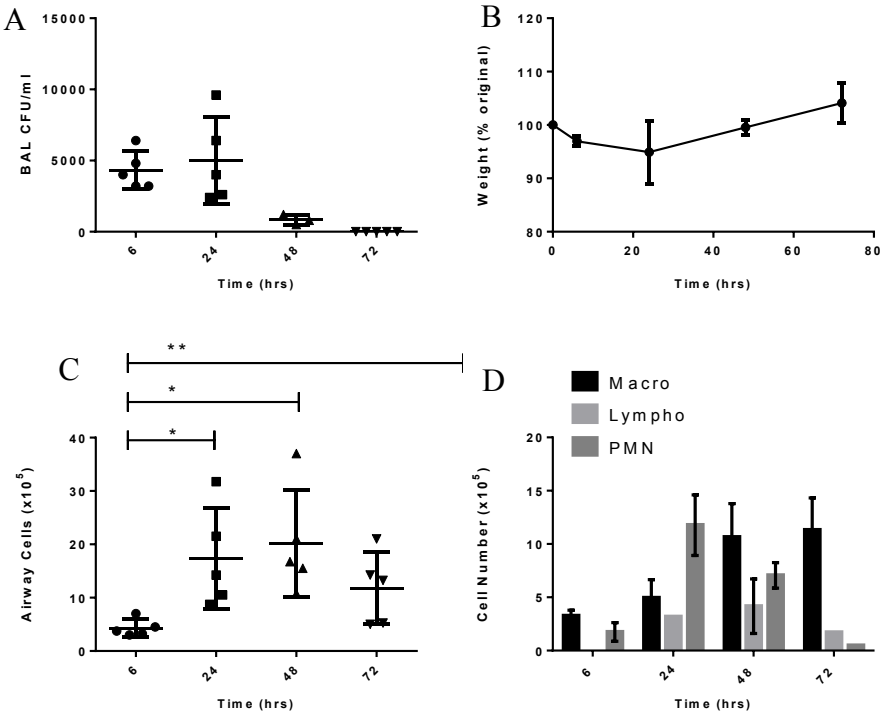


Figure 3.13 Acute infection over 72 Hours

C57BL/6 mice were infected with 10^6 CFU/mouse PAO1 intranasally and culled at 4 time points post infection. BAL was performed to determine airway CFU/ml (A) and percentage weight loss was compared over 72 hours (B). Airway cells counts (C) and population counts (D) were calculated from BALF and H&E staining. * p≤0.05, ** p≤0.01*** p≤0.001 by t-test.

3.10. Infection with live PAO1 induces an acute respiratory infection

To determine if the hallmarks associated with acute respiratory infections were due to *Pseudomonas* infection, naïve mice were compared to mice intranasally given LB medium only, heat killed PAO1 and live PAO1. As expected, the only group with weight loss were mice given live PAO1 (Figure 3.14A) and bacteria were also recovered from the BALF and homogenised lungs in this group (Figure 3.14B-C). There was also an increase in recovered airway cells and neutrophils in this group (Figure 3.14D,E) confirming that infection with live PAO1 is responsible for the observed inflammatory response and infection.

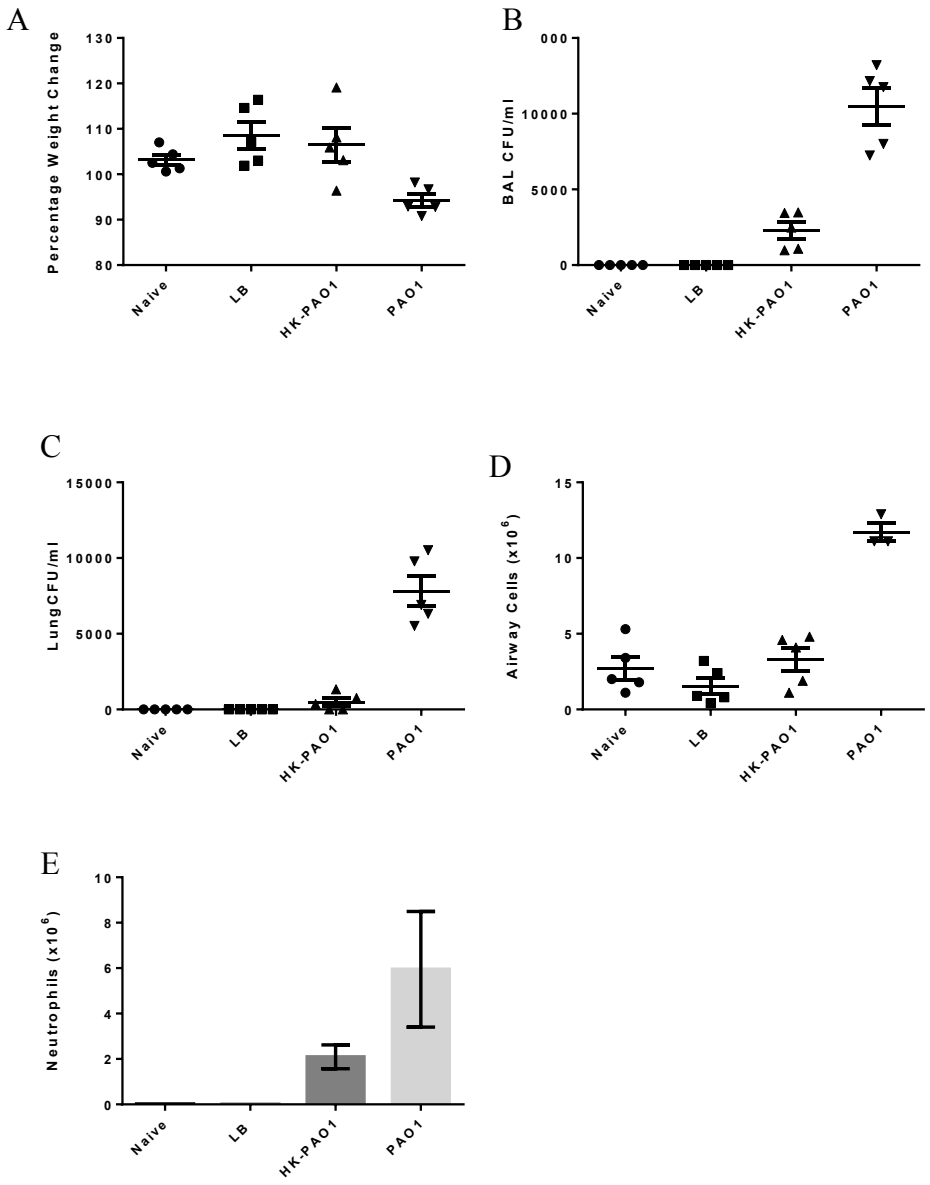


Figure 3.14 Live PAO1 causes acute infection

C57BL/6 mice were infected with sterile LB, heat killed (HK) or live PAO1 at a dose of 106 CFU/mouse intranasally. 24 hours post infection mice were sacrificed by injecting 100 µl IP pentobarbital and percentage weight change was calculated (A). BALF was used to determine airway CFU/ml (B). Homogenised lungs were used to determine lung CFU (C) and airway cells (D) and neutrophil counts (E) were compared.

3.11. Streptozocin induces hyperglycaemia in mice and increases bacterial burden after 24 hours

Once the *in vivo* infection model was established the hyperglycaemic model was developed to determine if glucose promotes bacterial growth *in vivo* as observed *in vitro*. However there were several optimisation steps with glucose and weight monitored (Figure 3.15A). Publications used as guidance suggested 5 mg of streptozocin injected IP would be sufficient to cause hyperglycaemia however after 7 days mice were not hyperglycaemic (Figure 3.15B).

To achieve hyperglycaemia, streptozocin, was injected IP at 0.25mg/g two times, the second dose 7 days after the first dose. Injections were done in the mornings so female C57BL/6 mice could be monitored over the course of the day.

Injection led to an increase in blood glucose after 14 days, this 14 day time point was then used for *P. aeruginosa* infections. The increase in airway glucose was also confirmed (Figure 3.15D). This also is line with the World Health Organisation's classification of hyperglycaemia 2 hours after eating which is 11 mmol/l.

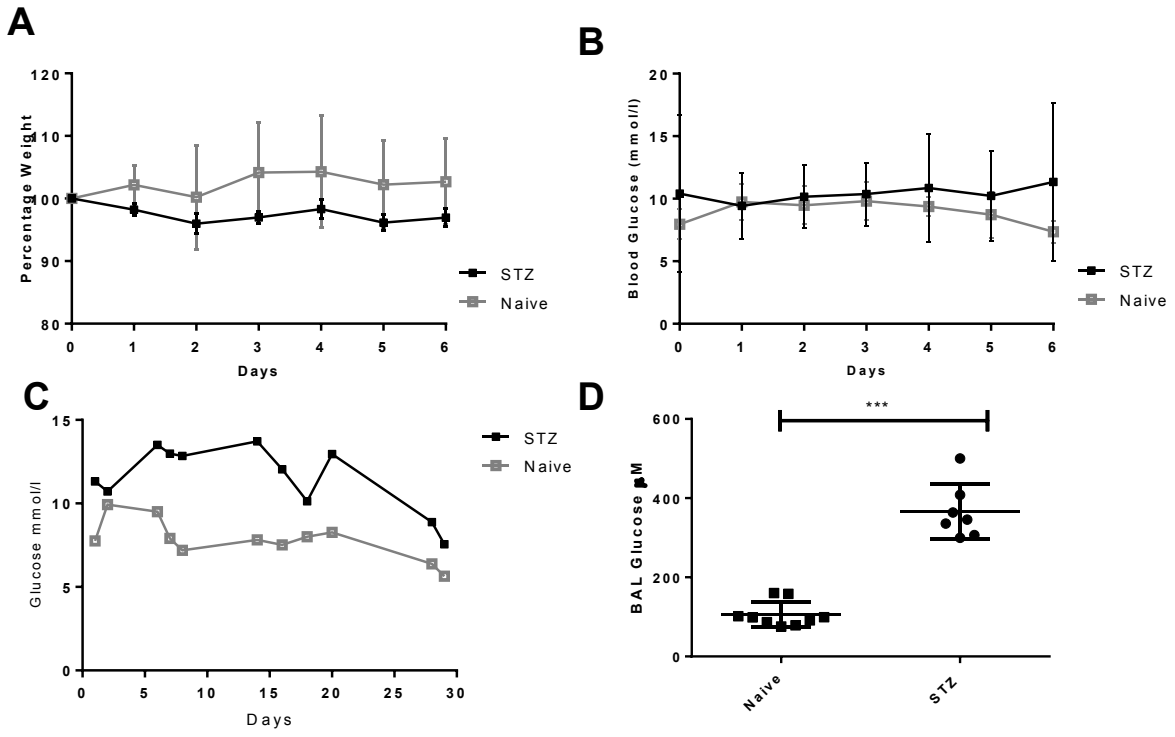


Figure 3.15 Streptozocin injection induces hyperglycaemia

Female C57BL/6 mice were injected IP with 5 mg streptozocin in 100 µl which was equivalent to 0.25 mg/g and weight loss was monitored (A). Blood glucose was recorded over 7 days (B). After optimisation and a second dose 7 days after the first dose, glucose peaked on day 14 there was less overlap with untreated mice (C) which correlated with BAL glucose from mice 14 days post the first STZ dose. (D). *** p≤0.001 by t-test.

3.12. Hyperglycaemia increases bacterial load and duration of infection

To determine if hyperglycaemia has a positive effect on *in vivo* growth, naïve mice and STZ treated mice were infected with 10^6 WT PAO1/mouse. Mice were culled at 24, 48 or 72 hours after which bacterial burden and markers of acute infection were compared. After 24 hours, hyperglycaemic mice had significantly higher CFU/ml from BALF compared to naïve mice (Figure 3.16A), which was also observed at 48 and 72 hours post infection (Figure 3.16A). Hyperglycaemic mice also lost more weight after 24 hours which is used as a measure of infection but this increases up to WT levels at 48 hours (Figure 3.16B). There was also a slight increase in airway cells after 24 hours in hyperglycaemic mice compared to naïve mice (Figure 3.16C) but no difference between groups in neutrophil counts (Figure 3.16D) After culling mice, blood glucose levels showed there was significantly higher glucose in blood taken from mice 24 hours after infection which decreased over the period of 72 hours (Figure 3.16E) and there was little difference BAL glucose levels post infection (Figure 3.16F). There was no difference between naïve and STZ treated mice in after 48 hours in cytokine and chemokine levels suggesting that there was no compromised immune response (Figure 3.16G).

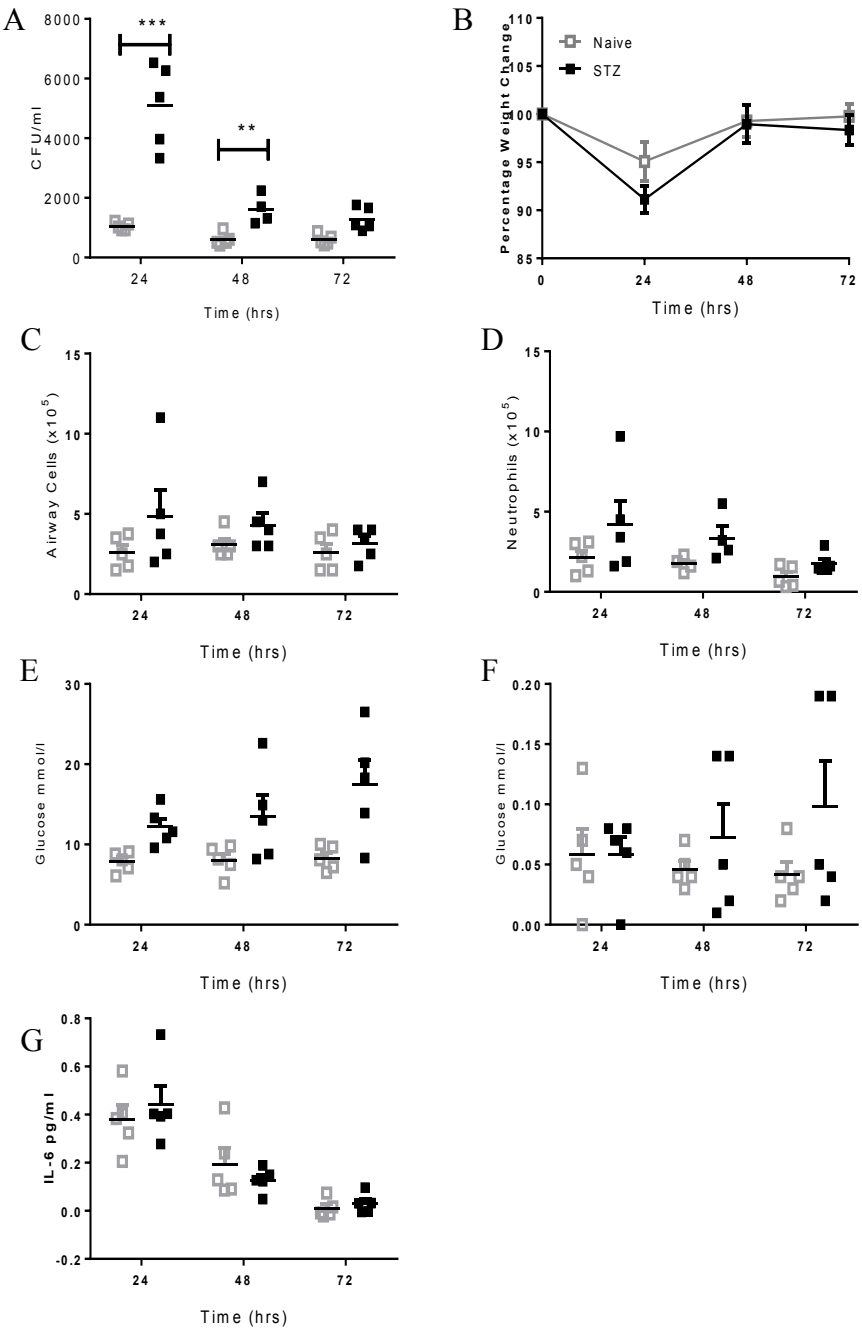


Figure 3.16 Hyperglycaemia increases bacterial burden and duration of acute infection

C57BL/6 mice treated with STZ and infected with 10^5 CFU PAO1 I.N. Infection was followed over the course of 72 hours with BALF (A) and weight loss compared (B) as well as airway cells and neutrophils (C,D). Blood glucose was taken (E) and airway glucose was measured from BALF (F). IL-6 levels were compared by ELISA using BALF (G). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ by t-test. Grey open squares – naïve mice, black closed squares – STZ treated mice.

3.13. Hyperglycaemia increases acute infection in mice infected with WT but not mutant PAO1 strains.

To investigate the importance of glucose directly on bacterial growth in vivo, naïve and hyperglycaemic mice were infected with 10^6 CFU/mouse of WT or glucose uptake / utilisation mutants. After 24 hours mice were culled and severity of infection was compared. As observed previously hyperglycaemic mice infected with WT PAO1 had a significantly higher bacterial burden in the lungs compared to naïve mice (Figure 3.17A,B). This was the opposite for naïve and STZ mice infected with the 4 mutant strains where bacterial burden averaged less than naïve mice although not by statistical significance (Figure 3.17A). This was more evident when fold change in CFU/ml recovered from lung fluid was compared between naïve and hyperglycaemic mice (Figure 3.17B). The same pattern was observed in CFU counts from lung homogenates (Figure 3.17C). For other markers of acute bacterial infection there was no difference in airway cells (Figure 3.17D) and neutrophils (Figure 3.17E) or in immune responses, and KC ELISAs (Figure 3.17F) or IL-6 (Figure 3.17G). Elevated levels of blood glucose were confirmed in infected mice (Figure 3.17H).

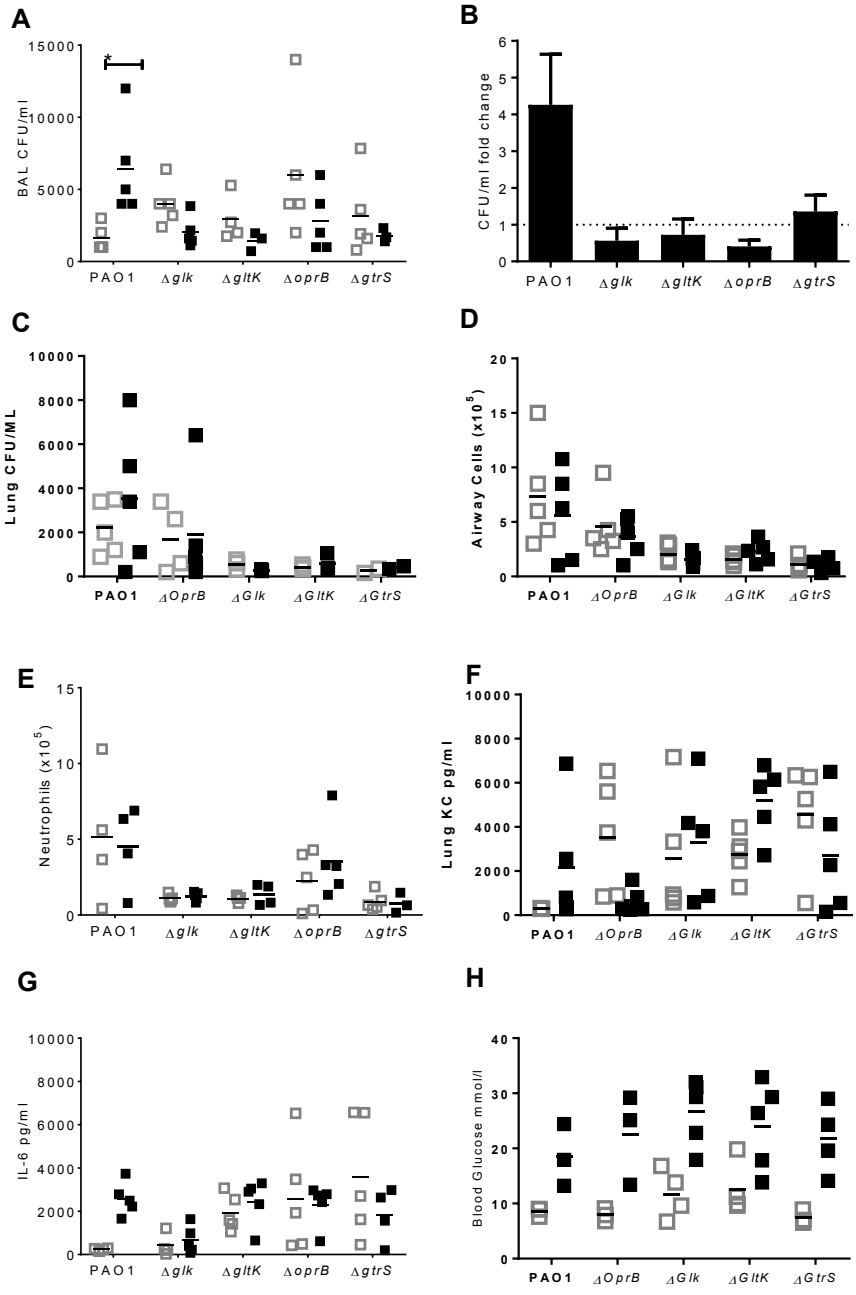


Figure 3.17 Hyperglycaemia does not promote growth *in vivo* of mutant strains

Naïve and STZ treated C57BL/6 mice were infected IN with 10^6 CFU/mouse WT PAO1 or mutant strains of PAO1 and culled after 24 hours. BALF and lung CFU/ml were calculated (A-C). Airway cells were determined from BALF (D) and neutrophil counts (E), lung KC (F), IL-6 (G) and blood glucose (H) measured after infection. n=5 * p=<0.05 by t-test. Grey open squares – naïve mice, black closed squares – STZ treated mice.

3.14. Compounds to reduce bacterial burden and airway glucose levels

The results suggest that glucose uptake or utilisation gene products could be used as novel targets to reduce bacterial burden and acute infection. Identification of genes offers the potential of targets which could be essential for *in vivo* survival and colonisation.

Additionally, targeting glucose in the host rather than bacterial gene products is a potential strategy to combat bacterial growth and infection. As hyperglycaemia induced by STZ increased bacterial burden the effect of administering an anti-diabetic drug after infection with 10^6 CFU/mouse WT PAO1 was assessed to determine if lowering glucose levels lowers bacterial burden. Prior to STZ treatment glucose levels were the same between all mice, however after STZ treatment blood glucose levels started to rise as well as airway glucose levels (Figure 3.18A,B).

There was no difference in disease profile between treated and untreated animals after 24 hours (Figure 3.18C). Hyperglycaemic mice had an increased bacterial burden in the airway (Figure 3.18D) or lungs compared to naïve mice (Figure 3.18E). Mice given insulin, I.P. 0.5 units /kg (10 µl /unit) had a lower recovery of bacteria from BALF than untreated hyperglycaemic mice but this effect was greater in mice given 4 mg/ml of metformin in 200 µl (Figure 3.18D). Lung CFU/ml (Figure 3.18D) and airway cells (Figure 3.18E) were comparable between groups. Mice given insulin had a greater number of neutrophils recovered from BALF (Figure 3.18G) and elevated levels of IL-6 (Figure 3.18H) which was not observed in mice given metformin.

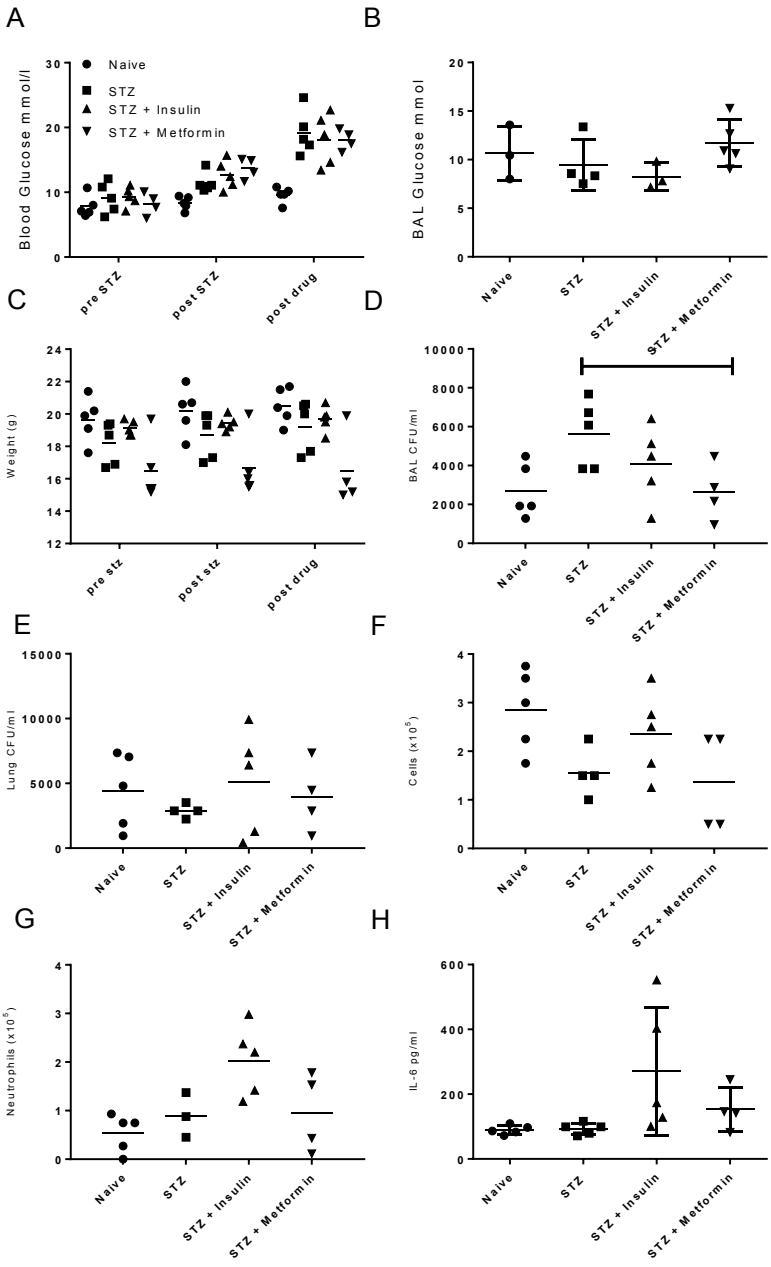


Figure 3.18 Metformin reduces bacterial burden in the lung

Naïve and STZ mice were treated with metformin or insulin. Blood glucose (A) and airway glucose (B) were determined using a commercial glucose kit and the amplex red glucose assay. Weight loss (C) was compared as well as BALF (D) and lung (E) CFU/ml were determined by serial dilution and plating. Total cell numbers (F) neutrophil counts (G) were determined by staining and IL-6 by ELISA (H). Pre STZ 14 days before infection, post STZ 2 days before infection and post drug day of infection. Metformin was administered IP 0.25/g in 200 µl and insulin was injected IP 0.5 units/kg (~10ul/unit). *= $p<0.05$ by t-test. Circles Naïve, Squares STZ treated, upward triangles STZ/ insulin treated, downward triangles STZ/ metformin treated.

3.15. Metformin as an antimicrobial strategy

To further explore the role of metformin, naïve mice, hyperglycaemic mice and metformin treated were infected with WT PAO1. Metformin was delivered I.P. at a concentration of 4 mg/ml in 200 µl. In hyperglycaemic mice not given metformin, there was an increase in blood glucose over the period of 14 days (Figure 3.19A) which is not lowered by metformin. Airway glucose was also compared in naïve mice, mice treated with STZ and STZ treated mice given metformin prior to infection. In contrast to the change in blood glucose, metformin successfully reduces airway glucose (Figure 3.19B) As observed before there was an increase in bacteria recovered from BALF in STZ treated mice and reduction in bacteria recovered from BALF in mice given metformin (Figure 3.19C). There was no difference in neutrophils between groups (Figure 3.19D) and IL-6 levels (Figure 3.19E).

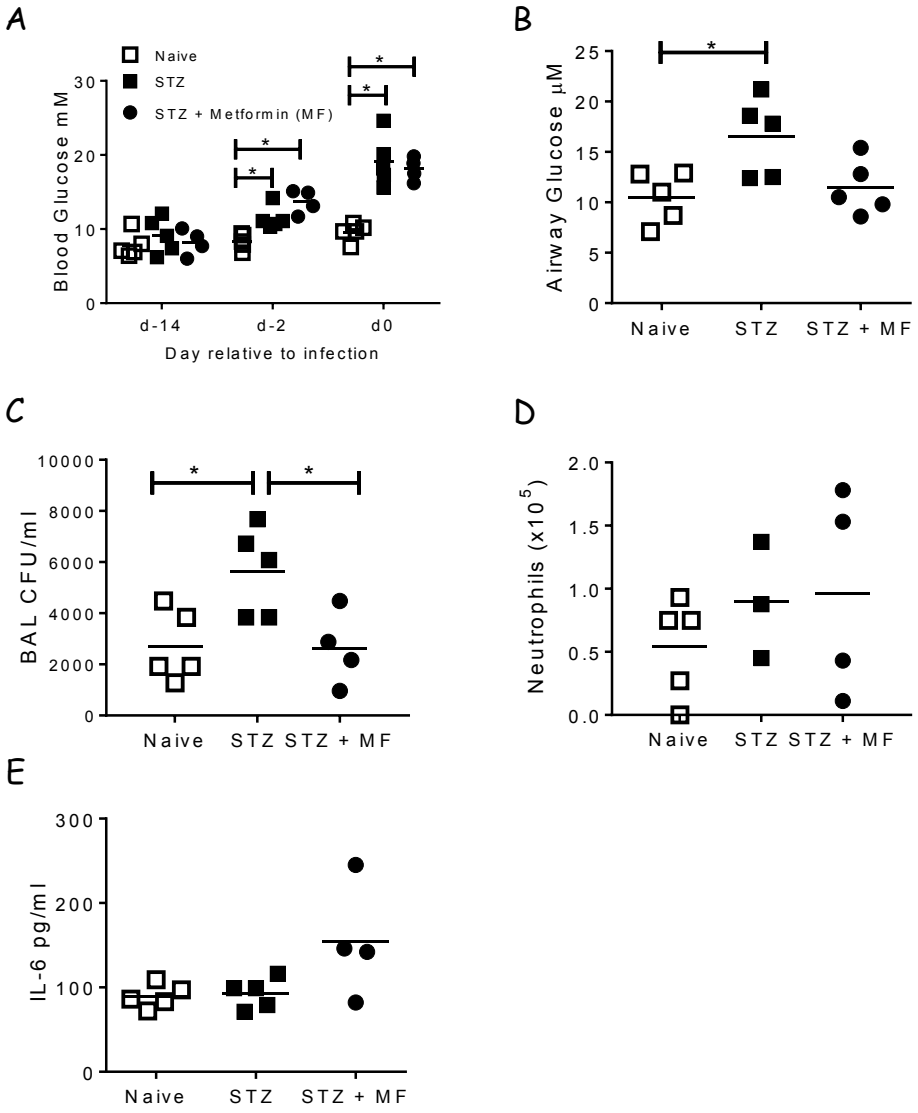


Figure 3.19 A reduction in airway glucose leads to a significant reduction in airway bacterial burden

Blood glucose of naïve, STZ and STZ + metformin mice was monitored over a period of 14 days (A) and airway glucose prior to infection was compared (B) BALF CFU/ml was compared between naïve, STZ and STZ + metformin mice (C). Neutrophil counts were compared by H&E staining (D) and IL-6 by ELISA (E). * = $p<0.05$ by t-test. Open squares – naïve mice, black squares – STZ treated mice, black circles STZ/ metformin treated mice.

3.16. Single Transposon Mutant Library Growth

To identify genes involved in growth in the presence of glucose, a library of single transposon mutants in the PA14 background was made available by Prof Alain Filloux. As this library consists of thousands of mutants, a rapid and efficient screening method of growth was developed to identify potential genes of interest. This involved using growth in 96 well growth plates adapted from a 384 well growth assay Campbell (2011).

3.17. High throughput 96 well screening of Transposon Mutants

To determine if growth in 96 wells would be a suitable mode of growth, a comparison between cultures in falcon tubes, which were previously used for growth and characterisation of mutants generated in this study, and 96 well plates was performed over a period of 8 hours. 96 well plates could be used to screen a greater number of mutants and to identify mutants which display reduced growth in minimal medium supplemented with glucose but not with an alternative source such as succinate. Growth in falcon tubes and 96 well culture plates were similar over a period of 8 hours which was determined by both CFU/ml and OD_{600} values (Figure3.20). This validation suggested that that this method would be suitable for screening growth of a large number of mutants simultaneously under different nutrient conditions. In addition to comparable results between growth methods, these results also support bacterial growth in falcon tubes with adequate aeration and nutrients which was used throughout this study.

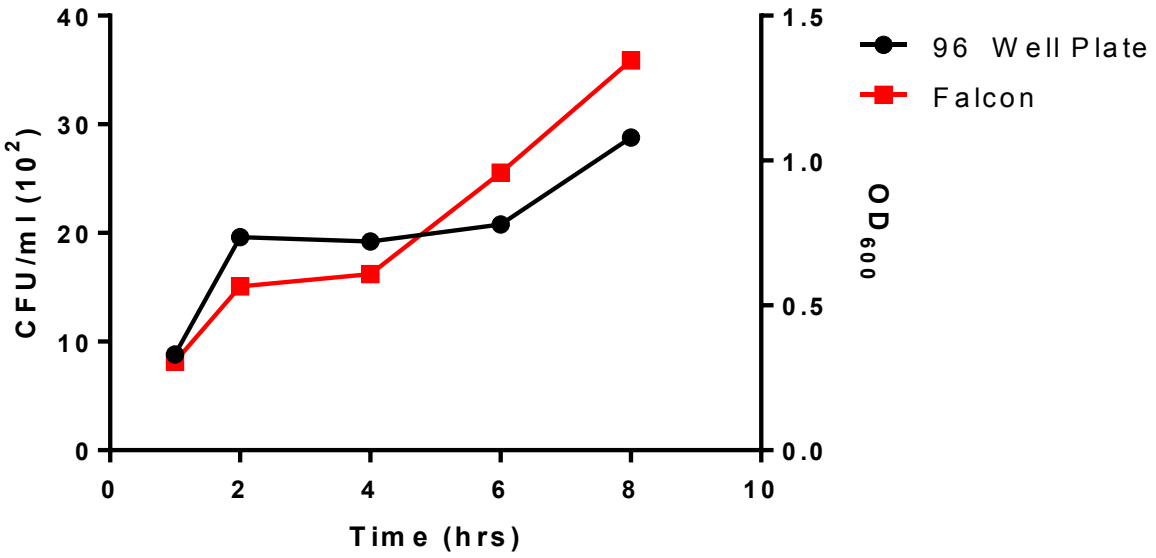


Figure 3.20 200 µl 96 well growth compared to growth in 5 ml cultures Overnight cultures of PAO1 grown in 5 ml LB were used to inoculate fresh LB at a ratio of 1:100 either in a falcon tube with 5 ml LB or in a 96 well U bottom plate with 200 µl LB. Samples were incubated at 37 °C, 200 r.p.m and OD_{600} measurements were taken over the course of 8 hours. CFU/ml was determined by serial dilution and plating on LB plates incubated overnight at 37 °C. n=1

3.18. Transposon Library Growth in Minimal Medium

Single transposon mutants, which were identified from a literature search of genes involved in glucose utilization were obtained from Prof Alain Filloux and used to confirm that the observations of growth in minimal medium were the same for similar mutants in a different *P. aeruginosa genetic* background, strain PA14 as well as to identify other genes of interest.

Mutants included previously published genes such as *zwf* and *edd* as well as a variety of genes hypothesized to be involved in glucose utilisation identified from computational and experimental evidence. Each mutant was grown in minimal media supplemented with glucose and OD_{600} was used as a measure of growth to determine the effect on gene deletion compared to wild type PA14 (Fig 3.21). Of the mutants screened, most displayed similar growth to wild type PA14 or reduced growth in the presence of glucose suggesting that these genes are involved in glucose uptake and utilisation and could be used to identify novel genes of interest as well as assign novel gene function. There were several mutants with no assigned gene annotation, which when deleted displayed an increase in growth in the presence of glucose compared to the wild type further indicating the complex glucose ultilisation process employed by *P. aeruginosa.* These are identified by PA reference numbers from the Pseudomonas database.

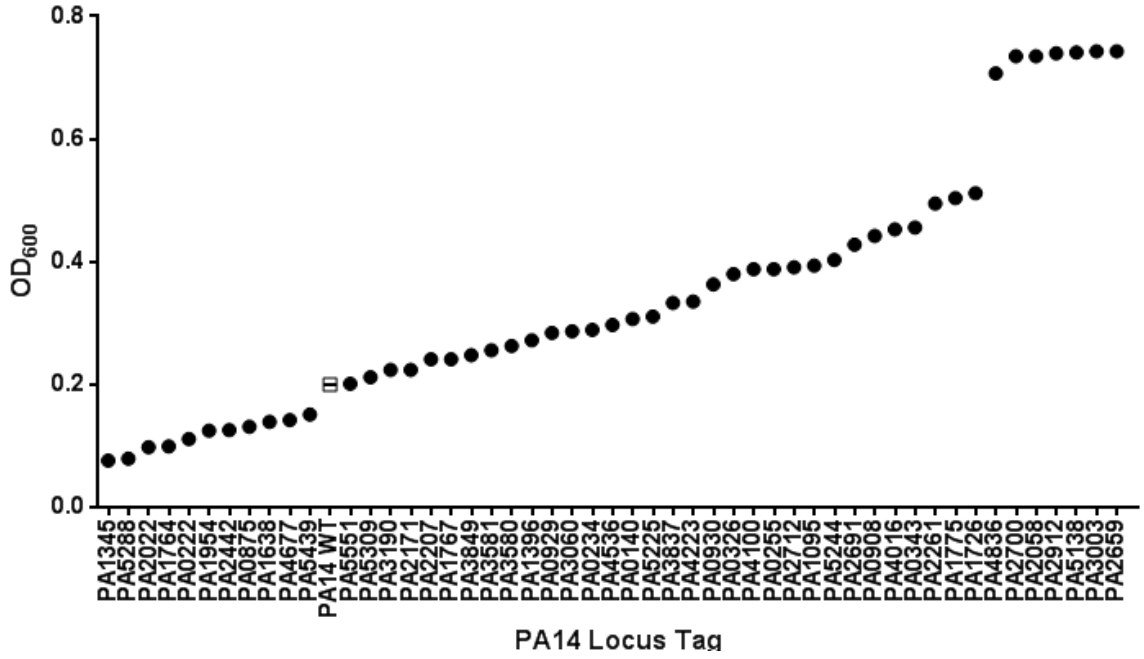


Figure 3.21 Single Transposon Mutant Growth in M9

Single transposon mutants in the PA14 background were grown in minimal medium supplemented with glucose at a final concentration of 20 mM. Cultures were grown at 37 °C, 200 rpm after which optical density was measured determine growth. n=3 average shown.

3.19. Biofilm Formation in the Presence of Glucose

Chronic infection, particularly in CF patients is associated with biofilm formation and it has previously been suggested that biofilm formation is promoted by certain available nutrients specifically glucose. Using the 96 well growth assay, biofilm formation of WT PAO1 was compared in minimal medium with glucose or succinate without shaking to permit biofilm formation.

Figure 3.22 compares CFU/ml on the left y axis with OD of crystal violet after staining, used as a measure of biofilm production, on the right y axis in minimal

medium supplemented with either glucose or succinate to determine if glucose had a greater effect on growth determined by CFU/ml and biofilm production. Optical density (OD_{600}) measurements were used to quantify the amount of biofilm produced by crystal violet staining and as expected there was an increase in CFU/ml and biofilm formation (Fig 3.22), with increasing levels of glucose compared to succinate, suggesting that in this model limiting available glucose could reduce biofilm formation and therefore reduce the severity of chronic infection. However after 8 hours of growth, the difference in CFU/ml and biofilm formation determined by crystal violet was negligible, suggesting that biofilm formation is more complex than the scope of this experiment.

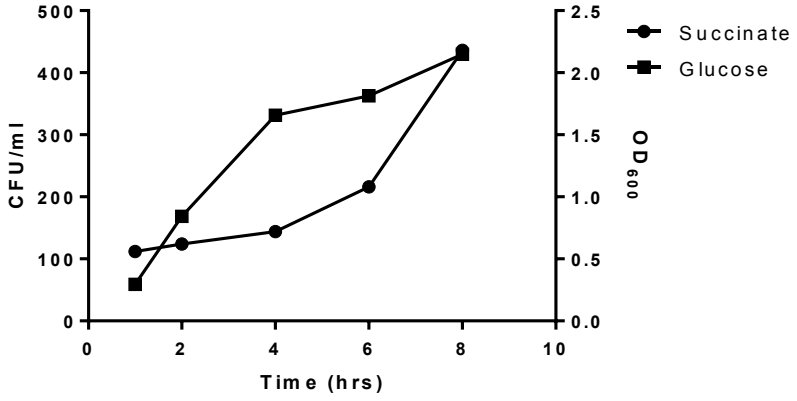


Figure 3.22 Biofilm formation in the presence of glucose

Overnight cultures of PAO1 grown in LB was used to inoculate M9 medium 1:100 and supplemented with glucose or succinate (50 mM) and cultures were grown at 37 °C in stationary culture. Cultures were dried and stained with 0.01 % crystal violet and incubated at room temperature for 15 minutes. Biofilms were washed, and dried at room temperature and crystal violet was resuspended using 30 % acetic acid and OD_{600} and CFU readings were compared.

3.20. Clinical Isolates from Diabetic and non-Diabetic Cystic Fibrosis Patients

P. aeruginosa is commonly isolated from CF patients and is the dominant species in chronic infections. Through collaboration with Prof. Jane Davies (Imperial College London), clinical isolates from CF patients were available from the Royal Brompton Hospital and from patients who were diabetic and non-diabetic. 35 *P. aeruginosa* strains from non-diabetics and 23 *P. aeruginosa* strains from diabetics were grown in M9 minimal medium supplemented with glucose and LB in a 96 well format to allow rapid testing of *P. aeruginosa* strains.

For these strains the only available data was diabetic status therefore a detailed analysis on the demographics of the patients from whom the strains came from was not possible. However from the data, there was no pattern or trend between isolates taken from diabetic or non-diabetic patients compared to WT *Pseudomonas* for either growth (Figure 3.23A) or biofilm formation (Figure 3.23B) indicating that these clinical isolates did not preferentially grow in the presence of glucose compared to the laboratory strains PAO1 and PA14 using this model.

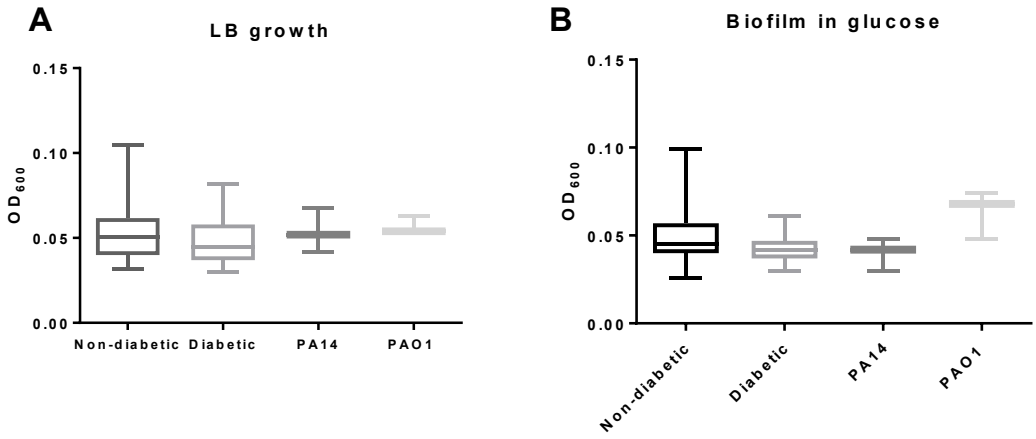


Figure 3.23 Clinical isolates displayed similar growth and biofilm formation despite diabetic status

Strains were grown in LB in a 96 well for 8 hours at 37 °C with shaking for growth in rich LB medium (A) or M9 minimal medium in 96 well culture plates overnight for biofilm staining with crystal violet, which was resuspended in 30 % acetic acid and quantified by OD_{600} (B).

4 Discussion

Overall the data from this thesis supports the hypothesis that glucose is a critical factor in airway bacterial growth and that elevated glucose seen in hyperglycaemia leads to elevated bacterial loads. I will now discuss some of the issues raised by this work.

4.1. Mutant Strains of P. aeruginosa

Deletion mutants of *P. aeruginosa* were generated, using the background strain PAO1, prior to access to mutant libraries in the PA14 library. PAO1 has an easily accessible published genome (Winsor et al., 2011). PAO1 is a strain of *P. aeruginosa* originally isolated from a wound (Holloway, 1955) and is the most widely used *P. aeruginosa* lab and reference strain. It is also the strain which was used to provide the first complete genome of *P. aeruginosa* and is capable of using a wide range of nutrients including glucose which was highly relevant for this project (Stover et al., 2000).

From previously published studies and genes sourced from references on the online database, this study identified four genes, which are theoretically essential for growth in the presence of glucose. Two genes previously associated with glucose were *gltK* and *oprB* (Wylie and Worobec, 1995) but there are no published data for *glk* mutants. Additionally where *gltK* and *oprB* mutants were shown to be important for growth in the presence of glucose in growth assays these mutants had not been tested *in vitro* or *in vivo.*

Previous publications have identified *grtS* as an activator of T3SS as well as having a role in host responsiveness and environmental sensing (O'Callaghan et al., 2012,

Lewenza et al., 2005. However in all previous publications with the four mutants used in this study, none had a comparison of bacterial growth in a hyperglycaemic environment which this study used to identify the role of the gene on glucose uptake and utilisation.

The four mutants generated in the initial stages of this project were suitable for use in the *in vitro* and *in vitro* models as there was no attenuation of mutant growth when alternative nutrients were available and mutants in nutrient rich environments were able to grow to comparable levels to the wild type suggesting that gene deletion did not affect overall ability to grow.

However one major caveat of the study is that the mutants were not complemented where the gene would be inserted into the mutants created. This would have confirmed that the observed attenuation of growth of mutants in the presence of glucose was due to gene deletion and further supported the role of the selected genes in glucose uptake and utilisation.

4.2. Effect of glucose on bacterial growth in vitro

This project tested four mutants in an *in vitro* co-culture model which previous studies have not done as robustly. For instance, Pezzulo et al. (2011) hypothesised that glucose restriction contributes to airway sterility by limiting bacterial growth and used *in vitro* co-cultures using wild type and a mutant strain. However, Pezzulo et al. (2011) did not perform a bacterial growth kinetics study with the *edd* mutants alone. The previous study demonstrated through the use of glucose transport inhibitors *in vitro* that glucose flux has a role on bacterial growth and glucose promotes apical growth in the same manner as this project. This thesis shows with greater specificity

that glucose directly promotes bacterial growth and supports previously published data where bacteria are able to utilise glucose transported across the epithelial layer to promote growth (Pezzulo et al., 2012).

However, this could be confirmed using radio labelled [C^{14}] glucose to measure intracellular levels of glucose in mutants to confirm reduced uptake and to determine transport of glucose across the epithelial layer in a similar manner to the study performed by Garnett et al. (2013).

The co-culture model used in this study supported previous publications that glucose in the basolateral compartment is transported to the apical side due to an increased gradient, which cannot be adjusted for by epithelial uptake (Kalsi et al., 2009b). Previous studies have shown that the excess glucose promotes wild type bacterial growth, which causes damage to the epithelial layer and increases the amount of available nutrients. The co-culture data also supports the hypothesis of a cycle of cell damage caused by bacterial growth due to elevated glucose levels which is able to pass to the apical side, although as discussed previously, this was not studied in detail in this project. The cycle of 'cell damage promoting bacterial growth hypothesis' could explain why glucose did not promote apical recovery of mutants which was significantly less compared to the WT suggesting that WT is able to cause cell damage resulting in increased paracellular diffusion leading to more glucose on the apical side. The results from the *in vitro* studies also support previous observations which suggest ASL with low glucose levels does not support bacterial growth alone and other nutrients are required for growth and in this case this was glucose.

The inability of mutant strains to grow in the presence of glucose was confirmed by the lack of bacterial growth following the addition of glucose to the apical side of the transwell co-culture. Only when succinate added to the apical side was mutant growth restored to WT levels which demonstrates that even when glucose was directly available on the apical side, mutants were not able to utilise it as a carbon source and the observed reduction in *in vitro* growth was not a growth defect. Taken as a whole, the data further supports the hypothesis that excess glucose promotes bacterial growth.

The co-culture model developed in this study used airway cells and bacteria supplemented with glucose to determine how glucose supports bacterial growth. The importance of glucose for host responses has been explored previously but how glucose could affect the host response and potentially contribute to a detrimental immune response to *P. aeruginosa* infection was not performed in this study. A triple cell culture with the addition of neutrophils or macrophages could have been performed to better understand the host requirement for glucose and the effect of hyperglycaemia on host cell function as well as determining what effect a hypoglycaemic environment could have on host responses which could potentially be induced by drugs used to lower glucose as an alternative antimicrobial strategy.

Tight junction regulation also plays a role in inflammation, which has been demonstrated previously through tight junction regulation and is known to play a role in host defence by limiting transport of glucose into the airway surface liquid. The role of tight junction regulation was highlighted by Pezzulo et al. (2011) in a study suggesting that by limiting glucose in the airway surface liquid, bacterial growth would be limited. However patients who are at risk from *P. aeruginosa* infection,

such as CF patients, patients with COPD as well as obese hyperglycaemic individuals, are more likely to have deregulated tight junctions and epithelial integrity contributing to excess available nutrients in the lungs (Hallstrand et al., 2014).

The hypothesis of a vicious cycle was first suggested by Cole (1989) where bacterial colonisation leads to an inflammatory response of bacteria which damages the lung leading to further infection and lung damage and could explain the observed increase in bacterial growth due to paracellular flux of glucose. However the effect of secreted bacterial toxins, such as Exotoxin A leading to damage to the epithelium could also contribute to increased paracellular flux of glucose. Previous studies have demonstrated that strains expressing the type three secretion system and exotoxin A producing strains have increased virulence which causes host cell death by apoptosis (Miyazaki et al., 1995) and coupled with an increased bacterial burden due to excess nutrients this could cause greater lung damage.

The *in vitro* co-culture was developed using previously published techniques and adapted using A549 cells which were suitable to use to study the interaction between airway epithelial cells and *P. aeruginosa* (Hermanns et al., 2004), however there were limitations to this study. For example, the use of primary cell cultures could have provided a better model of airway epithelial cell (Wu et al., 2005) and *P. aeruginosa* interaction however extensive model development, optimisation and obtaining primary cells would have been time consuming and for this reason primary cells were not used. Additionally, to support previously published data demonstrating

a clear link between CF and *P. aeruginosa* infection, the use of a CFTR cell line as an *in vitro* model of infection would have been useful to determine the role of glucose and CF on *P. aeruginosa* growth (Stanton et al., 2015, Trinh et al., 2015)

4.3. Effect of glucose on bacterial growth in vivo

Murine lung infection models have greatly increased the understanding of pathogenicity of *P. aeruginosa* but have mostly focused on the chronic infection model using bacteria embedded onto the surface of agarose beads which serves as an immobilising agent (Ding et al., 2012). This model can be used to study long term infections because PAO1 is rapidly cleared from the lungs during acute infections but artificial chronic infection models using beads do not represent a realistic model of human infection. In addition to this, the models used supported previous studies demonstrating the suitability of the mouse strain used (Tam et al., 1999). Acute infection models have been used to explore the early events of *P. aeruginosa* infection and the role of virulence toxins produced by *P. aeruginosa* (Mijares et al.,2011, Sullivan et al., 2015).

The *in vivo* hyperglycaemic model was designed to determine the role of glucose in acute infection. The acute infection models that have been used previously include intranasal delivery of *P. aeruginosa* and involve the analysis of the hallmarks of pulmonary infections including weight loss, airway cell counts and bacterial burden in the airways and lung which were the markers used in this study Liu et al. (2011). As discussed in relation to the *in vitro* data, Pezzulo et al. (2011) performed *in vivo* infections using leptin receptor deficient *db/db* and leptin deficient *ob/ob* mice where hyperglycaemia promoted wild type but not *edd* mutant *P. aeruginosa* growth.

However, the *in vivo* model involved an acute 6 hour time course of infection whereas this thesis shows peak infection at 24 hours. There are also complications with using leptin receptor and leptin deficient mice which is discussed in greater detail later. Additionally, the volume used in the Pezzulo study may not have been sufficient to fully reach the lungs as control mice for each group had no bacterial recovery from the lung, whereas in the current study bacteria was recovered from the lungs of wild type animals.

The *in vivo* infection models described in this study support the rapid clearance of *P. aeruginosa* after 72 hours which has been observed previously. The development of this model shows that intranasal administration of 10^6 CFU/C57BL/6 mouse in 100 µl was suitable as there was good recovery of bacteria from the lungs after this time with no associated mortality. In addition to this, the model reflects the acute stages of infection as hallmarks – influx of neutrophils, high cell counts from BALF and weight loss were observed after 24 hours.

In contrast to the acute 24 hours infection model used, a chronic infection would have been unsuitable for this study as agar beads hypothetically could contribute to damage to the lining of the lung and cause an influx of glucose into the airways unrelated to bacterial damage which would therefore skew the observations. Additionally, the chronic bead model of infection is artificial and therefore bacterial clearance in mice would have been more difficult to compare between naive and hyperglycaemic mice (Heeckeren et al., 1997).

4.4. Effect of Hyperglycaemia on Respiratory Bacterial Infection

There have been numerous models of hyperglycaemia to study diabetes as well to study complications associated with diabetes and replicate type 1 or type 2 diabetes (King, 2012). Streptozocin was selected as the drug to induce hyperglycaemia as it has previously been used to study chronic wound infections in diabetes (Goren et al., 2006).

Publications exploring bacterial infection in diabetes model type 2 diabetes using *ob/ob* mice which lack the hormone leptin (Zhang et al., 1994) or *db/db* mice which lack the leptin receptor (Lee et al., 1996). Mice which lack leptin or the leptin receptor rapidly become obese but also display characteristics associated with type 2 diabetes such as hyperglycaemia, increased plasma insulin and an intolerance to insulin (Wang et al., 2014).

An advantage of using leptin or leptin receptor deficient mice is that the effect of obesity over a long period can be explored and as well as the similarities with human type 2 diabetes. Therefore leptin and leptin receptor deficient mice can be used to explore complications associated with obesity leading to a greater understanding of how obesity and type 2 diabetes are risk factors for infection. However as the use of *db/db* or *ob/ob* mice has its limitations as immune complications such as supressed innate and acquired immune response have been associated with *ob/ob* mice (Anstee and Goldin, 2006) which would complicate the data. Leptin has numerous functions and regulates food intake, glucose uptake by cells as well as influencing inflammatory immune responses. For example, in mouse

models it has been demonstrated that leptin increased the production of IFN-γ which suggests that mice which lack leptin or the leptin receptor could have an impaired innate immune defence (Lord et al., 1998). It has also been demonstrated that leptin deficiency in mice leads to a decrease in lymphocytes and monocytes and increased susceptibility to LPS (Faggioni et al., 2000) adding to a more complex animal model with immune deficiencies.

Additionally, hyperglycaemia in type 2 diabetics in humans is not caused by leptin or leptin receptor deficiency so using *ob/ob* or *db/db* mice adds another factor to consider when directly assessing the role of glucose on bacterial growth.

In light of the complications associated with leptin deficiency, it was necessary to develop an alternative to use instead of *ob/ob* and *db/db* mice to exclude genetic defects, complex intracellular complications associated with leptin deficiency and underlying difference between mice and humans when determining the role of glucose on bacterial growth *in vivo*. The use of streptozocin was more favourable than using *db/db* or *ob/ob* mice for the reasons listed above but it also was more cost and time effective as well as being more convenient for experimental design. The model developed was also more appropriate to directly assess the role of elevated glucose in airways on bacterial growth in the lungs alone. Streptozocin acts by targeting β islet cells of the pancreas and is transported into the cells by GLUT2 transporters (Szkudelski, 2001, Schnedl et al., 1994) and acts only on the production of insulin which eventually leads to hyperglycaemia. The *in vivo* hyperglycaemic model developed in this study involved 2 injections of STZ and development of the hyperglycaemic model

involved several optimisation steps as a single dose was not effective in inducing hyperglycaemia. In addition to this, there were animal ethics issues associated with a single 10 mg dose of STZ with high mortality rates which was consistent with the literature where hyperglycaemia was unstable in mice and multiple low doses induced steady state of hyperglycaemic (Ventura-Sobrevilla et al., 2011). The final method used to induce hyperglycaemia in this study resulted in acute hyperglycaemia and no deaths or mice which exceeded the severity limits.

A recent review article highlighted the difficulty associated with the development of new hyperglycaemic models using STZ (Deeds et al., 2011) which supports the optimisation during the early stages of this project. Additionally high single doses contributed to high levels of mortality during the development of the hyperglycaemia model in this study and this was not in line with the 3 Rs – reduction, refinement and replacement which are followed as part of Home Office Animal Licensing regulations so methods with the lowest mortality were used.

Streptozocin can induce a type 1 and type 2 diabetic phenotype and after optimisation, 2 doses were sufficient to induce hyperglycaemia without any other side effects. C57BL/6 mice need approximately 200 mg/kg dose of STZ (Sakata et al., 2012) as a guide this dose was followed but required extensive dose and drug preparation optimisation to reduce mortality as discussed.

The use of streptozocin to develop a hyperglycaemic *in vivo* model was advantageous as hyperglycaemia was acute in comparison to *db/db* or *ob/ob* models where hyperglycaemia is sustained for a longer period of time which could contribute to morbidity. Additionally, genetically hyperglycaemic mice used in previous

publications exploring the relationship between hyperglycaemia and bacterial infection are phenotypically similar to type 2 diabetics and so have the associated obesity and immune defects. The use of STZ to induce hyperglycaemia accounts for this as mice became hyperglycaemic approximately 7 days prior to infection and were not obese which is known to contribute to immune defects. The data presented in this study demonstrates that glucose in the airways drives *P. aeruginosa* growth and this supports previous publications on the associated risk between hyperglycaemia and bacterial infection.

The effect of hyperglycaemia on the immune system has been discussed in detail in the introduction and the advantage of the STZ model in this study was that the immune complications associated with genetic diabetes and obesity related diabetes does not impact on determining the role of glucose**.** This study demonstrated that STZ increased blood and airway glucose, which promotes wild type bacterial growth in the airways, and the acute infection model used in this study demonstrated increased bacterial burden in the lung as well as reduced clearance compared to naïve mice. Wild type and glucose uptake and utilisation mutant bacterial growth was similar in untreated mice but the same increase in recovered CFU/ml from hyperglycaemic mice was not observed with mutant strains. This confirms the *in vitro* observations that the deletion of the 4 genes (*glk, gltK, gtrS* and oprB) does not cause global growth defects and instead only attenuates growth when there is an excess in available glucose.

Analysis of immune responses shows that IL-6 levels were comparable between naïve and STZ mice as well as neutrophil responses. Interestingly, STZ treated mice have elevated levels of KC/CXCL1, the mouse analogue of IL-8 (CXCL8), which is

even greater in mice infected with mutant strains compared to WT. This could suggest that the mutants, like the wild type, trigger an IL-8 response, but mutants are unable to use the excess glucose in the airways so there is less bacterial growth meaning fewer neutrophils are recruited to the site of infection. The excess in glucose could also lead to impaired neutrophil function and could also explain the low levels of neutrophils recovered from the lung. This relates to the immune defects associated with diabetes and hyperglycaemia as discussed in detail in the introduction (Geerlings and Hoepelman, 1999).

Previous data has demonstrated that a reduction in glucose by insulin to restore normal glucose levels resulted in reduced mortality in ICU patients. The majority of these studies focus on aberrant immune responses in hyperglycaemia whereas this study used hyperglycaemic mice with no apparent difference in the immune responses providing a stronger link between glucose and bacterial infection. However the effect of deletion of *glk, gltK, gtrS* and *oprB,* genes on virulence was not assessed in this project and gene deletion could cause a change in gene expression and the secretion of virulence toxins or expression of virulence genes. This would be an important factor to consider when determining the role of glucose in acute bacterial infection.

Overall, these studies support the idea that airway glucose is a critical determinant in bacterial lung infection.

4.5. Anti-Diabetic Drugs as Antibacterial Agents

This study identified 4 genes and the associated gene products which could potentially act as targets which when blocked can attenuate bacterial growth in

hyperglycaemia. By identifying glucose as a key driver of bacterial growth, targeting bacterial glucose uptake or utilization could offer novel treatment alternatives for bacterial infections of hyperglycaemic patients, with *P. aeruginosa* but potentially also with other bacterial pathogens.

The *in vivo* model developed was used to test whether anti-diabetic drugs could reduce bacterial growth and therefore act as novel antimicrobial drugs against respiratory bacterial infections. Though the direct effect of metformin on *Pseudomonas* growth needs to be assessed, previous studies have shown that it has no effect on *S. aureus* growth (Garnett et al., 2013).

In healthy people, insulin is released by the pancreas in response to increased blood glucose levels but is not produced by people with type 1 diabetes and people with type 2 diabetes are insulin resistant. Insulin is typically given to type 1 diabetics and metformin to type 2 diabetics when lifestyle changes are not effective (Nathan et al., 2009). Metformin is the most commonly prescribed drug for type 2 diabetics and acts to lower blood glucose as well as increasing insulin sensitivity (Witters, 2001). Using the hyperglycaemic *in vivo* model developed in this project, insulin and metformin were given to mice with the intention of reducing bacterial burden in the lung. Insulin and metformin did not lower blood glucose in this study but airway glucose was reduced in hyperglycaemic mice given metformin. In this first study comparing insulin and metformin administration on bacterial lung infection; only metformin had a significant effect on reducing bacteria recovered from the airways of hyperglycaemic mice. Insulin use in this study was therefore discontinued due to the fact that dosing of mice was not as safe as metformin, due to the risk of hypoglycaemia. Previous use of insulin in a diabetic wound model of infection tested the impact of insulin

administration on wound resolution in chronically infected mice and found that although insulin improved the diabetic status of mice it did not contribute to wound healing or bacterial clearance (Watters et al., 2013) support the use of metformin instead of insulin in this thesis. This was not the expected result and could be due to several factors. In addition to this, the results from this model show that insulin did not lower blood glucose or airway glucose which could be due to only one insulin dose suggesting that the experimental dose of insulin in this study was not sufficient to be effective. The use of insulin was expected to result in lower glucose levels in the blood and airways, which in turn would result in a lower bacterial burden. However as this was not the case the use of insulin was discontinued.

Metformin activates adenosine 5'-monophosphate-activated protein kinase (AMPK) (Zhou et al., 2001) which is activated when energy stores are low (Hardie, 2011) and in addition to reducing airway glucose, metformin is known to influence the immune response through the activation of AMPK which leads to more alternatively activated M2 macrophages which contributes to tissue repair (Kato et al., 2010). Taken together the data suggest that the reduction in bacterial burden with metformin treatment in this study could be multifactorial with a positive impact on the resolution of inflammation leading to less epithelial damage to the lung and could explain the observation that insulin did not reduce bacterial burden in this study

4.6. Clinical Isolates

Clinical isolates of *P. aeruginosa* from CF patients were used to determine if there were distinct growth characteristics dependent on diabetic status of the host. There were no differences between isolates or lab isolates when grown in LB, however a

slight trend observed in biofilm formation suggests that clinical isolates from diabetic patients display a tighter growth phenotype and there is a greater spread the biofilm formation in isolates from non-diabetics. This is most likely due to the complex adaptation process which *P. aeruginosa* undergoes during chronic infection in the CF lung (Hogardt and Heesemann, 2013) which again highlights the opportunistic nature of *P. aeruginosa*.

In contrast to tighter growth phenotypes whereby isolates are greater adapted to growth in the presence of glucose, there is evidence which suggests that isolation of *P. aeruginosa* in the lung drives diversity which means that isolates from chronically infected patients are likely to be adapted to a wide range of nutrient conditions. When these patients are diabetic isolation in specific areas of the lung would drive diversification but this diversification would be skewed driving isolates to be better adapted to glucose utilisation and the level of diversity would not be as high compared to CF patients (Jorth et al., 2015). Another factor is that at the point of the samples being taken the glucose levels of patients were not recorded only the diabetic status so it was not confirmed whether the diabetic patients had controlled glycaemia or were hyperglycaemic. Irrespective of this, the diabetic status of a person can still influence susceptibility to bacterial infection as there are likely to be fluctuations in glucose level control.

The development of chronic infection in susceptible CF patients and the formation of biofilms could be significant in diabetic patients. For example, biofilm formation of *P. aeruginosa* in the presence of glucose has been previously tested and demonstrated that high glucose promotes biofilm formation determined by thickness and height of biofilms using flow cell chambers with varying nutrient gradients (Song et al., 2013).

In support of this, there is also evidence suggesting that available nutrients such as glucose have an impact on quorum sensing which is important in early biofilm development (Rasamiravaka et al., 2015).This could suggest that in patients with elevated levels of glucose, there could be an increased risk of biofilm formation and the development of chronic infection.

4.7. Future Work

Using the models developed in this project numerous collaborative experiments within Imperial College London, St Georges University London and Astra Zeneca have been conducted using different strains of *P. aeruginosa* as well as several *in vitro* experiments linked to the role of glucose on bacterial growth.

4.7.1. Transposon Library

This study identified 4 genes which were involved in growth in the presence of glucose which was confirmed using the *in vitro* and *in vivo* infection models developed during this project. The generation of these mutants was effective for this project but required extensive optimisation and was suitable for small scale mutant generation. For large scale identification of more essential genes – genes which when deleted cause a more detrimental growth phenotype, which could be used for drug targets, a better screening method was developed.

By screening a pool of a large number of mutants under different growth conditions, or in the *in vivo* models developed and performing next generation sequencing on a TraDIS (Transposon directed insertion site sequencing) library there is the opportunity to identify novel gene products for drug targets which would inhibit growth. The resulting library of mutants contains strains with random insertions of a

transposon across the genome. The importance of identifying novel genes involved in growth under specific conditions is highlighted by the fact that on average 30 – 40 % of genes in a bacterial genome have unknown functions or hypothetical functions (Judson and Mekalanos, 2000).

In vitro and in vivo models using a TraDIS library could also be developed to identify genes involved in the formation of biofilms under hyperglycaemic conditions. This technique has been used previously to compare *Salmonella typhi* virulence genes, (Langridge et al., 2009), to identify novel gene function by deep sequencing techniques (Opijnen and Camilli, 2013) and to identify gene function in *P. aeruginosa* (Lewenza et al., 2005).

By generating a TraDIS library, specific genes and their associated products could be identified as potential drug targets for the prevention of bacterial growth not only in hyperglycaemic conditions but also during chronic infections and biofilm formation. Mutants can then be screened using the higher throughput growth assay developed to identify which genes can be potential drug targets. Through the use of the *in vitro* and *in vivo* models developed, antimicrobial peptides or compounds designed to target potential essential genes could lead to alternative treatment strategies to antibiotic resistance.

4.7.2. P. aeruginosa growth in Bronchoalveolar Lavage

This project identified glucose as a risk factor for bacterial infection and the assays developed in this project will be used determine whether glucose levels in sputum samples from COPD patients correlated with bacterial growth.

Glucose in BAL samples isolated from COPD patients will be used as the culture medium for *P. aeruginosa.* As the data presented in this thesis demonstrates that increased levels of glucose promote *P. aeruginosa* growth it will be interesting to see if BAL from COPD patents with increased levels of glucose results in higher bacterial growth and if the findings from this thesis can be used to better identify which patients will be at greater risk of *P. aeruginosa* infection.

This data is currently being prepared for inclusion in a publication with a collaborator acquired during the course of this thesis.

4.8. Concluding Remarks and Limitations

This project had two major themes, which involved the identification of glucose as a driver of *P. aeruginosa* growth and the models used to develop this and expand on results for future work.

There are alternative models to use in *P. aeruginosa* infection, such as gut colonisation (Okuda et al., 2010, Nelson et al., 2013) and ocular infections (Gowda et al., 2015, Heimer et al., 2013) but this study had the sole aim of exploring the role of glucose in lung infections. Wound models have been used to show that people with diabetes and hyperglycaemia have increased bacterial infection (Noor et al., 2016) but these models do not reflect the hypothesis and aims of this project, which are directly on airway glucose and increased *P. aeruginosa* infection. As previous studies, and as yet unpublished data I have contributed to throughout this study, have demonstrated that airway glucose levels are elevated in patients admitted to intensive care as well as people with COPD and viral infections (Baker at al., 2009)

the main aim of this project was to develop models to assess the role of glucose on bacterial growth without diabetes or obesity.

The link between glucose and bacterial infection has been eluded to previously (Dooley and Chaisson, 2009, Gan, 2013, Bader, 2008, Jacobsson et al., 2007) but complications such as immune deficiency and how hyperglycaemia contributes to immune deficiency have made linking exactly how glucose promotes bacterial growth *in vivo* difficult to conclude. For example, the main question has been is it the underlying immune deficiencies associated with diabetes that contribute to susceptibility or is it glucose alone that drives the initial colonisation. One conclusion that can be drawn from this study is that glucose alone does contribute to an increase in *in vivo* bacterial growth which could suggest that where patients have elevated levels of glucose in their airways, bacterial growth during the acute stages of infection is promoted. However, whether the immune deficiency observed in susceptible patients then contributes to greater disease severity has not been explored in this project.

Previous studies which have aimed to determine the role of elevated glucose levels on infection have used alternative models to those developed during this project (Garnett et al., 2013; Pezzulo et al., 2011). Although this study links airway glucose to bacterial growth there are limitations associated with the models used to demonstrate this. One major limitation is that the host response to bacterial infection under hyperglycaemic conditions was not explored which could have further determined whether glucose alone is a risk factor or it is the combined effect of a compromised immune system with hyperglycaemia. By further exploring the host requirement for glucose, the tipping point between too much glucose having a

negative effect on the host can be determined leading to better treatment of hyperglycaemic patients.

The data from the *in vitro* and *in vivo* models from chapter 3 clearly demonstrate the positive role of glucose in acute *P. aeruginosa* infection and this hypothesis could be applied to other bacterial infections, with increasing antimicrobial resistance and are common opportunistic pathogens, especially in hyperglycaemic conditions. The *in vivo* model demonstrated that in a non-immunocompromised model of hyperglycaemia airway glucose directly promotes bacterial growth and this can be lowered through metformin delivery which is safe and effective in reducing airway glucose. However this could also be the result of altered virulence factor expression which was not explored in this thesis or an effect of metformin on bacterial growth.

However, the mechanisms of metformin were not determined in this study which could have explained the observations in changes in airway glucose levels. The observed decrease in airway glucose but not blood glucose after metformin administration could be explained by the various effects of metformin. Metformin has other functions in addition to being used to treat type 2 diabetes and is known to supress TNF-α production by bronchial epithelial cells and an anti-inflammatory role has been previously suggested (Park et al. (2012). The same study also demonstrated tissue remodelling after metformin administration suggesting that in addition to the lowering airway glucose which lowers bacterial recovery, there could be physiological effects which were not explored in this project and could influence the effect of metformin when administered to hyperglycaemic individuals in a clinical setting. This could suggest that using metformin to reduce to paracellular diffusion

could reduce flux of glucose in to the airways and provide a favourable outcome for the host.

A major component of this study was the development of *in vivo* infection models to test the hypothesis of this project. The acute model of infection over a 24 hour period was optimised for infectious dose where a balance was achieved between acute respiratory infections with no mortality. The acute model was developed to take into consideration duration of infection where peak hallmarks of infection could be compared without clearance of *P. aeruginosa.*

The final *in vivo* model, infection over a period of 24 hours after intranasal inoculation of 10^6 CFU/ml PAO1, resulted in a model with reduced disease severity and mortality of mice infected with *P. aeruginosa.*

Model development also extended to a hyperglycaemic model which required extensive optimisation to reduce side effects of the drug which would affect the outcome of bacterial infection which could be used with other pathogens as part of collaborative research.

The final streptozocin treatment protocol resulted in mice which were hyperglycaemic with no other underlying health defects except for elevated blood and airway glucose which is more beneficial than using *db/db* mice or genetic knockouts as this was an acute hyperglycaemic mouse model to specially determine the role of glucose alone. Another favourable reason for using streptozocin is that chemically induced diabetes is more financially viable compared to commercially

available diabetic mice when using a high number of animals which was an important factor in this project.

Additionally, the role of hyperglycaemia on innate immune responses during acute bacterial infection could be better determined by generating streptozocin treated mice which are subsequently infected with other pathogens. This could be important as it has been hypothesised that hyperglycaemia leads to aberrant neutrophil function which could increase susceptibility to other respiratory pathogens including viruses.

However, despite streptozocin inducing hyperglycaemia and reflecting an acute hyperglycaemic environment in the lung, this differs from a chronic state of hyperglycaemia observed in patients who are prone to infections which has other complicating factors which were not explored in this project. In addition to this a major disadvantage of using streptozocin is that there is a high degree of toxicity and during development of the model this became apparent and should be carefully considered when using chemicals to induce diabetes.

Another key finding from this study is that the deletion of 4 genes which are involved in glucose uptake and utilisation resulted in reduced bacterial burden in the airways suggesting that by targeting genes involved in glucose metabolism by blocking uptake or activity could be an attractive antimicrobial approach bacterial growth in hyperglycaemic patients.

Using the 96 well growth assay developed in this project, the identification of other genes which could act as potential drug targets can be accelerated which could lead to novel drug development and forms the basis of several ongoing projects.

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