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Pseudomonas aeruginosa
In Tasmania

Richard Bradbury

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

The University of Tasmania, September 2009

Dedicated to God and to my Father

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Richard Bradbury

Date

Abstract

Pseudomonas aeruginosa is an organism commonly found in the environment, and one of the most common causes of infectious disease in humans. Infections caused by *P. aeruginosa* may present in many forms, reflective of the great versatility of this organism. *P. aeruginosa* infection occurs more commonly in patients with some form of immunocompromisation, and this is particularly significant in the nosocomial setting and as a cause of chronic infection in the cystic fibrosis (CF) lung.

Tasmania is an island state in the southernmost portion of Australia. It has a relatively small, isolated population with a greater balance of the population living in rural and regional areas than any other state in Australia. The state has a single tertiary care referral hospital in the capital city, Hobart and amongst the highest number of CF births per capita of anywhere in the world. Until 2003, CF patients in Tasmania did not attend any centralised clinics, and had little social or other contact with each other. These factors provided a unique opportunity to study the epidemiology of *P. aeruginosa* infections in a whole population sample.

In addition, a large number (n=184) of *P. aeruginosa* isolates from diverse clinical and environmental sources, including isolates from adult CF patients, were obtained from both within the major tertiary referral hospital in Tasmania and the wider community. Antimicrobial resistance testing was performed on all isolates by four separate methods and the results of these compared. A sub group of CF and hospital environment strains appeared to present with an increased propensity towards antimicrobial resistance and frank multi-drug resistance. Molecular epidemiological analysis of the CF strains revealed a single genotype of *P. aeruginosa* to be infecting over a quarter of the adult CF patients in the state. Isolates of the genotype concerned showed a greater propensity towards multi-drug resistance than any other cohort of *P. aeruginosa* strains included in the study, and were shown to

cause poorer clinical outcomes in infected patients. The strain was determined to be a new CF clonal complex, described as Australian Epidemic Strain 3 (AES3). The source of this strain appears to a CF summer camp which occurred more than ten years prior to this study. A further common genotype (infecting 11% of adult Tasmanian CF patients) was identified. This strain was described as the “Tasmanian CF cluster strain”.

Further molecular epidemiological analysis of *P. aeruginosa* strains from infected patients within the major tertiary referral hospital and the wider community, as well as environmental isolates from these and other sites showed that the AES3 and Tasmanian CF cluster strains are not common in non-CF patients, and do not have an obvious environmental source either in the hospital or the wider community.

A survey of the virulence factor genes associated with all isolates in this study was performed. This represented one of the most comprehensive studies of virulence genes over a wide range of *P. aeruginosa* isolates ever performed. The study found no specific difference in the prevalence of these genes between AES3 strains and other CF strains. CF strains were less likely to carry the low prevalence, horizontally transferred, *exoU* virulence gene. Conversely, isolates recovered from environmental sampling in the hospital intensive care and neurosurgical wards showed an increased propensity towards both antimicrobial resistance and *exoU*⁺ genotype.

A selected group of CF, non-CF clinical and environmental isolates were subjected to an assay of global cellular virulence in a novel modification of the *Dictyostelium discoideum* eukaryotic virulence assay employing two

D. discoideum mutants. The majority of clinical CF isolates supported the growth of *D. discoideum*. *D. discoideum* was unable to grow on any other isolates of *P. aeruginosa*, except one environmental isolate which supported the growth of only one of the two *D. discoideum* mutants tested. No difference in the capacity of clonal complex strains and unique CF strains to support *D. discoideum* growth and development was identified. Variations in the capacity of *D. discoideum* to develop beyond the amoeboid stage were noted within the CF isolates. No significant differences were noted between assays performed in the presence or absence of azithromycin, ceftazidime or tobramycin.

This was the first study of which we are aware to demonstrate the capacity of wild-type CF *P. aeruginosa* strains to support the growth of *D. discoideum*, and has provided significant findings with regard to “whole cell” virulence of this organism, and its down-regulation in the CF lung. Differences in the degree of development of *D. discoideum* on CF isolates may also lead to new insights into the mechanisms of virulence in such strains in the human disease setting.

The work presented in this thesis has found new information regarding the epidemiology of *P. aeruginosa* infections. It has also provided new information regarding the distribution of the *exoU* gene in hospital environmental isolates, and the association of this genotype with hospital intensive therapy wards. Finally, in describing a novel modification of the *D. discoideum* virulence model, and applying this model to multiple clinical and environmental isolates of *P. aeruginosa*, this work has added to the body of scientific knowledge regarding the expression of virulence by *P. aeruginosa* isolates from different clinical and environmental sources.

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List of Abbreviations

16S rRNA: 16S Ribosomal Ribonucleic acid

AAC: Aminoglycoside acetyltransferase

ADP: Adenosine Di-phosphate

ADPRT: Adenosine Di-phosphate Ribosyl Transferase

AES1: Australian Epidemic Strain I

AES2: Australian Epidemic Strain II

AES3: Australian Epidemic Strain III

ANT: Aminoglycoside Nucleotidyltransferase

Apr: Alkaline Protease

APH: Aminoglycoside Phosphoryltransferase

ATCC: American Type Culture Collection

AX2: *Dictyostelium discoideum* Axenic strain 2

AX4: *Dictyostelium discoideum* Axenic strain 4

BAL: Broncho-alveolar lavage

BMI: Body Mass Index

BSAC: British Society of Antimicrobial Agents and Chemotherapy

COPD: Chronic Obstructive Pulmonary Disease

CLSI: Clinical Laboratory Standards International

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Regulator

CHO: Chinese Hamster Ovary cell line

DH1-10: *Dictyostelium discoideum* wild type strain DH1-10

DIG: Digoxigenin-dUTP

DNA: Deoxy-ribonucleic Acid

ExoA: Exotoxin A

ESBL: Extended Spectrum β -lactamase

FAFLP: Fluorescent Amplified Fragment Length Polymorphism

FEV1%: Forced Expiratory Volume in one second

FVC%: Predicted Forced Vital Capacity

G+C: Guanine and Cytosine

GTP: Guanosine Tri-phosphate

HCN: Hydrogen Cyanide

HIV: Human Immunodeficiency Virus

HSL: Homoserine Lactone

ICU: Intensive Care Unit

IDSA: Infectious Diseases Society of America

IV: Intravenous

LasB: Elastase

LES: Liverpool Epidemic Strain

LPS: Lipopolysaccharide

MBC: Minimal Bactericidal Concentration

MDR: Multi-drug Resistant

MES: Manchester Epidemic Strain

MM β L: Mobile Metallo- β -lactamase

MDCK: Madin-Darby Canine Kidney cell line

MIC: Minimal Inhibitory Concentration

NATA: National Australian Testing Authority

NCTC: National Collection of Type Cultures

NPV: Negative Predictive Value

NSU: Neurosurgery Unit

PA14: *Pseudomonas aeruginosa* laboratory control strain 14

PAI-1: N- (3-oxododecanoyl)-L-homoserine lactone

PAI-2: N-butyral-L-homoserine lactone

PAO1: *Pseudomonas aeruginosa* laboratory control strain 01

PA14: *Pseudomonas aeruginosa* laboratory control strain 14

PA99: *Pseudomonas aeruginosa* laboratory control strain 99

PA103: *Pseudomonas aeruginosa* laboratory control strain 103

PAK: *Pseudomonas aeruginosa* laboratory control strain K

PCN: Pyocyanin

PCR: Polymerase Chain Reaction

PFGE: Pulsed Field Gel Electrophoresis

PPV: Positive Predictive Value

PQS: 2-heptyl-3-hydroxy-4-quinolone

QS: Quorum Sensing

RAPD: Random Amplified Polymorphic DNA

RFLP: Restriction Fragment Length Polymorphism

RHH: Royal Hobart Hospital

T1SS: Type I Secretion System

T2SS: Type II Secretion System

T3SS: Type III Secretion System

TFP: Type IV Pili

TLR4: Toll-like Receptor 4

UK: United Kingdom of Great Britain and Northern Ireland

UTI: Urinary Tract Infection

X22: *Dictyostelium discoideum* Xenic strain 22

CHAPTER ONE

Review of Literature

1.1 Introduction

Pseudomonas aeruginosa is a common cause of bacterial infection in humans, animals and plants worldwide. Infection may vary in severity from a mild, self-limiting illness through to severe and debilitating systemic disease, associated with significant morbidity and mortality. *P. aeruginosa* is recognized as the causative agent of a remarkable spectrum of diseases in both healthy and immunocompromised patients, and is the primary cause of morbidity and mortality in cystic fibrosis (CF) patients. It is ubiquitous within the environment, having been recovered from a wide range of aqueous and vegetative environmental sources (Sekiguchi, Asagi *et al.* 2007). *P. aeruginosa* will often merely colonise (the presence and multiplication of an organism within or upon the host, without tissue invasion or damage) a host. In cases where a breach in the integrity of host defences occurs, strains may cause infection (the presence and multiplication of an organism within the host, leading to tissue damage through local cellular injury, secretion of toxins or antigen-antibody reactions).

The incidence of severe infections caused by *P. aeruginosa* has risen dramatically in recent decades, a change that has been proposed as being prompted by a number of factors including: a) a significant increase in the longevity of severely ill and immunocompromised patients, b) a concurrent significant increase in the use of broad spectrum antimicrobials in patients (which in many cases will select for *P. aeruginosa* colonisation and infection), c) a significantly increased capacity for the development or acquisition of mechanisms to resist the action of antimicrobials and d) the recent acquisition of new virulence factors by horizontal gene transfer (Kulasekara, Kulasekara *et al.* 2006).

1.2 General characteristics

P. aeruginosa is a non-spore forming, non-fermentative Gram negative bacillus (Kiska and Gilligan 2003). The organism is catalase positive and oxidase positive and has traditionally been considered a

strict aerobe; unable to grow in anaerobic conditions due to its requirement for oxygen as a terminal electron acceptor in biochemical pathways. However anaerobic growth of *P. aeruginosa* has been demonstrated to be possible in the presence of nitrate or arginine, which the organism may also utilise as terminal electron acceptors (Kiska and Gilligan 2003). *P. aeruginosa* has a wide growth temperature range, growing equally well at room temperature and 37°C. Slower growth rates are seen at 4°C (Baron 2001; Pier and Ramphal 2005). *P. aeruginosa* is distinguishable from other clinically significant *Pseudomonas spp.* by its capacity for growth at 42°C (Baron 2001; Kiska and Gilligan 2003).

Colonies of *P. aeruginosa* are round, matt, butyrous colonies 2-3 mm in diameter after 24 hours growth on blood agar at 37°C, with irregular edges and a floccular internal structure. Commonly described are smaller colonies, resembling coliforms, and raised, rough, umbonate or even rugose colonial morphologies have been described (Pitt 1998). *P. aeruginosa* may dissociate into multiple colonial morphotypes, which are regularly observed following sub-culture of an isolated colony (Zierdt and Schmidt 1964). Most distinctive of the differing morphotypes are the mucoid strains first described by Sonnenschein in 1927 (Çetin, Töreci *et al.* 1965) often recovered from patients with CF and other chronic respiratory infections with *P. aeruginosa*. Mucoid isolates have also been recovered from contact lens associated corneal infections and urinary tract infections (particularly those UTIs associated with in-dwelling catheters), the common factor between all of these infections being growth in biofilm (Engel 2003).

1.3 Environmental range

P. aeruginosa is ubiquitous in the environment, being found in soil, water, animals and on plant matter (Schmidt, Tummler *et al.* 1996). It is a well recognised phytopathogen (Kiska and Gilligan 2003), and

has a predilection for moist environments, and thus is often cultured in very high numbers from drains and similar semi-aquatic environments (Grundmann, Schneider *et al.* 1995; Talon, Cailleux *et al.* 1996; Brisse, Milatovic *et al.* 2000). It has displayed an ability to use varied substrates as a basis for growth. The organism is particularly common in hospital environments having been isolated from soap, disinfectants, respiratory equipment, mattresses, endoscopes, distilled water and suction apparatus (Pier and Ramphal 2005). Thus, avoidance of exposure to *P. aeruginosa* in the general environment is impossible. Isolates from hospital environments generally display greater resistance to antimicrobials than environmental samples and are common causes of opportunistic infection in patients (Talon, Cailleux *et al.* 1996; Brisse, Milatovic *et al.* 2000).

1.4 Infection with *Pseudomonas aeruginosa*

1.4.1 Immunocompetent patients in the community setting

P. aeruginosa causes an extremely wide range of disease in both immunocompetent and immunocompromised patients. In immunocompetent patients, the organism generally requires a breach in host defences such as a skin abrasion, or placement of a urinary catheter, in order to establish infection. In previously healthy patients, *P. aeruginosa* infection is generally limited to skin and soft tissues. Contaminated swimming pools, whirlpools and hot-tubs are a recognised cause of folliculitis and are associated with urinary tract infection in females (Stryjewski and Sexton 2003). One report described *P. aeruginosa* folliculitis consequent to the use of a contaminated loofa sponge (Bottone and Perez 1993). Folliculitis associated with depilation of the legs has also been described (Stryjewski and Sexton 2003). Otitis externa (swimmer's ear) may be caused by *P. aeruginosa* following exposure to contaminated water (Kiska and Gilligan 2003). While colonisation of the upper respiratory tract occurs commonly, this rarely progresses to clinical disease in previously healthy individuals (Pier and Ramphal 2005). Risk factors for community acquired *P. aeruginosa* pneumonia are a history of

smoking, exposure to aerosolised bacteria in water sprays or welding dust and near-drowning in fresh water or whirl pools (Stryjewski and Sexton 2003). *Pseudomonas aeruginosa* has been implicated as a cause of community-acquired gastroenteritis (Adlard, Kirov *et al.* 1998), and a cluster of community acquired sepsis cases in previously healthy infants in Taiwan was reported, associated with prior community acquired gastroenteritis (Stryjewski and Sexton 2003). *P. aeruginosa* wound infections following trauma are common. Corneal ulcers in association with contact lens use and other ocular infections following trauma are well recognized, and endocarditis in intra-venous drug users has been recorded (Kiska and Gilligan 2003). Calcaneous osteomyelitis in children has been associated with penetrating injuries through footwear colonised with *P. aeruginosa* (Stryjewski and Sexton 2003; Pier and Ramphal 2005). *P. aeruginosa* infections appear to be increasing in the community setting (Seigel 2008), whilst the cause of this is not completely understood, increased virulence in environmental strains through acquisition of new virulence genes (such as *exoU*) via horizontal transmission is a feasible explanation.

1.4.2 Immunocompromised patients in the community setting

P. aeruginosa is a common cause of infection in the immunocompromised patient, such as those with burns, diabetes mellitus or more severe immunocompromisations such as cancer or HIV. Infection in such cases may occur through both nosocomial acquisition and by exposure in the community setting. *P. aeruginosa* is second only to *Staphylococcus aureus* as a cause of burn infections in the United States (Stryjewski and Sexton 2003). It has been shown to be the second most common cause of bacteraemia subsequent to burn infections (Vostrugina, Gudaviciene *et al.* 2006). The severe and invasive progression of *P. aeruginosa* otitis externa to malignant otitis externa is particularly associated with the diabetic patient, with up to 94% of cases being observed in diabetics (Johnson and Ramphal 1990). Indwelling catheters and drains may easily be colonised by *P. aeruginosa*, which will form

biofilms on such devices, rendering eradication difficult or impossible. In such cases, patients are often asymptomatic, but without removal of the devices, progression to true infection often results (Stryjewski and Sexton 2003). Physically disabled patients and those in long term nursing institutions are predisposed to *P. aeruginosa* infection, as such patients are often unable to personally attend to basic hygiene needs, are often catheterised, and may have concurrent physical difficulties in bladder flushing, swallowing, coughing and other protective reflexes (Stryjewski and Sexton 2003). HIV positive status predisposes to *P. aeruginosa* infection, a Spanish study finding that in 189 cases of *P. aeruginosa* bacteraemia, HIV was the most common underlying defect of the immune system (Stryjewski and Sexton 2003). In those with advanced disease, chronic *P. aeruginosa* lung infection is a common and intractable infection (Asboe, Gant *et al.* 1998).

Transplant and chemotherapy patients are exposed to a large number of risk factors for *P. aeruginosa* infection. Such patients are given long-term immunosuppressive therapy and often concurrently have neutropenia, indwelling catheters, frequent hospital stays and repeat courses of antibiotics. Thus it is unsurprising that high rates of *P. aeruginosa* pneumonia, bacteraemia and UTI are seen in these patients. Mortality rates are 20-25% for bone marrow transplant patients with *P. aeruginosa* bacteraemia (Stryjewski and Sexton 2003). Profoundly immunosuppressed patients are advised to avoid fresh vegetables, due to the probability of contamination with *P. aeruginosa* and other Gram negative bacilli, consumption of which may lead to intestinal colonisation and the risk of subsequent sepsis (Correa, Tibana *et al.* 1991; Kiska and Gilligan 2003). Despite these measures, that *P. aeruginosa* may be found even in bottled water (Rosenburg 2003) demonstrates its ubiquity in the environment and the associated difficulty in avoiding contact with the organism.

In the community setting, chronic lung disease accounts for a large amount *P. aeruginosa* mediated disease. Much of this burden is carried by CF patients (discussed below), but chronic respiratory infections with *P. aeruginosa* are also commonly seen in chronic obstructive pulmonary disease (COPD) and bronchiectasis. Predisposing factors for such disease are long term smoking, or inhalation of other harmful chemicals. The damaged and dysfunctional lung becomes an ideal site for *P. aeruginosa* colonisation and chronic infection. Prevalence of *P. aeruginosa* in COPD patients is 4%, increasing to approximately 13% in those with advanced disease (Maciá, Blanquer *et al.* 2005). In such cases, *P. aeruginosa* isolates often show phenotypic similarity to those recovered from the CF lung, including conversion to mucoid phenotype in up to 40% of cases (Pujana, Gallego *et al.* 1999), increased phenotypic diversification and long term colonisation with a single genotype (Maciá, Blanquer *et al.* 2005).

1.4.3 Nosocomial infections

P. aeruginosa is a recognised nosocomial pathogen, responsible for between 10% to 20% of hospital-acquired infections (Dinesh, Grundmann *et al.* 2003; Matar, Chaar *et al.* 2005). Indeed, *P. aeruginosa* is the second most common cause of nosocomial pneumonia, and shows the highest mortality for this type of infection (30%) (Dinesh, Grundmann *et al.* 2003). *P. aeruginosa* is also highly associated with burn wound infections, sepsis, meningitis and infections associated with indwelling devices amongst hospitalised patients (Dinesh, Grundmann *et al.* 2003; Stryjewski and Sexton 2003). Given the large number of severely ill and immunosuppressed patients in hospitals, the widespread use of antimicrobial agents and disinfectants in these patients and their surrounds, it is unsurprising that a positive selection for *P. aeruginosa* in the hospitalised patient occurs. These pre-disposing factors are particularly applicable in the context of the ICU, as 29% of infections in ICUs may be attributable to *P. aeruginosa*,

ranking it as the most common cause of Gram negative infection in this setting (Deplano, Denis *et al.* 2005).

Nosocomial isolates of *P. aeruginosa* display markedly higher rates of antimicrobial resistance and multi-drug resistance than those recovered from community acquired infections (Brisse, Milatovic *et al.* 2000), particularly amongst ICU patients (Guitierrez, Juan *et al.* 2007). One study found that of 419 isolates of *P. aeruginosa* from 19 separate Canadian ICUs, 12.6% showed an MDR phenotype, the closest frequency of MDR phenotype in other Gram negative rods isolated in this study was that of 0.6% in *Enterobacter cloacae* (Zhanel, DeCorby *et al.* 2008). Infection control practices play an important role in the control of *P. aeruginosa* infections within the hospital setting (Saiman and Siegel 2004).

1.4.3.1 Molecular epidemiology

P. aeruginosa is an environmental organism, and it is generally accepted that the overwhelming majority of community acquired infections are caused by environmental strains. Seven percent of healthy individuals carry *P. aeruginosa* in their throats. Other sites of colonisation include the skin (particularly in warm and moist areas such as the axilla) and the gastrointestinal tracts (variable carriage rates reported; up to 24% of healthy volunteers), where rates of carriage may be dependent upon diet (Speert 2002; Pier and Ramphal 2005). In other cases, the source of infection is exogenous, such as traumatic implantation in calcaneus osteomyelitis or cases of contact colonisation such as swimmers ear and whirlpool folliculitis (Pier and Ramphal 2005). The administration of narrow spectrum antibiotics greatly increases rates of colonisation of *P. aeruginosa*, and may select for resistant strains (Speert 2002). In hospitalised patients, where antibiotic use is greatly increased, intestinal colonisation rates of up to 60% of patients have been reported (Speert 2002). In the majority

of cases *P. aeruginosa* strains imported into hospitals in the bowels of patients are considered to be the source of their infections (Speijer, Savelkoul *et al.* 1999), an observation supported by the panmictic (multiple and diverse genotypes present) state of *P. aeruginosa* infections in most hospitals (Speert 2002; Guitierrez, Juan *et al.* 2007). Despite this, transmission of *P. aeruginosa* via environmental sources such as contaminated water and on the hands of health care workers may occur (da Silva, Filho *et al.* 2008).

In some cases, nosocomial outbreaks of *P. aeruginosa* infections are associated with genotypically indistinguishable strains. Reports of epidemics of MDR strains in ICUs and NSUs have been published (Deplano, Denis *et al.* 2005; Sekiguchi, Asagi *et al.* 2005), such strains will often spread rapidly within a hospital (Brisse, Milatovic *et al.* 2000) and may spread between hospitals (Guitierrez, Juan *et al.* 2007; Sekiguchi, Asagi *et al.* 2007). Nosocomial outbreaks are not always associated with resistant strains, a study of nosocomial clinical and environmental strains in a hospital in Beirut found a single antibiotic sensitive genotype to be the predominant isolate (Matar, Chaar *et al.* 2005).

1.4.4 Infection in cystic fibrosis patients

1.4.4.1 Demographics

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians (Tatterson, Poschet *et al.* 2001). The disease is rarely encountered in Africans, and is exceptional in those of Asian descent, but is more common in African Americans and natives of the Middle East and Indian sub-continent (Rubin and Farber 1994; Bosque and Asensio 2003). The genetic mutations leading to CF are particularly common in people of Anglo-Saxon descent (Bosque and Asensio 2003). Tasmania has a significantly higher incidence of CF per head of population than the mainland states of Australia and most other countries of the world, with approximately 110 cases in the state (Blest 2000). This is primarily

attributed to the predominantly Caucasian population with a largely Anglo-Celtic ethnic background and the low genetic diversity in the community resultant from geographic isolation (Blest 2000).

1.4.4.2 Epidemiology in respect to cystic fibrosis

P. aeruginosa may display a differing epidemiology in the case of CF patients compared to other types of infection. It is most probable that colonisation of the CF lung occurs following environmental exposure, and once established, a strain will generally remain present in a patient for the remainder of their life. Further genotypes may colonise an individual patient's lung and exist alongside the original colonising strain (Anthony, Rose *et al.* 2002). Person to person transmission in most cases is rare, although this may be seen in patients who spend a great deal of time in each other's company, such as siblings, and is associated with intimate contact such as kissing (Speert, Campbell *et al.* 2002; Saiman and Siegel 2004). Despite this, a recent study found that at a CF Summer camp in the Netherlands, of 80 children attending, there were 18 cases of possible transmission, and three cases of probable transmission of *P. aeruginosa* between attendees (Brimicombe, Dijkshoorn *et al.* 2008). Thus, summer camp attendance may be considered a risk factor for acquiring new *P. aeruginosa* infections or colonisation with new strains of the bacteria in CF children.

In-patient transmission of *P. aeruginosa* is also considered to be a rare event, and when it does occur was commonly associated with break down in infection control techniques such as staff hand washing and sterilisation of respiratory equipment (Geddes 2001; Saiman and Siegel 2004). Interestingly, while desiccated saline suspensions of non-mucoid *P. aeruginosa* can survive on inanimate surfaces for 24 hours, mucoid strains will survive for 48 hours. When strains are suspended in CF sputum, their survival on dry surfaces extends to 8 days (Saiman and Siegel 2004), raising the potential for *P. aeruginosa* acquisition from fomites. Mucoid isolates were found to survive longer in aerosolised

droplets than non-mucoid isolates, but no significant differences were observed between clonal complexes (LES and MES) and unique CF isolates (Clifton, Fletcher *et al.* 2008).

CF summer camps and family camps are a well recognised source of cross-infection with respect to *B. cepacia* (Honicky, Harden *et al.* 1993), and the potential for *P. aeruginosa* cross-infection at such camps has been investigated. The incidence of permanent conversion to an acquired genotype following attendance at a CF holiday camp was found to be only 1.9% in one study, and thus considered that the risks of cross-infection with *P. aeruginosa* were outweighed by the psychological benefits of such camps in CF patients (Hoogkamp-Korstanje, Meis *et al.* 1995).

1.4.4.3 *P. aeruginosa* clonal complexes in CF patients

A relatively recent and concerning development in the epidemiology of CF *P. aeruginosa* infections has been the emergence of clonal complexes of *P. aeruginosa* in both the United Kingdom and Australia. These strains have often spuriously been referred to as “clonal”, a description which cannot correctly be applied without whole genome sequencing. Indeed, in many cases these strains only display only a degree of genotypic similarity beyond that attributable to random events, rather than displaying completely identical patterns upon genotypic analysis (Wiehlmann, Wagner, *et al.* 2007; Rakhimova, Wiehlmann, *et al.* 2008). Finally, such strains often display a marked degree of intra-strain phenotypic variability, due to mutation and horizontal transfer of new genes. It was therefore considered more correct to refer to such isolates as “epidemic strains”. Given that such strains are almost exclusively limited to CF infections, and do not spread at an overwhelmingly rapid rate, the latter description is also strictly incorrect. Such strains are now being referred to as “CF clonal complexes of *P. aeruginosa*” (Lavenir, Sanroma, *et al.* 2008).

The first report of a potential clonal complex of *P. aeruginosa* amongst CF patients occurred in Denmark in 1984 (Pederson, Koch *et al.* 1986), this study reported an MDR clonal complex based upon serotyping and phage typing of the isolates. Later PFGE typing of these isolates showed the cluster to be composed of two genotypically distinct strains (Saiman and Siegel 2004). Römmling *et al.* described the presence of a genotypically indistinguishable strain (clone C) from 12 (37%) of 32 patients (3 female siblings and 9 unrelated patients) at a CF clinic in Hannover, Germany in 1994. Two of these patients appeared to lose this strain over the 2.5 year period of the study (Römmling, Fiedler *et al.* 1994). It appears that the significance of these findings may have been overlooked upon their first publication. The first report of a CF clonal complex associated with hospital CF clinics using reliable molecular epidemiology techniques was published by Cheng *et al.* (1996), describing the identification of a genotypically indistinguishable and ceftazidime resistant strain of *P. aeruginosa* in a number of CF patients at a CF clinic in Liverpool, described at the time as the “Alder Hey strain”.

The strain formerly known as “Alder Hey” has since been renamed as the “Liverpool epidemic strain” (LES). LES is amongst the most studied of the currently identified CF *P. aeruginosa* clonal complexes. The strain was originally identified due to its ceftazidime resistant phenotype, but isolates sensitive to this antibiotic have since been identified (Scott and Pitt 2004). MDR phenotype does however predominate amongst LES isolates (Scott and Pitt 2004). This strain may act as the primary colonising strain or super-infect patients that already carry one predominant, unique genotype, and over time supplant the previously dominant strain to become the only genotype present in the lung (McCallum, Corkill *et al.* 2001).

Following the first description of LES, a second clonal complex was identified in 24 (16%) of 154 CF patients attending a CF clinic in Manchester (Jones, Govan *et al.* 2001). This strain also showed a

greater propensity towards antimicrobial resistance when compared to genotypically unique isolates, with most isolates resistant to ceftazidime, piperacillin, ciprofloxacin, aztreonam, imipenem, and meropenem, and some showing additional resistance to the aminoglycosides (gentamicin, tobramycin and amikacin) (Jones, Govan *et al.* 2001).

The death in quick succession of five CF patients under the age of five years at CF clinic in Melbourne, Australia, prompted an epidemiological study of 27 patients. This identified *P. aeruginosa* clonal complex colonising all five of the deceased children as well as three other paediatric CF patients (Armstrong, Nixon *et al.* 2002). This strain was described as “pulsotype 1”, and was shortly afterwards isolated from 10 (56%) of 18 CF patients in Sydney (Anthony, Rose *et al.* 2002). These findings lead to a study in Brisbane, which found another CF clonal complex (pulsotype 2) in 39 (39%) of 100 CF patients attending two centralised CF clinics (O'Carroll, Syrmis *et al.* 2004). Pulsotype 1 was identified in 8 of the 100 patients tested (O'Carroll, Syrmis *et al.* 2004), although half of these patients had previously resided in Sydney (Armstrong, Bell *et al.* 2003). It is probable that Pulsotype 2 represents a newly emerged strain, as typing of CF *P. aeruginosa* strains in Brisbane in 1991 by RFLP did not reveal any cross infection (Fegan, Francis *et al.* 1991). Since this time, the two strains have been re-named Australian Epidemic Strain 1 and 2 (AES1 and AES2).

A study in New Zealand study identified three patients with British or Australian CF clonal complexes, there was no evidence of transmission of these strains to new hosts (Schmid, Ling *et al.* 2008). However, in one CF centre a small cluster of strains existed in a number of patients above that which could be attributed to chance, and this cluster may represent a novel clonal complex (Schmid, Ling *et al.* 2008).

Further studies have been performed in Turkey (Yagci, Ciragil *et al.* 2003), Brazil (Da Silva Filho, Levi *et al.* 2001; Silbert, Barth *et al.* 2001), British Columbia (Speert, Campbell *et al.* 2002) and Belgium (Van Daele, Franckx *et al.* 2005), all of which did not identify the presence of strain transmission in the tested CF populations. The Canadian study did identify 5 (3%) of 174 patients who shared a genotypically indistinguishable strain whilst having no known contact with each other. Whilst not a finding considered during the publication of the Canadian study, it may be that these five isolates represent a potential new CF clonal complex that has not yet become widespread in the Canadian CF population (Speert, Campbell *et al.* 2002).

In response to the presence of LES and the Manchester epidemic strain (MES), Scott and Pitt (2004) performed the most widespread genotypic study of *P. aeruginosa* infections in CF patients to date, drawing upon 1225 isolates (1 per patient) from 31 centres across England and Wales representing sampling of 20% of the entire CF populations of these areas. This study found LES in 11% of patients from 15 separate centres throughout the geographic range of the study, only 11 isolates from just 3 centres were identified as MES. A new CF clonal complex, termed “Midlands 1” was identified in 86 patients from 9 centres. This strain showed increased resistance to antimicrobials, and was the only type in the study to include a colistin (polymyxin class antimicrobial) resistant isolate. FAFLP analysis showed greater heterogeneity in LES strains than midlands 1 isolates, suggesting a greater temporal space since the emergence of LES when compared to midlands 1. Also of noted in this study was the presence of “clone C” in 15 patients from 8 centres (Scott and Pitt 2004).

The presence of clone C in a large number of patients so far from its site of original isolation in Germany leads to interesting considerations regarding the epidemiology of this particular strain in relationship to other strains of *P. aeruginosa* and other clonal complexes thus far described. Clone C

does not show the propensity towards a resistant antibiogram that is so closely associated with other clonal complexes. It has been isolated from infections other than those in the CF lung, having been found causing otitis externa in one patient and isolates showing 75% or greater similarity by PFGE have been recovered from a CF patients in Toulouse, France, the peritoneal dialysate of a non-CF patients in Birmingham, UK and in the urine of a non-CF ICU patient in Durham, also the UK. Clone C has also been widely recovered from environmental aqueous sources throughout Europe, including swimming pools, drinking water and rivers (Dinesh, Grundmann *et al.* 2003).

While little work has been performed thus far on the virulence of clone C, the effect on patient outcomes of infection with a number of other CF clonal complexes has been investigated. McCallum, *et al* (2002) reported the transmission of LES from an infected CF child to both of her parents, in which it caused significant disease. The father was known to have COPD, and was infected with both LES and a unique *P. aeruginosa* strain, whilst the mother was physically well apart from mild asthma (McCallum, Gallagher *et al.* 2002). Transmission of *P. aeruginosa* from CF individuals to healthy patients has not previously been described (McCallum, Gallagher *et al.* 2002), and this suggests that in at least the case of LES, the strain is significantly more capable of colonising and causing disease in the human lung. These findings are supported by the unusual report of LES causing sinusitis in the pet cat of an LES colonised CF adult (Mohan, Fothergill *et al.* 2008).

Further study of LES by subtractive hybridisation has found that these strains all belong to serotype 06 and elaborate pyoverdine III, an unusual form of pyoverdine. All LES strains tested possessed *exoS* (compared to a mean *exoS* positivity of only 76% in *P. aeruginosa*), are not hypermutable and all possess the PAG-1 pathogenicity island (overall *P. aeruginosa* mean positivity 81%) (Parsons, Panagea *et al.* 2002). Studies of two LES isolates and PA01 both in exponential phase and under oxidative

stress by microarray identified the loss or down regulation of a number of genes and bacteriophage related clusters (Salunkhe, Smart *et al.* 2005). Of particular interest amongst these results was that many of the differences in genes were associated with QS function. However, no significant results were forthcoming from this analysis due to both the small sample size, and the variability within the LES isolates possession of specific genes, and response to oxidative stress (Salunkhe, Smart *et al.* 2005). Patients infected with LES showed a greater reduction in pulmonary function and BMI over four years compared to age matched controls infected with unique strains (Al-Aloul, Crawley *et al.* 2004).

Work on the phenotypic characteristics of MES has been performed. As previously discussed, these isolates are generally highly resistant to antimicrobials, but also are exclusively non-pigmented and non-motile. The presence of mucoidy in isolates is variable, while 22 out of 24 isolates belonged to an unusual pyocin type. In the first report of the existence of MES, patients harbouring this clonal complex had almost universally (23 out of 24) had recent stays (<2 years) in the Manchester hospital in which it was first isolated (Jones, Govan *et al.* 2001). A comparison of 22 patients with MES against 56 patients harbouring unique strains showed that those infected with this clonal complex had more exacerbations and required more courses of IV antibiotics, but showed no significant difference in BMI, FEV1% and FVC%. MES has not been isolated from any non-CF patients (Jones, Dodd *et al.* 2002). MES isolates suspended in saline were found to be less resistant to desiccation than unique strains or LES isolates (which showed equal resistance) (Panagea, Winstanley *et al.* 2005), however, in clinical practice such strains would be suspended in CF sputum and the resistance of these isolates to desiccation when suspended in this matrix has not been investigated.

With regard to the Australian CF clonal complexes of *P. aeruginosa*, both AES1 and AES2 have been shown to have increased resistance to antimicrobials when compared to unique strains. AES1 isolates were predominantly mucoid on first isolation (Armstrong, Nixon *et al.* 2002), whilst AES2 showed no greater propensity towards a mucoid phenotype compared to unique isolates (O'Carroll, Syrmis *et al.* 2004). AES2 carriers in Brisbane were younger (mean average age 16.8 vs 24.6 for patients with unique strains), and showed poorer lung function as measured by FVC% and FEV1% (O'Carroll, Syrmis *et al.* 2004). An unpublished study by the University of Sydney using PA01 microarray chips (Affymetrix) to compare four AES1 and four matched unique isolates yielded some novel results, although the sample size was too small to reach statistical significance. The study found that QS genes in AES1 were all upregulated and that all strains of AES1 harboured the *pf4* gene, which was not present in any of the unique strains. The entire PAO632-39-bacteriophage locus was absent in all AES1 isolates tested, and a putative virulence factor gene of MES, *pao724* was not present in the AES1 strains tested (Munro 2005). A similar loss of an entire bacteriophage gene locus (PAO632-48) was observed in microarray analysis of two LES strains (Salunkhe, Smart *et al.* 2005).

1.5 Laboratory identification

CLSI guidelines maintain that in cases of uncomplicated infection, the characteristic oxidase reaction, pigment and smell of *P. aeruginosa* is sufficient for laboratory identification (Baron 2001). However, due to the potential for severe adverse patient outcomes based upon incorrect bacterial identification further identification is required in isolates from sterile sites, (Baron 2001). In practice, the majority of laboratories in Australia employ at least one further test to identify any isolates of *P. aeruginosa*. Characteristic morphology, pigment, and a capacity to grow at 42°C are considered sufficient for identification. Diagnostic discs impregnated with the compound C390 may be effectively used for separation of *P. aeruginosa* from other *Pseudomonas spp.* (Anthony, Rose *et al.* 2002). A variety of

commercial phenotypic testing kits and automated systems are available for the identification of non-fermentative Gram negative rods, these may be effectively employed to identify *P. aeruginosa*.

CF and other chronic respiratory *P. aeruginosa* isolates may represent a diagnostic dilemma for clinical laboratories. Many of these isolates do not conform to traditional *P. aeruginosa* phenotypes (including pigmentation variability), and may grow very slowly, limiting the effectiveness of identification methods based on biochemical properties (Miller and Gilligan 2003). In response to this, a number of PCR based applications have been developed specifically for the identification of *P. aeruginosa* and other *Pseudomonas spp.* isolates from CF respiratory samples (Spilker, Coenye *et al.* 2004; Anuj, Whiley *et al.* 2009). A review was conducted in Australia of 2, 267 bacterial isolates from the CF lung identified by routine phenotypic methods in clinical laboratories as *P. aeruginosa* using a duplex *gyrB* and *ecfX* PCR (Anuj, Whiley *et al.* 2009), followed by partial 16S rRNA sequencing of any those providing negative results. This study found a misidentification rate of 2.3%; the most common organisms misidentified as *P. aeruginosa* being *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and *Inquilinus limosus* (Kidd, Ramsay *et al.* 2009). This highlights the importance of genotypic identification methods for *P. aeruginosa* from the CF lung in the routine laboratory setting.

1.6 Phenotypic and Genomic Variability

By far the most phenotypic variability of *P. aeruginosa* isolates is seen in chronic infections of the CF lung. Alterations in the *P. aeruginosa* phenotype in the CF airway occur as chronicity of infection develops. The most striking of these alterations is the conversion to a mucoid phenotype, an event which is associated with significantly increased morbidity in the affected patient (Lyczak, Cannon *et al.* 2002; Davidson, Currie *et al.* 2003). It has been suggested that a correlation exists between biofilm growth and mucoidy (Nivens, Ohman *et al.* 2001).

P. aeruginosa has an unusually large genome (~6.3 Mb) (Projan 2007). Global genome variation between *P. aeruginosa* isolates is estimated to be at a level of 11% on average (Shen, Sayeed *et al.* 2006). Between individual isolates, genomes may vary by up to 30% (Morales, Wiehlmann *et al.* 2004), representing a massive level of intra-species genome variation. It appears that very little change has occurred in core genome over the past 50 years (Morales, Wiehlmann *et al.* 2004).

1.7 *Pseudomonas aeruginosa* in Biofilm

The formation of biofilms is considered to be a major factor in *P. aeruginosa* persistence in the CF lung (Prince 2002). Biofilms are large masses of bacteria growing in organised structures that allow symbiosis between cells within the structure to provide greater economy in cell functions than would be possible when growing alone in a planktonic state. Biofilms may be found in the natural environment, adherent to surfaces within an extracellular polysaccharide matrix (Lewis 2001; Engel 2003) and in human infection, such as when growing on stationary mucous within the CF lung or upon the airway surface liquid (Worlitzsch, Tarran *et al.* 2002; Engel 2003). Biofilm formation occurs in four stages; attachment and replication, microcolony formation, differentiation into a structured community and release of planktonic cells (Engel 2003).

1.8 Quorum sensing

Within *P. aeruginosa* biofilms, cell differentiation and formation of the biofilm itself is regulated by quorum sensing (QS) cell signalling mechanisms, autoinduced by acyl homoserine lactone molecules. The QS system of *P. aeruginosa* is a complex series of positive and negative feedback pathways that consummately reflects the great complexity of gene expression within this species. The expression of genes in response to QS autoinducers is a factor of autoinducer density in the surrounding environment,

and thus proportional to the density of bacterial cells secreted autoinducer molecules (De Kievit, Gillis *et al.* 2001; Engel 2003). As a consequence of this, planktonic cells will only begin biofilm formation when a critical density of cells is present within any given area (Dale and Park 2004). In the case of *P. aeruginosa*, biofilm formation and differentiation is controlled by the *las* and *Rhl* signalling pathways (De Kievit, Gillis *et al.* 2001; Engel 2003). The *las* pathway consists of the *lasR* transcriptional regulator and the *lasI* synthetase. *LasI* synthesises the N-(3-oxododecanoyl)-L-homoserine lactone (PAI-1), which also has regulation activity in virulence factor expression, the activation of *Rhl* pathway and expression of *LasI* itself (Engel 2003). The *rhl* QS pathway is composed of the *RhlR* transcriptional activator and the *RhlI* synthetase, directing production of N-butyral-L-homoserine lactone (PAI-2) (Engel 2003). The *rhl* QS system also plays a role in both biofilm and virulence factor regulation (Engel 2003). A third QS autoinducer in *P. aeruginosa* has recently been described; 2-heptyl-3-hydroxy-4-quinolone (PQS), positively regulated by *las* and negatively regulated by the *rhl* QS systems (Wade, Calfee *et al.* 2005). PQS is an autoinducer of its own negative regulator, *LasR*, and also functions in regulation of expression of genes involved in iron acquisition and oxidative stress response (Bredenbruch, Geffers *et al.* 2006), as well as a number of virulence factors, including pyocyanin and *LasB* (Engel 2003; Jensen, Lons *et al.* 2006).

1.8.1 Azithromycin as a disruptor of quorum sensing

Macrolide antibiotics have MICs to *P. aeruginosa* far above achievable levels *in vivo*. Despite this, a role for the new macrolide azithromycin has been proposed in the treatment of chronic *P. aeruginosa* lung infection. Azithromycin has been shown to have an anti-inflammatory effect and up-regulate T-helper 1 lymphocyte function, as well as disrupting QS in *P. aeruginosa* (Nguyen, Louie *et al.* 2002; Parnham 2005). As QS is essential for the formation and maintenance of biofilms, these findings have had significant implications for the treatment of chronic *P. aeruginosa* lung infections.

Azithromycin has also been shown to display a bactericidal effect on *P. aeruginosa* during certain growth phases due to its interaction with the cell's outer membrane. CF patients treated over a 3 month period showed significant improvement in both FEV1 and FVC1% following treatment (Jaffé and Rosenthal 2002). The achievable level of azithromycin within the lung is only 8 mg/L (Gillis and Iglewski 2004), while the MBC of PA01 in exponential phase of growth is 128 mg/L (Imamura, Higashyama *et al.* 2005). However, in biofilm, the majority of cells are thought to be in stationary phase, and the azithromycin MBC of PA01 dropped to just 1 mg/L when the cells were in stationary phase (Imamura, Higashyama *et al.* 2005). Gillis & Iglewski found that clinically achievable levels of 8 mg/L had no effect on PA01 biofilm formation *in vitro*, whilst lower levels of 2 mg/L did delay biofilm formation (Gillis and Iglewski 2004). Sub-MIC concentrations of various macrolides have been shown to reduce the expression of virulence factors, including alginate, elastase, lecithinase, protease, leukocidin, exotoxin A, phospholipase C and pyocyanin without affecting bacterial growth rates (Tateda, Ishii *et al.* 2007). Very recent work has identified the potential for not just azithromycin, but also ceftazidime and ciprofloxacin (commonly used in the treatment of *P. aeruginosa* infections) to inhibit QS, and lead to a consequent decrease in virulence factor expression (Skindersoe, Alhede *et al.* 2008).

Certainly, azithromycin therapy seems to have improved patient outcomes in those with chronic *P. aeruginosa* lung infections, with the 10 year survival rate for diffuse panbronchiolitis patients rising from below 50% prior to the institution of azithromycin therapy to above 90% following its introduction (Schultz 2004). Azithromycin is now also used in the treatment of CF patients with chronic *P. aeruginosa* infections (McCormack, Bell *et al.* 2007).

1.9 Antimicrobial resistance

Treatment of *P. aeruginosa* with antimicrobial agents may be hampered by the large number of both inherent and acquired resistance mechanisms (table 1.1) associated with the organism. Rates of acquired resistance to antimicrobials vary throughout the world, with higher rates seen in Europe and parts of the third world than in the United States, and nosocomial *P. aeruginosa* isolates tend to be more resistant to antimicrobials than community acquired isolates (Kiska and Gilligan 2003). CF isolates of *P. aeruginosa* demonstrate more overall resistance to antimicrobials when compared to isolates from other types of infection, and these isolates provide peculiar difficulties in antimicrobial resistance testing due to their hypermutable state. A concerning trend towards multi-drug resistance is emerging worldwide, which has grave implications for the capacity of current therapies to eradicate *P. aeruginosa* infections in the future (Kiska and Gilligan 2003).

1.9.1 Intrinsic resistance mechanisms

P. aeruginosa is resistant to a range of antimicrobials commonly used to treat Gram negative infections. This was until recently considered to be due to cell membrane impermeability, but in fact the large membrane porin OprF of *P. aeruginosa* will easily allow access of these agents into the cell. More recent work has identified the presence of the efflux pump system MexAB-OprM, which actively removes β -lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline and trimethoprim from the cell, as well as a number of dyes, detergents, inhibitors of fatty acid biosynthesis and solvents (Hirakata, Srikumar *et al.* 2002; Livermore 2002).

1.9.1.1 The chromosomal β -lactamases

P. aeruginosa possesses a chromosomal β -lactamase, AmpC, which is both induced by and hydrolyses a wide spectrum of β -lactam agents, including third generation cephalosporins and aztreonam, but not

imipenem (Livermore 2001; Livermore 2002). Three AmpC types have been described in *P. aeruginosa*; CMY-1, Fox-1 and MOX-1, although with the emergence of plasmid mediated AmpC resistance more types may be found (Rice, Sahm *et al.* 2003). The chromosomal gene, *ampC* exists naturally in a repressed state, and thus levels of AmpC production are below clinically significant levels. However, exposure to specific β -lactam agents, particularly ceftazidime, ampicillin and imipenem will result in the de-repression of *ampC* and subsequent hyperproduction of the AmpC enzyme, with resultant treatment failure.

The activity of AmpC is not significantly inhibited by clavulanic acid (Rice, Sahm *et al.* 2003), but the anti-pseudomonal penicillin classes; carboxypenicillins (ticarcillin, carbenicillin) and ureidopenicillins (piperacillin), are effective despite the activity of AmpC, as while they are hydrolysed by this enzyme (carbenicillin may be resistant depending on AmpC type), they do not induce its hyperproduction (Pitt 1998). AmpC hyperproduction is a mutation which occurs relatively infrequently without induction (approximately 1 in 10^{-8}) (Pitt 1998).

1.9.1.2 Active efflux pumps

As previously discussed, the active efflux system MexAB-OprM plays a major role in the intrinsic resistance of *P. aeruginosa* to many antimicrobial agents. This active efflux is composed of the MexB broad spectrum pump, MexA linking protein and the OprM outer membrane porin. In its natural environment, it is thought that this pump may play a role in removing toxic substances from the bacterial cytoplasm. Mutations in *nalB*, a gene of the *mexR* locus, result in high frequency up regulation of MexAB-OprM. This up regulation of active efflux will raise MICs to penicillins, cephalosporins, tetracycline and chloramphenicol. Selection of this mutation will occur *in vitro* during therapy, and it is the predominant mutation involved in resistance to ticarcillin and carbenicillin, but

may also confer resistance to ceftazidime and meropenem. A much less common mutation of *nfxC* of the *mexT* locus, induced by fluoroquinolone therapy will induce up regulation of MexEF-OprN, raising MICs to both carbapenems (possibly due to co-regulation with OprD) and fluoroquinolones (Livermore 2001). A further uncommon mutation of *nfxB* upregulates MexCD-OprJ, resulting in a similar resistance phenotype to mutant MexAB-OprM (Livermore 2002).

A fourth mutant active efflux pump system, MexXY-OprM (syn. AmrAB) is implicated in resistance to β -lactam agents, macrolides, glycyclcyclines, lincosamides, chloramphenicol, fluoroquinolones and aminoglycosides, but not imipenem (Livermore 2002). This phenotype is by far the most concerning in clinical practice, as it confers resistance to a very wide range of antimicrobial agents in one mutation. Expression of *mexXY* is regulated by MexZ, and mutation of *mexZ* gene results in up regulation of this gene. Studies have found that mutation of *mexZ* *in vitro* did not confer aminoglycoside resistance, and it is suggested that another as yet unknown factor may be required for complete up regulation of MexXY-OprM (Livermore 2001). Genome sequencing of *P. aeruginosa* has identified at least five other active efflux pump systems which are yet to be characterised (Livermore 2002).

1.9.1.3 Membrane changes

Changes in the membrane structure of *P. aeruginosa* to reduce permeability plays a major role in the resistance of this organism to antimicrobials. The outer membrane protein OprD is a porin for the passive uptake of amino acids, but which is also permeable to carbapenems. Loss of OprD expression raises the imipenem MIC of *P. aeruginosa* strains to clinically resistant levels, but will only reduce susceptibility to meropenem. This change does not affect the MICs of non-carbapenem antimicrobials. It has been found that MexEF-OprN is co-regulated with OprD, and thus decreased expression of OprD and up regulation of MexEF-OprN may occur simultaneously upon exposure to fluoroquinolones or

less commonly, imipenem (Livermore 2001). Also notable is a significant correlation between the presence of OprR and resistance to quarternary ammonium compound disinfectants, such as cetylpyridium chloride and benzalkonium chloride (Tabata, Nagamune *et al.* 2003).

The operon *oprH-phoP-phoQ*, regulated by the concentration of Mg^{2+} ions and PhoP, codes for products involved in both aminoglycoside and polymixin resistance. These genes are expressed in conditions of low Mg^{2+} concentration, and it is thought that OprH may be involved in maintaining outer membrane stability, whilst PhoP and PhoQ are involved in regulation of cationic ion levels. PhoP and PhoQ have been shown to play a role in resistance to both polymixin B and aminoglycoside antibiotics. The mechanisms by which this occurs are not yet fully understood, although it is known that elevated Mg^{2+} decreases the sensitivity of *P. aeruginosa* to aminoglycoside antibiotics (Macfarlane, Kwasnicka *et al.* 2000).

1.9.1.4 Mutations in type II topoisomerase enzymes

The fluoroquinolone antibiotics (ciprofloxacin, levofloxacin) act upon the type II isomerase enzymes. These enzymes control the topological conformation of DNA during replication and transcription. In *P. aeruginosa*, fluoroquinolone resistance may occur either through multidrug efflux pumps (see previous) or alterations in the type II topoisomerase II enzymes (DNA gyrase and topoisomerase IV) which render them resistant to the action of the fluoroquinolones. DNA gyrase is composed of two A and two B sub units, encoded by *gyrA* and *gyrB*. Topoisomerase IV bears the same heterotetramer formation as DNA gyrase, with one double sub-unit coded by *parC* and one by *parE*. Mutation in any one of these genes may result in resistance to fluoroquinolone therapy, and this mechanism is the most commonly identified cause of fluoroquinolone resistance in clinical strains of *P. aeruginosa* (Akasaka, Tanaka *et al.* 2001). Mutations in both *gyrA* alone resulted in 4 to 64 times higher MICs to

fluoroquinolones when compared to wild-type, while a combination of *gyrA* and *mexR* (MexAB-OprM pump) mutations resulted in resistance levels 1,024 times higher than wild type (Nakajima, Sugimoto *et al.* 2002).

1.9.1.5 Aminoglycoside modifying enzymes

Three broad classes of aminoglycoside modifying enzymes have been identified in *P. aeruginosa*, these being the aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) enzymes. These enzymes may be either chromosomal or mobile on plasmids and transposons. Depending on sub type and activity, they are capable of hydrolysing either a specific and limited number of antimicrobials or a broad spectrum of agents of the aminoglycoside class (Poole 2005).

The most common aminoglycoside hydrolysing enzymes in *P. aeruginosa* are the ANT (2'')-I enzymes, hydrolysing aminoglycosides at the 2' position. These enzymes will hydrolyse gentamicin and tobramycin, but not netilmicin or amikacin. This enzyme has been shown to be significantly more common in AES2 CF clonal complex isolates than in genotypically unique CF isolates (Syrmis, Bell *et al.* 2008). Other common enzymes are AAC (3)-I (gentamicin only) and AAC (6')-II (gentamicin, tobramycin and netilmicin). *aac(3)* and *aac(6')* genes may be found on transposons and integrons, often in association with narrow spectrum, extended spectrum or metallo β -lactamases (Poole 2005).

1.9.2 Mobile β -lactamases

Acquisition of resistance genes on mobile genetic elements is common in many bacteria, and no less so in *P. aeruginosa*. Genes coding for resistance enzymes are often found on plasmids and transposons, with some even carrying multiple resistance genes on a single integron (Walsh, Toleman *et al.* 2005).

The presence of multiple resistance gene cassettes on a single mobile element is of particular concern, given the capacity of such mobile elements to create a multi-resistant *P. aeruginosa* infection literally overnight. Exposure to antimicrobial agents selects for and amplifies the presence of resistant bacterial populations within a patient, providing a source for the spread of resistance throughout a hospital. In this way, hospitals and other institutions may harbour highly resistant and difficult to treat strains of *P. aeruginosa* unless very thorough infection control procedures are adhered to.

1.9.2.1 Narrow spectrum β -lactamases

P. aeruginosa strains commonly acquire OXA (Ambler class D) and/or PSE (syn. CARB - Ambler class A) β -lactamases (Bert, Branger *et al.* 2002; Rice, Sahm *et al.* 2003; Weldhagen, Poirel *et al.* 2003), which will hydrolyse carboxy-penicillins and ureidopenicillins, narrow spectrum cephalosporins and expanded spectrum cephalosporins, but not extended spectrum cephalosporins (Bert, Branger *et al.* 2002). These enzymes are commonly found in *P. aeruginosa*, but are considered very rare in the enterobacteriaceae. Conversely, narrow spectrum Ambler class A β -lactamases, such as TEM and SHV, commonly found in enterobacteriaceae are found only rarely in *P. aeruginosa* (Weldhagen, Poirel *et al.* 2003). A French study found that only 10% of ticarcillin resistant *P. aeruginosa* isolates (representing just 1.9% of *P. aeruginosa* isolates overall) had acquired a TEM type β -lactamase (Bert, Branger *et al.* 2002).

1.9.2.2 Extended spectrum β -lactamases

A relatively recent occurrence (1983) in the history of antimicrobial resistance was the discovery of extended spectrum β -lactamases (ESBLs). These enzymes were identified shortly after the introduction of the extended spectrum (or third generation) cephalosporins, such as ceftazidime, cefotaxime and ceftriaxone. ESBLs are narrow spectrum β -lactamase enzymes which have mutated to

allow the binding of extended spectrum cephalosporins. Thus, these enzymes are often active against all penicillins, and cephalosporins below the fourth generation (cefepime and cefpirome) as well as the monobactam, aztreonam (Jiang, Zhang *et al.* 2006). Some of these enzymes show a greater affinity for specific antimicrobials, such as the CTX-M ESBLs, which more efficiently hydrolyse cefotaxime and ceftriaxone than they do ceftazidime (Rice, Sahm *et al.* 2003). The activity of ESBLs may be inhibited by exposure to clavulanic acid, sulbactam or tazobactam. Consistent with their spectra of activity, the effect of a specific inhibitor is dependent upon what type of enzyme is involved. For instance, the CTX-M enzymes are more readily inhibited by tazobactam than by clavulanic acid, whereas some TEM ESBLs are resistant to all β -lactamases inhibitors (Rice, Sahm *et al.* 2003).

Many different ESBL types have been identified in *P. aeruginosa*, some of which are associated with specific geographic regions, leading to the suggestion that these enzymes may perform a role within specific ecological niches. Although rates of ESBL positivity in *P. aeruginosa* are low compared to the enterobacteriaceae, this frequency appears to be increasing (Jiang, Zhang *et al.* 2006). It is thought that the TEM and SHV ESBLs found in *P. aeruginosa* have been acquired from enterobacteriaceae, whilst the PER and OXA classes are mutations of enzymes from *P. aeruginosa* itself. ESBL types described in *P. aeruginosa* thus far are; VEB, PER, SHV, TEM, GES, IBC and BEL class ESBLs (Weldhagen, Poirel *et al.* 2003; Bogaerts, Bauraing *et al.* 2007).

1.9.2.3 Mobile metallo- β -lactamases

Metallo- β -lactamases (Ambler class B) are metalloenzymes, relying upon metal ions rather than serine for catalysis of their reactions. These enzymes have a broad spectrum of activity including penicillins, cephalosporins (including the fourth generation cephalosporins) and imipenem, but not aztreonam. They are not inhibited by β -lactamase inhibitors, but will not function in the presence of ion chelators

due to chelation of metal ion enzymatic co-factors. The extremely wide spectrum of action of these enzymes causes serious difficulties in clinical practice, as the use of virtually all β -lactam antimicrobials is ineffective in all organisms expressing such enzymes.

A recent and extremely disturbing event has been the emergence of multiple types of new mobile metallo- β -lactamases (MM β LS), and their spread between the enterobacteriaceae and non-fermentative Gram negative rods (Rice, Sahm *et al.* 2003). The first MM β L identified in a strain of *P. aeruginosa* was IMP-1, found to be encoded on a mobile conjugative plasmid. Since this time, at least four other types of MM β L have been identified (GIM-1, VIM-1, VIM-2, SPM-1), conferring resistance to all β -lactam agents, including imipenem, with a greater or lesser degrees of activity against aztreonam. The rapid spread of these novel resistance genes worldwide has resulted in its appearance in most countries (including Australia) (Walsh, Toleman *et al.* 2005). SPM-1 MM β L producing strains accounting for 35% of clinical carbapenem resistant isolates of *P. aeruginosa* from hospitals throughout Brazil in 2003 (Gales, Menezes *et al.* 2003). Of even greater concern has been the identification of an integron carrying a partially deleted *qac* and *sul* genes, *bla*_{GIM-1}, *aacA4*, *aadA1* and *bla*_{OXA-2}. Thus, in one mobile genetic element, resistance to sulphonamides, quarternary ammonia compounds, β -lactam agents and aminoglycosides, treatment with any of which would select for bacterial isolates that had acquired this integron (Walsh, Toleman *et al.* 2005). The potential for the world-wide spread of MM β L resistance and accompanying multi-drug resistance cassettes in both the enterobacteriaceae and non-fermenting Gram negative rods, in a process analogous to the rapid spread of the ESBL phenotype remains an ominous prospect.

1.9.3 Multi-drug resistance

Multi drug resistance in *P. aeruginosa* is defined by the US CF foundation consensus guidelines as resistance to all agents in two or more of the following classes: β -lactams, aminoglycosides and fluoroquinolones (Armstrong, Nixon *et al.* 2002). The emergence of multi-drug resistant (MDR) strains in both CF and non-CF clinical isolates over recent years remains of great concern. The previously discussed multiple mechanisms of *P. aeruginosa* resistance and multi-drug resistance may be found in any combination within any given isolate. Multi drug resistance is more common in nosocomial isolates than in those contracted within the community, with rates of MDR isolates infection decreasing with patient age (Flamm, Weaver *et al.* 2004). Empirical treatment of *P. aeruginosa* infections and prolonged treatment with antibiotics were found to be pre-disposing factors in the emergence of MDR phenotypes (Seigel 2008). MDR isolates were found in one US study (in which multi-drug resistance was defined as resistance to three or more antimicrobial agents) to be most common in respiratory isolates from patients in ICUs (Flamm, Weaver *et al.* 2004). In many cases, treatment options become limited to single drugs, such as ciprofloxacin or colistin (Kiska and Gilligan 2003). Nosocomial infections with MDR *P. aeruginosa* are result in severe limitation of treatment options and marked negative impacts on patient outcomes (Zavascki, Gaspareto *et al.* 2005).

1.9.3.1 Hypermutation and multi-drug resistance

MDR isolates of *P. aeruginosa* in the CF context merit separate attention to those found in a nosocomial setting. Up to 37% of CF patients carried at least one hypermutable isolate (Oliver, Levin *et al.* 2004), similar levels of hypermutation were found in a study of non-CF chronic lung infection isolates (bronchiectasis, COPD) in which 57% of patients were found to possess hypermutable isolates (Maciá, Blanquer *et al.* 2005). These figures represent the highest percentage of hypermutation in any known bacterial population (Gutierrez, Juan *et al.* 2004). By comparison, less than 1% of 103 ICU

isolates investigated in one study were hypermutable (Gutierrez, Juan *et al.* 2004). In the natural environment, a stable mutator phenotype confers great survival advantage to a bacterial strain by allowing it to better adjust to novel environmental conditions. In the case of human infection, a hypermutable state not only allows the organism to adjust to assault by the host immune system, but also to quickly develop resistance to antimicrobial agents that may be used in therapy (Gutierrez, Juan *et al.* 2004).

It has been suggested that hyper-mutation may be induced by prolonged antimicrobial therapy (as is applied to CF patients) (Morosini, Garcia-Castillo *et al.* 2005), representing the “chicken and egg” dilemma in hypermutable MDR chronic lung infection isolates. Alternately, Ciofu, *et al* performed work which suggested that hypermutation in chronic lung infections may be caused by oxidation of Pseudomonal DNA by reactive oxygen species liberated from polymorphonuclear leukocytes during chronic infection (Ciofu, Riis *et al.* 2005). It is possible that both mechanisms may be involved in the evolution of a hyper-mutable genotype in *P. aeruginosa* during chronic lung infection.

Hypermutation of *P. aeruginosa* in the context of chronic lung infection isolates presents specific difficulties in treatment. Due to the prevalence of hypermutation in these strains, it is probable that in any chronically infected lung, isolates already resistant to all or a majority of anti-pseudomonal agents exist. A study of an apparently fully sensitive PA01 Δ *mutS* strain found that at concentrations of 5×10^4 exposure to any of 11 anti-pseudomonal agents resulted in clinically relevant levels of resistance within 24-36 hours (Oliver, Levin *et al.* 2004). Thus, it was recommended that monotherapy never be used in

| Mechanism | Tic | car | pip | Tim | caz | fep | ipm | mer | atm | tob | cn | Ak | cip | Col |
|----------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|-----|-----|
| <i>bla</i> _{OXA} | • | • | • | | | | | | | | | | | |
| <i>bla</i> _{PSE} | • | • | • | | | | | | | | | | | |
| <i>bla</i> _{AmpC} | • | • | • | • | • | | | | | | | | | |
| ESBL | • | • | • | | • | | | | | | | | | |
| MMβL | • | • | • | • | • | • | • | • | • | | | | | |
| MexAB-oprM | • | • | • | • | • | • | | • | • | | | | • | |
| MexEF-oprN | • | • | • | • | • | • | • | • | | | | | • | |
| MexCD-oprJ | • | • | • | • | • | • | | • | | | | | | |
| MexXY-oprM | • | • | • | • | • | | | • | | • | • | • | • | • |
| ΔoprD | | | | | | | • | • | | | | | | |
| ΔPhoP/ΔPhoQ | | | | | | | | | | • | • | • | | • |
| ΔII topoimerase | | | | | | | | | | | | | • | |
| AAC (6')-I | | | | | | | | | | • | | • | | |
| AAC (6')-II | | | | | | | | | | • | • | | | |
| AAC (3)-I | | | | | | | | | | | • | | | |
| AAC (3)-II,III,VI | | | | | | | | | | • | • | | | |
| ANT (2'')-I | | | | | | | | | | • | • | | | |
| ANT (4')-II | | | | | | | | | | | • | • | | |
| APH (3')-VI | | | | | | | | | | | | • | | |

Table 1.1: Potential *P. aeruginosa* antibiogram against antimicrobial agents commonly employed in therapy by resistance mechanism.

tic=ticaracillin, car=carbenicillin, pip=piperacillin, tim = timentin, caz = ceftazidime, fep= cefepime, ipm = imipenem, mer = meropenem, azt=aztreonam, tob=tobramycin, cn=gentamicin, ak=amikacin, cip-ciprofloxacin, col=colistin(Macfarlane, Kwasnicka *et al.* 2000; Akasaka, Tanaka *et al.* 2001; Livermore 2001; Bert, Branger *et al.* 2002; Rice, Sahm *et al.* 2003; Bell, Gatus *et al.* 2004; Juan, Macia *et al.* 2005; Poole 2005; Walsh, Toleman *et al.* 2005).

CF infections, and that levels of any single drug used in therapy should not be allowed to drop below MIC levels *in vivo*, to avoid the selection of an MDR phenotype (Oliver, Levin *et al.* 2004).

Hypermutable strains will often display multiple colonial morphotypes, despite these colonies being of identical genotype. One study found a mean number of four morphotypes per CF sputum. The same study performed antimicrobial susceptibility testing by the BSAC method and found an average of three differing antibiograms per morphotype (Foweraker, Laughton *et al.* 2005). This variation in antibiogram is also seen within genotypes, and thus antibiograms are considered to be a poor marker for use in epidemiological surveillance (Williams 1997). The antimicrobial susceptibility results generated by clinical laboratories based on assays of one morphotype may provide misleading results to a clinician, and the efficacy of these results in guiding treatment of chronic *P. aeruginosa* CF lung infections is suspect.

1.10 Virulence factors of *Pseudomonas aeruginosa*

To be described as a virulence factor of an organism, a molecule must first adhere to the molecular Koch's postulates, which state that any virulence factor must i) be encoded by a gene that is associated with bacteria that cause disease, but usually absent in or inactive in strains that fail to cause disease, ii) be required for virulence, as evidenced by a loss of virulence when disrupted or a gain in virulence when expressed in an avirulent strain and iii) be usually expressed during infection (Engel 2003). Many virulence factors in *P. aeruginosa* are horizontally acquired (Engel 2003), as reflected by the great diversity in virulence factors within the species. It should be noted that most studies of virulence are performed upon laboratory control strains, and it is likely that there are novel virulence factors found in clinical strains (or even the much studied control strains) that are yet to be identified. Also to be noted is that virulence factors are likely to perform a useful function for any organism within their

natural environment, and that as *P. aeruginosa* is not an obligate parasite of humans, many factors harmful to humans expressed by this organism will most probably perform a more innocuous function within the organism's natural habitat.

Virulence factors in *P. aeruginosa* are divided into specific groups dependent upon their mode of action or method of delivery to the host cell. Thus, these virulence factors may be described as belonging to adhesins and secreted toxins and enzymes dependent or independent of the type I secretion system (T1SS), type II secretion system (T2SS) and type III secretion system (T3SS); these are described in detail later.

1.10.1 Global regulation of virulence

Between 468 and 521 genes bearing characteristics of transcriptional regulators or environmental sensors were found during the sequencing of the PA01 genome (Engel 2003). Regulation of many *P. aeruginosa* virulence factors is carried out by complex interactions with and between QS molecules (discussed previously), but QS effector expression itself is under regulation from a number of global regulators of virulence. Global regulatory proteins identified thus far in *P. aeruginosa* include Vfr, GacA, RsaL, QscR, RsmA, mvfR, MvaT, VqsR, pprB and the sigma factors RpoS and RpoN (Dong, Zhang *et al.* 2005). To demonstrate the complexity of regulation interactions within *P. aeruginosa*, the example of one regulator alone, Vfr (virulence factor regulator), may be considered. Vfr is involved in the regulation of the *las* QS system, which in turn regulates the *rhl* and PQS QS systems. Vfr also acts directly upon *toxA* and *regA* to regulate synthesis of the toxin ExoA (Suh, Runyen-Janecky *et al.* 2002). Mutation in *vfr* alone affected the production of least 60 proteins within the cell (Suh, Runyen-Janecky *et al.* 2002). Expression of Vfr itself is dependent upon the co-factor cyclic-AMP, which is controlled by the adenylate cyclases CyaA and CyaB (Smith, Wolfgang *et al.* 2004). The ennuui of regulation in *P.*

aeruginosa is beyond the scope of this review, but it exemplifies the great complexity and inter-relationship between regulatory, signalling and virulence factors in the organism, an attribute that confers its tremendous phenotypic malleability, allowing successful colonisation and infection in a diverse range of environmental and clinical conditions.

1.10.2 Cell surface virulence factors

Alginate, an exopolysaccharide produced by all *P. aeruginosa* cells, and hyperproduced by mucoid variants, was considered to have a role in adherence and invasion of host cells (Engel 2003). However, no difference in capacity to adhere to and invade host cells has been demonstrated between non-mucoid and mucoid phenotypes (Massengale, Quinn *et al.* 2000). Alginate is considered to provide protection against opsonisation by complement and phagocytosis (May, Shinabarger *et al.* 1991) as well as providing a barrier to the diffusion of antimicrobials into the cell (May, Shinabarger *et al.* 1991; Zielinski, Maharaj *et al.* 1992). Further to these advantages, alginate hyper-producing mucoid strains of *P. aeruginosa* display far greater capacity to survive in aerosol droplets *in vitro* (Clifton, Fletcher *et al.* 2008).

Cell surface lipopolysaccharide (LPS) is involved in the adherence of *P. aeruginosa* to host cells, and subsequent internalisation. *P. aeruginosa* LPS is composed of the polysaccharide O and core antigens and lipid A composed of fatty acid and phosphate groups bonded to a glucosamine disaccharide (Ernst, Adams *et al.* 2006). Truncation of the O antigen structure (to form rough LPS) may occur in the case of chronic CF and HIV infection (Asboe, Gant *et al.* 1998; Davidson, Currie *et al.* 2003; Ernst, Adams *et al.* 2006). Truncation of the O polysaccharide side chains renders the cell more susceptible to complement mediated lysis, but may provide a selective advantage by conferring immunity to detection by host O antigen specific antibodies (Davidson, Currie *et al.* 2003). Speert, *et al.* showed that change

to the rough phenotype LPS could be induced in *P. aeruginosa* when grown under sub-optimal levels of nutrition, and therefore suggested that this change may be due to a lack of nutrients in the CF lung rather than interactions between the bacteria and host (Speert, Farmer *et al.* 1990).

The adhesion interactions of cell wall lipopolysaccharide (LPS) and CFTR in the host cell lung present a possible role of CFTR in defence against *P. aeruginosa* colonisation warrant particular attention. In the normal lung, CFTR is expressed on the surface of respiratory epithelial cells (Tatterson, Poschet *et al.* 2001). It is postulated that CFTR is a cellular receptor for *P. aeruginosa* LPS, and that attachment of LPS to CFTR is followed by cellular internalisation. Following epithelial cell internalisation, *P. aeruginosa* would then be cleared from the airway by epithelial detachment (Tatterson, Poschet *et al.* 2001) or epithelial cell apoptosis (Grassme, Kirschnek *et al.* 2000). Cells expressing rough LPS have been shown to be less effectively internalised by this process (Davidson, Currie *et al.* 2003).

Type IV pili (TFP), found in many Gram negative organisms, and composed of a single protein; PilA, play a major role in the adhesion of *P. aeruginosa* to host cell membranes and inanimate surfaces, a remarkable example of which is their capacity to adhere to stainless steel (Giltner, van Schaik *et al.* 2006). Analogous to the interactions of CFTR and LPS, it is hypothesised that TFP may adhere more specifically to $\Delta F508$ CF respiratory epithelial cells than to normal respiratory epithelium. Studies have suggested that glycoproteins on the outer membrane of $\Delta F508$ CF respiratory epithelial cells show decreased sialation (Tatterson, Poschet *et al.* 2001). Following LPS and TFP mediated adhesion, *P. aeruginosa* has the capacity to secrete a large number of virulence factors capable of causing cell damage and death.

1.10.3 Secreted virulence factors

Secreted exoproducts of *P. aeruginosa* account for a significant number of its known virulence factors. Secretion of virulence factors may occur by a number of different pathways, both active and passive. Virulence factors that passively diffuse across the cell membrane include the pigments pyocyanin and pyoverdinin, while many other virulence factors are actively secreted by the type I, type II (secretion dependent) or type III secretion systems, common to many Gram negative organisms.

1.10.4 Hyperactive efflux pumps

The hyperactive efflux pumps involved in MDR phenotypes of *P. aeruginosa* may also be involved in increased virulence. It has been shown that these pumps export HSL molecules involved in the QS system, which regulates the production of many virulence factors. A Japanese study found that MexAB-OprM, MexXY-OprM active efflux systems had a direct effect on invasiveness. Knockout mutants without these efflux mechanisms showed significantly decreased invasiveness in MDCK cell lines and decreased mortality in leukaemic mice when compared to wild-type strains (Hirakata, Srikumar *et al.* 2002). It was suggested that these efflux systems may actively export not only antimicrobial agents and QS molecules, but also virulence factors. Other studies have found that MexAB-OprM and MexCD-OprJ mutants demonstrate reduced expression of T2SS virulence factors (Evans, Passador *et al.* 1998; Sanchez, Linares *et al.* 2002), and that induced MexCD-OprJ mutants of PAO1 showed decreased activity of the T3SS virulence pathway (Linares, Lopez *et al.* 2005).

1.10.5 Phenazines, pyocyanin and other pigments

One of the most obvious and striking phenotypic traits of *P. aeruginosa* is its blue/green pigment, expressed by over 90% of isolates (Mavrodi, Bonsall *et al.* 2001; Engel 2003). This pigmentation is caused by the secretion of the phenazine compound pyocyanin (PCN), which represents a significant

virulence factor in this organism. PCN is easily recovered from the ear discharges and sputum of *P. aeruginosa* infected individuals (Lau, Hassett *et al.* 2004). It also has strong antibacterial action (Engel 2003), which may provide a selective advantage to *P. aeruginosa* over other bacterial species in both its natural environment and infections. Production of pyocyanin is regulated directly by the *las* and *rhl* QS systems, and to a lesser degree the global regulator *GacA-GacS* and *Vfr* (Lau, Hassett *et al.* 2004). The precursor molecules, chorismic acid, coded by the *phzI* operon and phenazine-1-carboxylic acid, coded by the *phzII* operon are modified into the final three tricyclic phenazine compounds passively secreted by *P. aeruginosa*; pyocyanin, 1-hydroxyphenazine and phenazine-1-carboxamide by PhzH, PhzM and PhzS (coded by *phzH*, *phzM* and *phzS*, respectively) (Mavrodi *et al.* 2001; Finnán *et al.* 2004; Lau, Hassett *et al.* 2004).

Phenazine compounds exert their pathogenic action by increasing intracellular oxidative stress through intracellular redox cycling of reducing agents and oxygen, producing superoxide and hydrogen peroxide (Mavrodi, Bonsall *et al.* 2001). By this mechanism, phenazines can inhibit mitochondrial activity and superoxide production in neutrophils and macrophages. Inhibition of cell proliferation and cytokine secretion is also observed (Engel 2003). Pyocyanin may be recovered in high concentrations from the respiratory tract of CF patients, and is believed to be involved with lung damage through interference with ion transport mechanisms and inhibition of ciliary beating as well as interference with mucous secretion through alteration of intracellular concentrations of Ca^{2+} (Mavrodi, Bonsall *et al.* 2001).

P. aeruginosa may produce a number of pigments other than pyocyanin, including pyoverdine (yellow), pyorubrin (red) and pyomelanin (brown/black). The most clinically significant of these appears to be pyoverdine, expressed by 70% of clinical isolates. Pyoverdine acts as a siderophore, expression of which

is regulated by its own concentration, but which also regulates the production exotoxin A (Engel 2003). Pyorubrin is a water soluble pigment resulting in the production of rust coloured colonies. This pigment is found in only 2% of clinical isolates, primarily those from urine and CF cultures. Pyomelanin is only seen in 1% of clinical isolates, the dark brown/black coloured colonies resulting from pyomelanin production have provided difficulties in identification to inexperienced laboratory staff (Pitt 1998).

1.10.6 Cyanide

Cyanide has long been accepted as a potent toxin, being a powerful inhibitor of cytochrome *c* oxidase, the terminal component of respiratory metabolism (Finnan, Morrissey *et al.* 2004). Thus, the cyanogenic properties of *P. aeruginosa* provide it with a potent virulence factor in organisms that obtain energy through aerobic respiration. It has been suggested that HCN production is the primary virulence factor involved in the *Cenorhabditis elegans* virulence model (Gallagher and Manoil 2001). Cyanide synthase is coded by three separate genes; *hcnA*, *hcnB* and *hcnC* (Ramette, Frapolli *et al.* 2003), each coding for separate sub units of the enzyme (Laville, Blumer *et al.* 1998). Cyanide production by *P. aeruginosa* is controlled by the RsmA/RsmZ system (Heurlier, Williams *et al.* 2004), and occurs primarily when the organism is grown in microaerophilic conditions (Laville, Blumer *et al.* 1998; Sanderson, Wescombe *et al.* 2008).

1.10.7 Rhamnolipid and cytotoxic lectins

Rhamnolipid is a rhamnose containing a glycolipid biosurfactant which solubilise phospholipids in the lung surfactant, increasing their accessibility to the type II secreted phospholipase C enzymes (Engel 2003). Rhamnolipid is a heat stable haemolysin (Pitt 1998), also acting as a surfactant for swarming motility (Kohler, Curty *et al.* 2000). Studies utilising purified rhamnolipid have found it to induce

cytotoxic blebbing and decrease phagocytic ability in macrophages, inhibit ciliary beating in respiratory epithelial cells and rapidly lyse the environmental slime mould *Dictyostelium discoideum* (Engel 2003).

Two cytotoxic lectins are associated with *P. aeruginosa*; the galactose specific lecA (*lecA*, synonym PA-IL) and the fucose specific lecB (*lecB*, synonym PA-III). These lectins act as both adhesions and cytotoxins in the respiratory tract, and are also required for QS and rpoS σ factor function (Engel 2003; Tielker, Hacker *et al.* 2005). LecB appears to have a function in biofilm formation as lecB deficient mutants show impaired capacity to form biofilms (Tielker, Hacker *et al.* 2005). Expression of rhamnolipid and lecA is positively controlled by the Rhl QS pathway (Engel 2003), and is negatively controlled by small ribosome binding protein RsmA (Heurlier, Williams *et al.* 2004).

1.10.8 The type I secretion system

1.10.8.1 Alkaline protease

Alkaline protease (Apr, coded by *apr*) represents the only toxin of *P. aeruginosa* transported out of the cell by the type I secretion system, a sec independent active transport mechanism to transfer molecules from the inner to the outer membrane of a cell. Alkaline protease has a wide range of substrates, including collagen, C1q and C3 of the complement pathway, serum protease inhibitors, fibrin, fibrinogen, laminen and elastin (Pitt 1998; Engel 2003). The ability of Apr to hydrolyse pulmonary elastin is markedly inferior to that of the elastase enzyme, secreted by the type II secretion system (Pitt 1998).

1.10.9 The type II secretion system

The type two secretion system (T2SS) apparatus is coded for by the *xcp* cluster. The apparatus itself transports molecules from the inner cell membrane using a sec dependent mechanism. Molecules are excreted through the outer membrane by means of an outer membrane type II secretion apparatus similar to, and sharing components with the TFP and some DNA uptake systems. The expression of both T2SS secreted factors and *xcp* itself is controlled by cell density dependent QS molecules (Lee, Smith *et al.* 2005).

1.10.9.1 Elastolytic Enzymes

Elastase (syn: pulmonary elastase, LasB) is the most powerful proteolytic enzyme secreted by *P. aeruginosa*, accounting for 90% of its proteolytic activity⁸ (Pitt 1998; Engel 2003). Elastase begins as a 53.6 kDa pre-protein, which is then cleaved in the periplasm during transport across the cell membrane to a 33 kDa zinc metallo-protease with pH optima of 7-8 (Pitt 1998; Engel 2003). The enzyme has a wide range of substrates, including elements of connective tissue such as elastin, collagen, fibronectin and laminen, as well as immune and host defence molecules such as fibrin, gastric mucin, transferrin, α -1 proteinase inhibitors, IgG, γ -interferon and components of complement pathway. Elastase is coded by *lasB*, the expression of which is controlled through the las QS pathway (Engel 2003), and is produced in greatest quantity when cells are in the late logarithmic phase of growth or at high cell density (Finnan, Morrissey *et al.* 2004).

P. aeruginosa also produces a second elastolytic enzyme, the serine protease LasA (coded by *lasA*). This enzyme was thought to nick elastin, exposing active sites for proteolysis by LasB (Engel 2003). Subsequent studies have however shown that the enzyme is capable of elastolytic activity independent of LasB and Apr (Toder, Ferrell *et al.* 1994).

1.10.9.2 Exotoxin A

Exotoxin A (ExoA, *tox4*) is a 66 kDa protein acts as a major virulence factor of *P. aeruginosa*, analogous in action to that of diphtheria toxin. ExoA is a highly virulent protein, exhibiting an LD₅₀ of 2.5 mg/kg in mice, it has been shown that Δ *tox4* mutants are less virulent than wild type strains, and that vaccination against ExoA confers partial immunity to *P. aeruginosa* infection in animals (Engel 2003). Injection of purified ExoA results in leucopaenia, hepatic necrosis, hypotension and shock when injected into test animals. On a microscopic level, collagen is disrupted, proteoglycan ground substance is lost and widespread endothelial and epithelial cell death is observed (Pitt 1998).

1.10.9.3 Phospholipase C and lipases

P. aeruginosa is known to produce two heat labile phospholipase C enzymes; Plc-H (coded by *plcS*) and Plc-N (coded by *plcN*) (Pitt 1998; Engel 2003). Mutants deficient in Plc-H and Plc-N display decreased alveolar destruction in rabbit pneumonia models, decreased virulence in a *Cenorhabditis elegans* model, and a *plcH* mutant showed decreased virulence in a burnt mouse model when compared to wild-type strains (Engel 2003). The release of diacylglycerol as a bi-product of lecithin hydrolysis is most probably involved in the inflammatory reaction to *P. aeruginosa* infection, as this compound is further reduced to the powerful inflammatory mediator arachidonic acid by bacterial or host cell lipases (Pitt 1998).

P. aeruginosa excretes two lipase molecules, LipA a pro-enzyme of 30 kDa, and a monomeric lipase of 29 kDa, LipC. The lipases of *P. aeruginosa* are active against a variety of fats as well as tween 20 and tween 80 (Pitt 1998). Lipase has been found to inhibit monocyte activity *in vitro* and may be a promoter of the inflammatory response *in vivo* (Engel 2003). LipA is secreted during late logarithmic growth phase and appears to be bound to LPS (Pitt 1998).

1.10.10 The type III secretion system

The type III secretion system (T3SS) of *Pseudomonas aeruginosa* is a sec independent molecular export system (Engel 2003). The T3SS requires direct contact with host cells to function, effector molecules being actively translocated from the prokaryotic to the eukaryotic cell cytosol by means of a complex secretory apparatus (Miyata, Casey *et al.* 2003). Rates of T3SS expression are greater than 80% in acute *P. aeruginosa* infections, while expression occurs to a much lesser degree in chronic CF respiratory infections (Soong, Parker *et al.* 2007).

The T3SS of *P. aeruginosa* is composed of 5 classes of protein; the secretion apparatus, factors requiring translocation, effector proteins, chaperones and transcriptional regulatory proteins. Twenty or more proteins, coded by three contiguous operons, form a flagella related “needle” apparatus that creates a translocation channel, allowing transport of molecules across the bacterial cell membrane. Following attachment of the needle apparatus to the host cell, the hydrophobic proteins PopB and PopD, excreted by the T3SS, form pores in the host cell lipid bilayer, allowing unimpeded transport of effector proteins from the bacterial cytoplasm into the host cell cytoplasm (Engel 2003).

The regulatory protein ExsA is required for optimal transcription of both the translocation and effector molecules of the T3SS (Engel 2003; Smith, Wolfgang *et al.* 2004), and ExsA has been shown to be required for full virulence in animal models of acute pneumonia (Shaver and Hauser 2004). Expression of the T3SS is calcium ion concentration dependent (Kim, Ahn *et al.* 2005), and may be negatively regulated by the Rhl QS pathway (Bleves, Soscia *et al.* 2005). However, more recent work has suggested that whilst T3SS expression is cell density dependent; this faculty is independent of QS signalling (Shen, Filopon *et al.* 2008). Furthermore, it is suggested that the negative regulation of

T3SS is not dependent on QS signalling, but is controlled by the expression of the activity of tryptophan synthase and the presence of its substrate, tryptophan. This finding is not in conflict with the previously findings regarding a correlation between QS signalling molecule prevalence and negative regulation of T3SS, as tryptophan is a precursor molecule for a number of such signalling molecules (Shen, Filopon *et al.* 2008). Significantly, the presence of CD95 on host cell macrophages appears to mediate host cell apoptosis by the T3SS (Engel 2003), suggesting that this may be a receptor site for the T3SS apparatus. Also of note is that antibodies to PopB and the T3SS effector exoenzyme S are detectable by ELISA in the serum of CF patients at the time of colonisation with *P. aeruginosa*, and screening for these antibodies has been suggested as a method of early detection of this important clinical event (Corech, Rao *et al.* 2005).

While the translocation machinery of the T3SS is highly conserved in *P. aeruginosa*, presence or absence the four identified T3SS effector genes is diverse. The protein products of these genes are translocated across the T3SS secretory apparatus bound to chaperones, which maintain their stability during transport (Engel 2003). Four T3SS effector enzymes have been identified thus far, these being exoenzymes S, T, Y and U (ExoS ExoT, ExoY and ExoU) (Finnan, Morrissey *et al.* 2004). Different strains will display differing combinations of T3SS effector enzyme genes. While most strains possess *exoT*, the presence or absence of *exoS*, *exoY* and *exoU* is variable between strains (Engel 2003).

When compared to isolates from other sources, CF strains appear to be significantly more likely to carry *exoS*, but not *exoU* (Engel 2003). It has also been asserted that *exoS* and *exoU* are mutually exclusive (Lee, Smith *et al.* 2005; Vance, Rietsch *et al.* 2005), despite these genes being located at different points along the *P. aeruginosa* genome. However, Finnan, *et al* identified eleven CF clinical strains and one isolated from a plant rhizosphere that were PCR positive for both *exoU* and *exoS* in

conjunction (Finnan, Morrissey *et al.* 2004) and the laboratory control strains PA99 has been shown to express both ExoU and ExoS simultaneously (Shaver and Hauser 2004). Further to this, a study of *exoU* and *exoS* prevalence found that 9% of 45 isolates of *P. aeruginosa* carried both *exoU* and *exoS* concurrently (Wong-Beringer, Wiener-Kronish *et al.* 2007). As *P. aeruginosa* strains deficient in all four known T3SS effector enzymes are capable of T3SS mediated induction of apoptosis in host cells, it is probable that more as yet unidentified T3SS effectors exist (Engel 2003).

1.10.10.1 ExoT and ExoS

The first identified, and most studied, of the T3SS effector enzymes, ExoT and ExoS share 75% homology at the amino acid level (Engel 2003). The amino terminal end of the proteins target G-protein activating protein, while the carboxy terminal ends are ADP ribosylases (Vance, Rietsch *et al.* 2005). The N-terminal targets Rho-like GTPases, resulting in actin cytoskeleton rearrangements with consequent host cell rounding, host cell signalling disruption, inhibition of internalisation and impaired wound healing(Engel 2003; Vance, Rietsch *et al.* 2005). The C-terminal ends of both enzymes show ADP ribosyltransferase (ADPRT) activity, with ExoT showing only 0.2% of the ADPRT activity of ExoS (Engel 2003). ExoS C-terminal domains target small Ras-like proteins Ra1 and Ra5, inhibiting DNA synthesis and internalisation, and inducing apoptosis (Engel 2003; Vance, Rietsch *et al.* 2005). The C-terminal domains of ExoT target CrkI and CrkII, host kinases involved in focal adhesion and phagocytosis (Vance, Rietsch *et al.* 2005).

It has been found that ExoS is the major cytotoxin involved in colonisation, invasion and dissemination during infection, while ExoT protects cultured cells from T3SS dependent lysis *in vitro* (Lee, Smith *et al.* 2005; Soong, Parker *et al.* 2007). It is suggested that ExoT may counteract any damage caused to the host cell by insertion of the T3SS needle apparatus, analogous to the role of YopE in the *Yersinia*

spp. T3SS (Lee, Smith *et al.* 2005). ExoT has been found to play only a minor role in the virulence of *P. aeruginosa* in the lung, although it has been associated with dissemination of disease from the lung to the liver in mice (Shaver and Hauser 2004), and may possess anti-internalisation activity (Soong, Parker *et al.* 2007). Despite its low cytotoxicity in comparison to ExoS, the expression of ExoT alone is sufficient to induce death in *Galleria melonella* (Miyata, Casey *et al.* 2003) and to induce apoptosis in HeLa cells via activation of the mitochondrial apoptosis pathway (Shafikhani, Morales *et al.* 2008). More than 90% of *P. aeruginosa* strains carry *exoT* (Lin, Huang *et al.* 2006; Shafikhani, Morales *et al.* 2008), whilst approximately two thirds of strains carry *exoS* (Jain, Ramirez *et al.* 2004; Wong-Beringer, Wiener-Kronish *et al.* 2007).

1.10.10.2 ExoY and ExoU

Approximately 89% of *P. aeruginosa* strains carry *exoY* (Vance, Rietsch *et al.* 2005), though it is notable that only 70% of *P. aeruginosa* strains isolated from urine have been found to harbour *exoY* (Lin, Huang *et al.* 2006). The structure and function of this genes product; ExoY, is still relatively unknown, it has been shown to be an adenylate cyclase of unknown molecular weight, requiring a presently unidentified host cell protein for activity (Engel 2003). While studies in mouse pneumonia models have shown little ExoY mediated pathology (Lee, Smith *et al.* 2005), other studies in MDCK cell culture have attributed significant cytotoxic effect to ExoY (Lin, Huang *et al.* 2006).

In contrast, the fourth and most recently described exoenzyme of the *P. aeruginosa* T3SS, ExoU, shows marked cytotoxic capabilities. ExoU is a phospholipase with remarkably rapid and fulminant cytotoxic effect (Engel 2003; Finnan, Morrissey *et al.* 2004; Vance, Rietsch *et al.* 2005). One study found that ExoU causes significant cytotoxicity in MDCK cell culture, but that it was not associated with colonisation and invasion in BALB/c mice (Lin, Huang *et al.* 2006). Deletion of *exoU* has been

shown to severely limit the toxicity of *P. aeruginosa* strains in the lung, and the enzyme has been implicated as an agent associated with septic shock and increased disease severity and mortality in pneumonia (Engel 2003; Schulert, Feltman *et al.* 2003; Vance, Rietsch *et al.* 2005; Wong-Beringer, Wiener-Kronish *et al.* 2007). The prevalence of *exoU* amongst clinical *P. aeruginosa* isolates was found by one study to be 27% (Wong-Beringer, Wiener-Kronish *et al.* 2007). The prevalence of *exoU* appears to vary depending on the site of infection, with approximately one third of non-CF respiratory isolates possessing the gene, whilst twice this frequency has been observed in isolates recovered from wound infections (Wong-Beringer, Wiener-Kronish *et al.* 2007), suggesting that *exoU* serves particular functions, depending on disease setting.

1.10.10.2.1 Fluoroquinolone resistance and *exoU*

It is suggested that fluoroquinolone resistance may linked to the increased expression of T3SS virulence (Wong-Beringer, Wiener-Kronish *et al.* 2007). It is postulated that mutations conferring resistance to fluoroquinolones develop either through exposure to fluoroquinolone antibiotics, or by genetic recombination of resistance and virulence genes. Alternatively, the effect of DNA gyrase mutations (resulting in resistance to fluoroquinolone antibiotics) on DNA supercoiling may alter T3SS expression. The latter effect has been documented in previous studies of other bacteria possessing a T3SS (Wong-Beringer, Wiener-Kronish *et al.* 2007). Studies previous to this have established a link between multi-drug resistance and expression of *exoU* (Zaborina, Kohler *et al.* 2006). Importantly, a significant correlation of *exoU* positivity and *gyrA* mutation has been reported (Wong-Beringer, Wiener-Kronish *et al.* 2007). It is known that *exoU* is acquired on a genomic island through horizontal gene transfer, and that this acquisition has an advantageous role in cell fitness within differing environments, and the same selective process may apply to development of the *gyrA* mutation (Wong-Beringer, Wiener-Kronish *et al.* 2007).

1.10.10.3 The type III secretion as a dynamic system

During the course of this review, the concept of virulence as a complex, dynamic and irrevocably intertwined system of multiple effectors and regulators should become clear. *P. aeruginosa* T3SS virulence needs to be considered in the context of the other virulence mechanisms to allow a broad overview of the complex interactions and appreciation of how they together convey the unique environmental and clinical versatility associated with this organism.

1.10.10.4 Action of type III secretion system effector enzymes in combination

There has been limited study of T3SS in the clinical setting. Much work has been performed using *P. aeruginosa* control strains, and to a lesser degree clinical isolates with known genotypes of T3SS effector enzymes in both wild type and with T3SS gene knockout mutants. It has been suggested that strains expressing *exoU* are predominantly cytolytic, whilst strains expressing *exoS* are invasive. Rounding of epithelial cell lines has been found to depend upon ExoS and ExoT, but lysis of macrophages may occur without the involvement of any of the T3SS effector enzyme genes. This suggests that cell lysis through membrane puncture by the T3SS needle apparatus or by an as yet unidentified T3SS effector molecule (Vance, Rietsch *et al.* 2005).

Work on T3SS knockout mutants of PAK, PA103 and PA14 in CHO cell culture and BALB/c mouse models suggested that ExoS was more responsible for cytotoxicity than ExoU, whilst also demonstrating a possible protective role for ExoT (Lee, Smith *et al.* 2005). Conversely, another study employing T3SS mutants of PA103 and PA99 in a mouse model of pneumonia found that the expression of ExoU had a very significant impact on disease severity. This same study showed ExoT as having only a minimal effect. Strains expressing only ExoS showed disease severity in between

ExoU and ExoT expressing mutants (Shaver and Hauser 2004). A study employing a *G. mellonella* caterpillar host virulence model with wild type and mutants of PA14 with deleted effector and translocation apparatus genes was carried out by Miyata, *et al.* This found that ExoS and ExoY were not required for disease in the presence of ExoU, and that either ExoU or ExoT alone was sufficient to induce death (Miyata, Casey *et al.* 2003). A $\Delta pscD$ mutant (unable to construct translocation needle apparatus) showed greatly attenuated virulence in comparison to wild type (Miyata, Casey *et al.* 2003), further suggesting that the T3SS needle apparatus itself is capable of mediating host cell damage, or that an as yet unrecognised T3SS effector molecule exists. A further explanation for this effect could be that lack of transcription of the T3SS effector genes may up-regulate expression of one of the many other virulence factors associated with *P. aeruginosa*.

1.101.10.5 ExsA and regulation of the type III secretion system

Expression of the T3SS effector proteins is positively regulated by the presence of the translation promoter ExsA, whilst the activity of the T3SS translocation needle apparatus is controlled by calcium ion concentration (figure 1.1).

1.10.11 Putative virulence factors

The product of the *nanI* gene of PA01 has been suggested as a potential virulence factor in *P. aeruginosa*. While the role of this product is unknown, it appears to encode a sialidase, which may liberate sialic acid from gangliosides and thereby increase the availability of the attachment site asialo-GM₁ on respiratory epithelial cells. The gene has a significantly different G+C content to the majority of other PA01 genes, and thus most probably was acquired by horizontal transfer from another organism (Lanotte, Watt *et al.* 2004). Lanotte, *et al* also found that the *nanI* gene was most prevalent in isolates from the CF lung and those from plants (Lanotte, Watt *et al.* 2004). A newly discovered

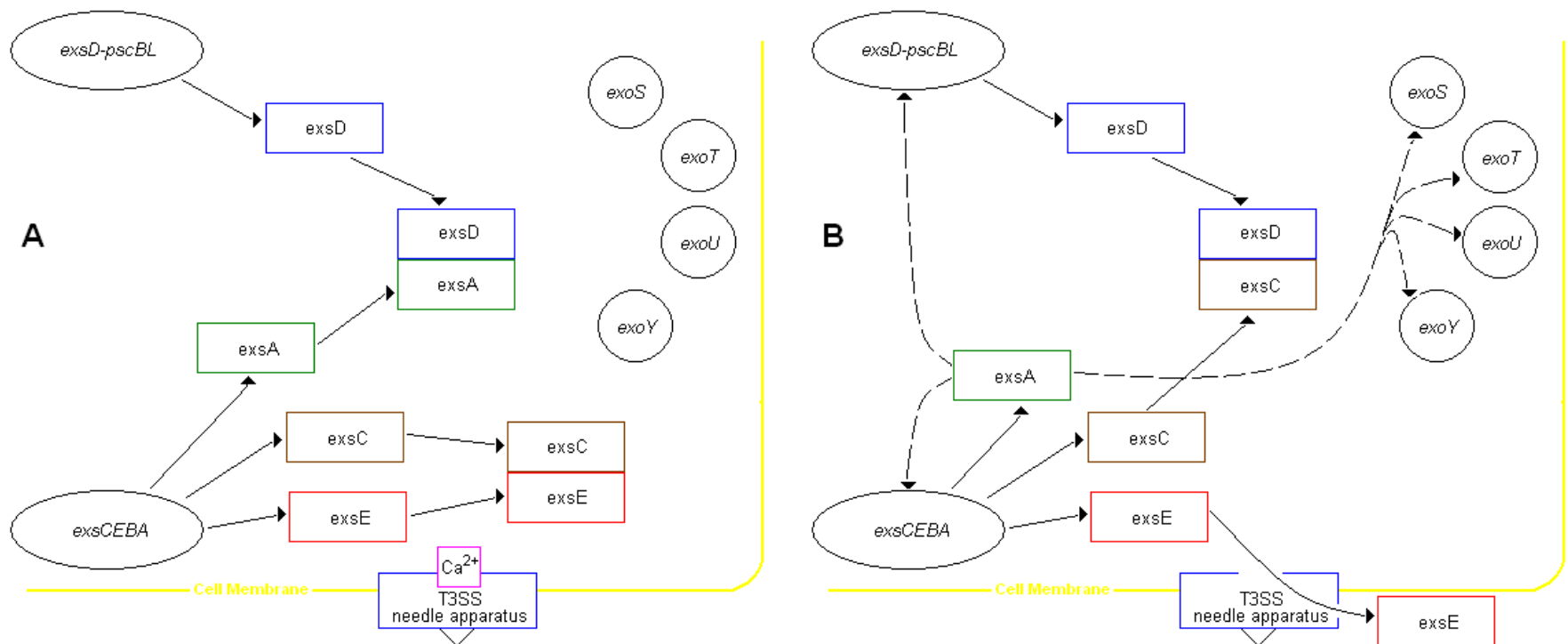


Figure 1.1: The availability of ExsA is regulated by interactions between the other products of the *exsCEBA* operon, the concentration of ExsA, and calcium ions.

A) The separate *exsD-pscBL* gene encodes ExsD, which forms a complex with ExsA, sequestering ExsA from the genes involved in T3SS effector synthesis. A second inactive complex is formed by the ExsC and ExsE products of *exsCEBA*. **B)** When an isolate is exposed to an environment with low concentrations of calcium ions, the T3SS apparatus becomes active and actively secretes ExsE, leaving unbound ExsC. ExsC has a greater affinity for ExsD than ExsA, and thus an ExsC-ExsD complex is formed, leaving unbound ExsA. Unbound ExsA then promotes the translation of *exoSTUY* as well as *exsD-pscBL* and *exsCEBA*, which through positive feedback causes an exponential increase in the intracellular concentration of T3SS effectors and promoters (Filopon, Merieau *et al.* 2006).

virulence factor transportation mechanism is the type VI secretion system (T6SS). This is a sec-independent mechanism for the transportation of effector proteins across the cell membrane. Although this mechanism was only recently discovered, and there is little knowledge of its mechanisms or effector proteins, genes corresponding to T6SS machinery have been found in *P. aeruginosa*, and serine-threonine phosphorylation has been proposed as the triggering mechanism for T6SS activity (Shrivastava and Mande 2008).

1.10.12 Temporal changes in virulence

Of particular importance when considering differing types of *P. aeruginosa* infection is the degree of expression rather than the presence or absence of virulence factors. Jain, *et al* carried out an extensive study of the expression of T3SS effectors in nearly 500 clinical CF isolates of *P. aeruginosa*. This study found that only 12% of isolates from chronically infected CF patients expressed ExoS, ExoU or ExoT (Jain, Ramirez *et al.* 2004). This finding correlated with those of Dacheux, *et al*, who found only 29% of 28 CF isolates from France expressed T3SS proteins (Dacheux, Toussaint *et al.* 2000) and Roy-Burman, who found only 41% of 37 CF isolates from the United States secreted one or more T3SS effector proteins (Roy-Burman, Savel *et al.* 2001). The study by Jain, *et al* found that 90% of environmental isolates (the presumed source of CF isolates) expressed T3SS effector proteins. In newly infected CF patients, only 49% of isolates expressed either ExoS, ExoU or ExoT, with this percentage dropping to 8% in chronically infected children and to only 4% in chronically infected adults (Jain, Ramirez *et al.* 2004). Lee, *et al* compared isolates collected from CF patients soon after colonisation with genotypically identical isolates collected years later. Early isolates (except highly mucoid strains) showed significant cytotoxicity, while later isolates were shown not to result in cytotoxicity in CHO cells (Lee, Smith *et al.* 2005). A study of chronic CF isolates found that this lack

of expression of T3SS effectors was irreversible (strains termed non-inducible) (Filopon, Merieau *et al.* 2006), suggesting that mutations had occurred in regulator genes rather than the effector genes themselves. Smith, *et al* carried out whole genome analysis on genotypically identical *P. aeruginosa* strains collected from the same CF patient 8 years apart and found 24 mutations in virulence factor genes, including one in *exsA*, which acts as a transcriptional regulator of the T3SS. In addition, sequencing of this gene in 91 isolates taken from 29 CF patients found mutations in *exsA* in eight isolates, with six separate patients harbouring *exsA* mutant strains (Smith, Buckley *et al.* 2006).

1.10.13 Eukaryotic virulence models

In recent years, attention has been focussed on whole eukaryotic cell models of virulence in *P. aeruginosa*. Currently, methods for the measurement of virulence may employ amoebae such as *Acanthamoeba*, the slime mould *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the plant *Arabidopsis thaliana* and the fruit fly *Drosophila melanogaster* (Pradel and Ewbank 2004). Such models provide an opportunity to measure the phenotypic virulence of an organism interacting with a eukaryotic host, and allow such work to be performed without the ethical concerns with mammalian models.

One such study compared the virulence properties of *P. aeruginosa* strains recovered from burn wounds and CF sputum in a *Drosophila melanogaster* model. Burns isolates were reliably lethal in both fly feeding (expected not to involve T3SS virulence) and fly nicking (expected to involved T3SS virulence) experiments. However, CF isolates showed great variability in their virulence towards the host using this model. Interestingly, multiple morphotypes of *P. aeruginosa* recovered from a single

CF patient displayed great variance in their respective capacities to cause fly death in the models employed (Lutter, Faria *et al.* 2008).

The *D. discoideum* models presently employed involve simple temporal killing assays; looking at the degree of killing affected by *P. aeruginosa* in mixed culture or *P. aeruginosa* culture supernatants at increasing concentrations over time (Cosson, Zulianello *et al.* 2002). These methods were found to show good correlation with mammalian host models, and certainly provide more information regarding virulence as a complex entity than simple single virulence factor tests.

The first published use of a *D. discoideum* virulence assay in the analysis of *P. aeruginosa* virulence was by Cosson, *et al* in 2002 (Cosson, Zulianello *et al.* 2002). This paper described assays involving the co-culture of *Klebsiella pneumoniae* with *P. aeruginosa* and *D. discoideum* wild-type strain DH1-10 on solid agar, culture of DH1-10 alone on *P. aeruginosa* lawn plates and the effect of *P. aeruginosa* cell culture supernatants on a *D. discoideum* and *K. pneumoniae* co-culture plate. It was found that wild-type strains of PAO1 did not allow the growth of *D. discoideum* plaques. By repeating the assays using virulence gene knockout mutants of the same PAO1 strain, virulence factors expressed through the *rhl* QS pathway, and specifically rhamnolipid were found to be major virulence factors in *D. discoideum*. This study also identified that the presence of multi-drug resistance efflux pump, MexEF-OprN significantly reduced the virulence of *P. aeruginosa*, whilst the presence of the MexAB-OprM and MexCD-OprJ pumps had no effect on virulence. It was found that the degree of virulence of *P. aeruginosa* in the *D. discoideum* virulence assay could be used to predict virulence of the bacterial strains tested in rats (Cosson, Zulianello *et al.* 2002).

Almost simultaneously, a study was published by Pukatzki, *et al* involving wild-type and mutants of PA14 and PA103, and their interactions with *D. discoideum* AX3. This found that AX3 did not grow on lawns of wild-type PA14 or PA103, whilst respective $\Delta lasR$ and $\Delta exoU$ mutants were permissive to plaque formation. Note that *lasR* is a precursor of the *rhl* QS pathway. It also found that pyocyanin production did not affect *P. aeruginosa* virulence in *D. discoideum*. Importantly, this study showed that the lack of plaque formation was due to killing of *D. discoideum*, and not starvation and that the lack of plaque formation in wild-type strains was not due to inhibited phagocytosis (Pukatzki, Kessin *et al.* 2002).

Only two further studies of *P. aeruginosa* virulence in *D. discoideum* have been performed; both utilising modifications of the original virulence assay method published by Cosson *et al* . One study used microarray to determine changes in host transcription shortly after exposure to PA14 and PAO1. This study found large and significant changes in the up-regulation and down-regulation of *D. discoideum* AX4 gene expression when exposed to *P. aeruginosa* compared to controls grown with *Klebsiella aerogenes*. It also found that strain AX4 would feed and grow on stationary phase cultures of PAO1, a finding not observed in PA14. Thus, it was asserted that PAO1 virulence in *D. discoideum* requires active bacterial cell growth. Indeed, this study found that virulence was generally increased in PA14 when compared to PAO1. One obvious source of error in this study was that a *K. aerogenes* co-culture test was used, and thus the virulence interactions of *K. aerogenes* with both *P. aeruginosa* and *D. discoideum* could not be excluded from the analysis (Carilla-Latorre, Calvo-Garrido *et al.* 2008).

The second study discussed identified three new genes involved in the virulence pathways of *P. aeruginosa*; *trp*, *pchH* and *pchI* by examining the permissiveness of mutagenised strains of a clinical

isolate of the bacteria in a *D. discoideum* DH1-10 model. This study also questioned the superiority of animal models compared to the *Dictyostelium* model due to the inability of changes in the virulence of *P. aeruginosa* mutant strains to be detected by *Drosophila* when ingested; inoculation of the fly by pricking with a needle was required to detect virulence. This study found the *D. discoideum* model to be comparable with a mouse model of pneumonia. Once again, the robustness of the *D. discoideum* virulence assay as a model for the identification of new virulence genes in *P. aeruginosa* was demonstrated (Alibaud, Kohler *et al.* 2008).

Despite the obviously significant findings of these studies, and the demonstrated usefulness of the *D. discoideum* assay in analysing the virulence of *P. aeruginosa*, this method has never to date been used to compare the virulence of clinical strains of *P. aeruginosa*. Potentially, this is because all wild-type control strains of the organism thus far analysed have been lethal to *D. discoideum* in co-culture. However, another amoeboid model previously found great variation in the virulence of clinical and environmental strains of *P. aeruginosa* (Fenner, Richet *et al.* 2006).

The virulence of clinical and environmental strains of *P. aeruginosa* has been successfully assayed in co-culture with *Acanthamoeba polyphaga* strain line AP-1. This assay employed culture of *A. polyphaga* in the centre of lawns of 83 community environmental and 69 hospital clinical isolates of *P. aeruginosa*. The majority (75%) of the clinical isolates were isolated from blood culture, with the balance being from BAL specimens, it is unknown if CF isolates were included in the BAL isolates. The distribution of size in plaques formed was bimodal, and a resistant/sensitive cut off was devised based upon this. Environmental strains were found to be significantly less virulent than the clinical isolates in this assay. Whilst no significant trend between resistance to any one of eight antimicrobials

tested and virulence noted in the isolates, it was found that isolates resistant to only one antibiotic appeared less virulent than fully sensitive strains, whilst those resistant to at least four antibiotics were significantly more virulent. One exception to this rule involved two genotypically indistinguishable BAL isolates recovered over a period of four weeks from the same patient, and assumed to represent the same infection, which over this time increased antimicrobial resistance, but decreased virulence towards *A. polyphaga* (Fenner, Richet *et al.* 2006).

There is great versatility and potential of such eukaryotic/prokaryotic models in the study of virulence. The aqueous environmental nature of *P. aeruginosa* would allow it many opportunities for contact with slime moulds and other amoebae in its natural habitat. Moreover, adaptation for interaction with such amoebae may then be reflected in the interaction of *P. aeruginosa* with higher eukaryotic cells, such as the human macrophage, as has been previously recorded in other organisms such as *Legionella pneumophila*. *P. aeruginosa* has not found to be a good food source for *Acanthamoeba castellanii*, and at high bacterial concentrations will kill this amoeba. However, *P. aeruginosa* and *Acanthamoeba* species have been recovered co-existing in contact lens fluid, and amoeba resistant *P. aeruginosa* strain surviving within free-living amoebae have been recovered from the environment (Grueb and Raoult 2004).

1.11 Geographic and demographic setting of study

Tasmania is the southernmost state of Australia, a large island lying between 40° and 45°S latitude, resulting in a significantly cooler climate and different environment to the rest of the country. The Tasmanian population (approximately 483, 000 people) is geographically dispersed and has a significantly more rural population base than the remainder of Australia. Another notable distinction between Tasmania and mainland Australia is the significant proportion of Tasmanians that do not

reside in coastal areas of the island. The population is primarily of Anglo-Celtic descent and there is little population movement either within the island itself or between the island and other parts of Australia or the world. The geographic isolation and lack of population movement in Tasmania has led to a lower degree of overall genetic diversity than may be seen in many other parts of Australia (Blest 2000). The state may be divided into three major population areas, the South (predominantly urban, population 238, 000), the North (urban and rural, population 137, 000) and the North (urban and rural, population 108, 000) (Farrell 2005). While there are seven private and public hospitals in the state, all the tertiary healthcare needs of the Tasmanian population are provided by a single 300 bed hospital in the capital, Hobart.

The low genetic diversity and predominant Anglo-Celtic origins of the Tasmanian population has resulted in the state exhibiting the second highest rate of CF births per head of population in the world (1 in 1600, compared to the average of 1 in 2500 Australia-wide). There are approximately 110 individuals with CF in Tasmania (Blest 2000). It should be noted that the significant mortality within any given CF population makes estimates of population size reliable for only short periods of time. Unlike other Australian states, there is no centralised CF clinic in Tasmania. Many patients in the South attend a clinic at the tertiary care hospital, but the majority of those in the North and North West do not attend a dedicated CF clinic. Many attend their own General Practitioner or Paediatrician and have no contact with other CF sufferers and no significant change in their place of residence. Due of its isolation, stable population demographics, single tertiary healthcare referral centre, lack of a centralised CF clinic, high CF birth incidence and differing ecology to mainland Australia, Tasmania provides a unique set of circumstances for the study the epidemiology and pathogenesis of *P. aeruginosa* infection in humans.

1.12 Conclusion

P. aeruginosa represents a major cause of morbidity and mortality in both the hospital and community setting. The significance of the organism is increasing due to novel adaptations in its virulence, resistance to antimicrobials and epidemiology within these settings and the greater environment. The purpose of this study is to gain a greater understanding of these processes and the mechanisms by which they are occurring within the epidemiological setting of the island state of Tasmania.

CHAPTER TWO

Materials

2.1 Control Strains

The AES1 CF isolate employed in this study was provided by Dr David Armstrong of the Department of Paediatrics, School of Medicine, Monash University, Monash Medical Centre, Clayton, Victoria, Australia. The AES2 strain was provided by Associate Professor Scott Bell of the Department of Respiratory Medicine, Royal Children's Hospital, Herston, Brisbane, Australia. The PA103 laboratory control strain was provided by Assoc. Prof. Iain Lamont of the Biochemistry Department, University of Otago, Dunedin, New Zealand. The PAO1 laboratory control strain was supplied by Professor Sylvia Kirov of the Department of Pathology, School of Medicine, University of Tasmania, Hobart, Tasmania, Australia.

Dictyostelium discoideum AX2 and *Dictyostelium discoideum* X22 were provided by Professor Paul Fisher of the Microbial Cell Biology Group, La Trobe University, Bundoora, Victoria, Australia.

Pre-prepared, lysed agarose plugs of PFGE international standard ACTC BAA-664 (*S. enterica* ser Braenderup H9812) were provided by Ms. Lyn O'Reilly of the Molecular Epidemiology Laboratory, Division of Microbiology and Infectious Diseases, PathWest Laboratory Medicine, Nedlands, Western Australia.

All community environmental strains of *P. aeruginosa* employed in this study were provided by the Public Health Laboratory of the Tasmanian Department of Health and Human Services, St. John's Park, New Town, Tasmania, Australia. The multi-drug resistant clinical isolate of *Klebsiella pneumoniae* and the clinical isolate of *Klebsiella aerogenes* used as controls in the *D. discoideum* assays were supplied by the Microbiology Department of the Royal Hobart Hospital, Liverpool Street,

Hobart, Tasmania, Australia. A number of type strains were also supplied by the Royal Hobart Hospital for use in these study, or used by them for the quality control of assays employed in this study, these were:

| | |
|------------------------------------|------------|
| <i>Aeromonas hydrophila</i> | ATCC 35654 |
| <i>Achromobacter faecalis</i> | ATCC 35655 |
| <i>Escherichia coli</i> | ATCC 11775 |
| <i>Escherichia coli</i> | NCTC 10418 |
| <i>Klebsiella pneumonia</i> | ATCC 13883 |
| <i>Proteus vulgaris</i> | ATCC 3427 |
| <i>Pseudomonas aeruginosa</i> | ATCC 10145 |
| <i>Pseudomonas aeruginosa</i> | ATCC 10662 |
| <i>Pseudomonas aeruginosa</i> | ATCC 27853 |
| <i>Pseudomonas aeruginosa</i> | NCTC 11560 |
| <i>Pseudomonas fluorescens</i> | ATCC 13525 |
| <i>Sphingobacterium multivoran</i> | ATCC 35656 |
| <i>Staphylococcus aureus</i> | NCTC 6571. |

2.2 Chemicals, Material and Suppliers

All chemicals, materials, software and suppliers are listed in table 2.1.

| Material | Manufacturer |
|--|---|
| 0.85 % Saline (2.5 mL vials) | Oxoid (Thebarton, SA, Australia) |
| 10 x PCR buffer for RAPD PCR | Ambion (Austin, TX, USA) |
| 10x PCR buffer | Fisher Biotech (Perth, WA, Australia) |
| 5% Horse blood agar plates | TasLabs (Launceston, TAS, Australia) |
| Acetone | David Craig Galenicals (Laverton, VIC, Australia) |
| Agar bacteriological (No. 1) LP0011 | Oxoid (Thebarton, SA, Australia) |
| Agarose for PCR gels | Promega (Madison, WI, USA) |
| Anaerobic gas generator | Oxoid (Thebarton, SA, Australia) |
| Antimicrobial disc dispenser | Oxoid (Thebarton, SA, Australia) |
| Antimicrobial susceptibility discs | Oxoid (Thebarton, SA, Australia) |
| API 20NE Bacterial identification kit | Biomerieux (Marcy l'Etoile, France) |
| API AUX Medium | Biomerieux (Marcy l'Etoile, France) |
| API on-line database (https://apiweb.biomerieux.com) | Biomerieux (Marcy l'Etoile, France) |
| API reagent james | Biomerieux (Marcy l'Etoile, France) |
| API reagent NIT1 | Biomerieux (Marcy l'Etoile, France) |
| API reagent NIT2 | Biomerieux (Marcy l'Etoile, France) |
| API zinc dust | Biomerieux (Marcy l'Etoile, France) |
| Autoclave | Atherton (Melbourne, VIC, Australia) |
| Azithromycin | Fluca (Buchs, Switzerland) |
| Bacteriological peptone L37 | Oxoid (Thebarton, SA, Australia) |
| Blank 8 mm diameter filter paper discs | Oxoid (Thebarton, SA, Australia) |
| Boric acid for PFGE solutions | ICN Biochemicals (Aurora, OH, USA) |
| Boric Acid | Merck (Darmstadt, Germany) |
| Bovine serum albumin | Promega (Madison, WI, USA) |
| C390 discs | Rosco (Taarstrup, Denmark) |
| Calcium Chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) | BDH AnalaR (Poole, UK) |
| Ceftazidime hydrate | Sigma (St. Louis, MO, USA) |
| Christensen's urea agar slopes | Oxoid (Thebarton, SA, Australia) |
| Conc. Hydrochloric acid | Merck (Darmstadt, Germany) |
| Crystal violet solution | Oxoid (Thebarton, SA, Australia) |
| D-glucose | BDH AnalaR (Poole, UK) |
| Dilute carbol fuschin solution | Australian biostain (Traralgon, VIC, Australia) |
| Dithiothreitol (Sputolysin) | Calbiotech (Spring Valley, CA, USA) |
| Diversity Database | BioRad (Hercules, CA, USA) |
| DMSO | Wak Chemie (Bad Homburg, Germany) |
| DNA 1 kb ladder | Geneworks (Adelaide, SA, Australia) |
| DNA cycler (GeneAmp 9600) | Applied Biosystems (Austin, TX, USA) |
| DNA Spectrophotometer (LKB Ultrospec III) | Pharmacia Biotech (Uppsala, Sweden) |
| dNTP | Finnzymes (Espoo, Finland) |
| dNTP for RAPD PCR | Ambion (Austin, TX, USA) |

| Material | Manufacturer |
|---|--|
| Easy-hyb | Roche Applied Science (Mannheim, Germany) |
| Easy-hyb 10x blocking solution | Roche Applied Science (Mannheim, Germany) |
| Easy-hyb anti-digoxigenin AP | Roche Applied Science (Mannheim, Germany) |
| Easy-hyb digoxigenin-dUTP (DIG) | Roche Applied Science (Mannheim, Germany) |
| Easy-hyb NBT/BCIP stock solution | Roche Applied Science (Mannheim, Germany) |
| EDTA | Sigma (St. Louis, MO, USA) |
| EDTA for PFGE solutions | Invitrogen (Carlsbad, CA, USA) |
| Electrophoresis powerpack (Powerpac 3000) | BioRad (Hercules, CA, USA) |
| Eppendorf tubes | Eppendorf (Hamburg, Germany) |
| Ethanol | Merck (Darmstadt, Germany) |
| Ethidium bromide | Sigma (St. Louis, MO, USA) |
| Gel loading buffer | Geneworks (Adelaide, SA, Australia) |
| Gel-Doc | BioRad (Hercules, CA, USA) |
| Glacial acetic acid | Merck (Darmstadt, Germany) |
| Glass test tubes | Corex (St. Louis, MO, USA) |
| Gram's iodine solution | Oxoid (Thebarton, SA, Australia) |
| Improved Neubauer haemocytometer | Hawksley crystalite (Leicestershire, UK) |
| Isopropanol | Merck (Darmstadt, Germany) |
| Low melting point agarose (Agarose Prep) | Pharmacia Biotech (Uppsala, Sweden) |
| MacConkey agar CM7 | Oxoid (Thebarton, SA, Australia) |
| MacConkey agar plates | TasLabs (Launceston, TAS, Australia) |
| Magnesium chloride (MgCl_2) for PCR | Fisher Biotech (Perth, WA, Australia) |
| Magnesium chloride (MgCl_2) for RAPD PCR | Ambion (Austin, TX, USA) |
| Magnesium sulphate (dried) (MgSO_4) | BDH AnalaR (Poole, UK) |
| Maleic acid | Ajax Chemicals (Melbourne, Australia) |
| McFarland 0.5 colorimeter control | Remel (Lenexa, KS, USA) |
| McFarland 0.5 turbidity control | Biomerieux (Marcy l'Etoile, France) |
| McFarland 1.0 colorimeter control | Remel (Lenexa, KS, USA) |
| Microbial DNA isolation kit | MoBio (Carlsbad, CA) |
| Microfuge tubes | Quality Scientific Plastics (Petaluma, CA, USA) |
| Mineral oil (Paraffin) | Faulding (Virginia, QLD, Australia) |
| mPAC agar plates | Oxoid (Thebarton, SA, Australia) |
| Mueller Hinton agar plates | Oxoid (Thebarton, SA, Australia) |
| Mueller Hinton agar plates for PFGE | Excel Laboratory Products (Perth, WA, Australia) |
| Neutralised bacteriological peptone L34 | Oxoid (Thebarton, SA, Australia) |
| N-lauryl sarcosine | ICN Biochemicals (Aurora, OH, USA) |
| Office Excel 2003 version (11.8220.8202) SP3 | Microsoft (Redmond, WA, USA) |
| Oxidase test paper strips | Oxoid (Thebarton, SA, Australia) |
| PCR Primer (RAPD 272) | Geneworks (Adelaide, SA, Australia) |
| PCR primers (<i>Pseudomonas aeruginosa</i> specific) | Geneworks (Adelaide, SA, Australia) |
| PCR primers (virulence factors) | Operon (Valencia, CA, USA) |

| Material | Manufacturer |
|--|---|
| PFGE Chef apparatus (Chef-DRIII) | BioRad (Hercules, CA, USA) |
| Phosphate buffered saline (dulbecco A) tablets | Oxoid (Thebarton, SA, Australia) |
| Pipette tips | Quality Scientific Plastics (Petaluma, CA, USA) |
| Pipettes | Labsystems (Helsinki, Finland) |
| Plastic PCR cycling tubes (8 cap) | Quality Scientific Plastics (Petaluma, CA, USA) |
| Polaroid Black and White 667 Polaroid film | Kodak (Rochester, NY, USA) |
| Polaroid Camera | Kodak (Rochester, NY, USA) |
| Potassium phosphate dibasic (anhydrous) (K_2HPO_4) | MP Biomedicals (Aurora, OH, USA) |
| Potassium phosphate monobasic (KH_2PO_4) | Sigma (St. Louis, MO, USA) |
| Proteinase K powder | Promega (Madison, WI, USA) |
| <i>Pseudomonas</i> agar CM0559 | Oxoid (Thebarton, SA, Australia) |
| QIAquick buffer CG | Qiagen (Hilden, Germany) |
| QIAquick buffer EB | Qiagen (Hilden, Germany) |
| QIAquick buffer PE | Qiagen (Hilden, Germany) |
| QIAquick buffer QG | Qiagen (Hilden, Germany) |
| QIAquick column | Qiagen (Hilden, Germany) |
| Restriction enzyme <i>Spe</i> 1 | Promega (Madison, WI, USA) |
| Restriction enzyme buffer A | Promega (Madison, WI, USA) |
| Sarcosyl | Sigma (St. Louis, MO, USA) |
| Sensitest agar | Taslabs (Launceston, TAS, Australia) |
| SKG agarose (Agarose Prep) | Pharmacia Biotech (Uppsala, Sweden) |
| Sodium Acetate (CH_3COONa) | Ajax Chemicals (Melbourne, Australia) |
| Sodium chloride (NaCl) | Scharlau Chemicals (Barcelona, Spain) |
| Sodium Citrate ($CH_6H_5Na_3O_7 \cdot 2H_2O$) | Sigma (St. Louis, MO, USA) |
| Sodium dodecyl sulfate | BioRad (Hercules, CA, USA) |
| Sodium Hydroxide (NaOH) Pellets | LabServ Biolab (Auckland, New Zealand) |
| Sodium phosphate dibasic ($Na_2HPO_4 \cdot 2H_2O$) | Sigma (St. Louis, MO, USA) |
| SPSS 12.0.1 for Windows, 2003 | SPSS (Chigaco, IL, USA) |
| Sterile plastic petri dishes | LabServ Biolab (Auckland, New Zealand) |
| Taq polymerase for RAPD PCR (Super Taq) | Ambion (Austin, TX, USA) |
| Taq polymerase (Fisher Taq) | Fisher Biotech (Perth, WA, Australia) |
| Tobramycin sulfate salt | Sigma (St. Louis, MO, USA) |
| Triptone LP0042 | Oxoid (Thebarton, SA, Australia) |
| Tris | Invitrogen (Carlsbad, CA, USA) |
| Tris for PFGE solutions | Merck (Darmstadt, Germany) |
| Tryptone soya broth CM0129 | Oxoid (Thebarton, SA, Australia) |
| UV light box | Alpha Innotech (San Leandro, CA, USA) |
| Vitek 1 Analyser | Biomerieux (Marcy l'Etoile, France) |
| Vitek GNI+ cards | Biomerieux (Marcy l'Etoile, France) |
| Vitek nephelometer | Biomerieux (Marcy l'Etoile, France) |
| Vitek sterile plastic straw | Biomerieux (Marcy l'Etoile, France) |
| Weight balance (A260 DeltaRange) | Mettler (Zurich, Switzerland) |
| Yeast Extract LP0021 | Oxoid (Thebarton, SA, Australia) |
| λ DNA Ladder | Roche Applied Science (Mannheim, Germany) |

Table 2.1: Chemicals, materials, software and suppliers

2.3 Solutions

2.3.1 General solutions

EDTA (0.5 M Stock, pH 8.0)

EDTA 186.1 g

Make up to 1 L with distilled H₂O

Correct to pH 8.0 with Sodium hydroxide pellets

Autoclave at 121°C for 20 minutes

Ethidium Bromide

1 tablet ethidium bromide

Sterile distilled H₂O 10 mL

Wrap container in aluminium foil to protect from light.

Phosphate Buffered Saline

Phosphate buffered saline (Dulbecco A) tablets 1 tablet

Make up to 100 mL with distilled H₂O

Autoclave at 121°C for 20 minutes

Proteinase K solution

Proteinase K powder 0.200 g

Make up to 10 mL with distilled H₂O

Ensure powder has completely dissolved in distilled H₂O. Dispense 160 µL aliquots in 0.6 mL microfuge tubes and store at -20°C.

0.85% Saline Solution

Sodium Chloride 8.5 g

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

20 % Sodium Dodecyl Sulfate

SDS 100 g

Make up to 500 mL with distilled H₂O

Autoclave at 121°C for 20 minutes

10% Sarcosyl

N-lauryl sarcosine 100 g

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

50x Tris Acetate EDTA (stock solution)

Tris 242 g

0.5 M EDTA (pH 8.0) 100 mL

Glacial acetic acid 57.1 mL

Make up to 1 L with Distilled H₂O

Autoclave at 121°C for 20 minutes

1 x Tris Acetate EDTA (working solution)

| | |
|--------------------------------------|-------|
| 50x Tris acetate EDTA stock solution | 20 mL |
|--------------------------------------|-------|

Make up to 1 L with Distilled H₂O

Tris Chloride (2.0 M Stock, pH 8.0)

| | |
|------|---------|
| Tris | 242.2 g |
|------|---------|

| | |
|-------------------------|--------|
| Conc. Hydrochloric acid | 100 mL |
|-------------------------|--------|

Correct to pH 8.0 with Conc. Hydrochloric acid

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

2.3.2 Pulsed field gel electrophoresis solutions**Cell Suspension Buffer**

| | |
|-------------------------------------|-------|
| Tris chloride (2.0 M Stock, pH 8.0) | 50 mL |
|-------------------------------------|-------|

| | |
|--------------------|--------|
| 0.5M EDTA (pH 8.0) | 200 mL |
|--------------------|--------|

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

5 x Tris Borate EDTA (stock solution)

| | |
|--|--------|
| Tris | 54 g |
| Boric acid | 27.5 g |
| EDTA (0.5 M Stock, pH 8.0) | 20 mL |
| Make up to 1 L with distilled H ₂ O | |
| Autoclave at 121°C for 20 minutes | |

0.5 x Tris Borate EDTA (working solution)

| | |
|--|--------|
| 5 x Tris borate EDTA (stock solution) | 250 mL |
| Make up to 2.5 L with distilled H ₂ O | |

Tris-EDTA Buffer

| | |
|--|--------|
| Tris chloride (2.0 M Stock, pH 8.0) | 100 mL |
| EDTA (0.5 M Stock, pH 8.0) | 100 mL |
| Make up to 1 L with distilled H ₂ O | |
| Autoclave at 121°C for 20 minutes | |

Cell Lysis Buffer

| | |
|--|--------|
| Tris chloride (2.0 M Stock, pH 8.0) | 25 mL |
| EDTA (0.5 M Stock, pH 8.0) | 100 mL |
| 10% Sarcosyl | 100 mL |
| Make up to 1 L with distilled H ₂ O | |
| Autoclave at 121°C for 20 minutes | |
| Proteinase K solution | 2.5 mL |
| Add proteinase K solution immediately prior to use | |

2.3.3 DNA hybridisation solutions**Antibody Solution (per 100 cm² of nylon membrane used)**

| | |
|---|--------|
| Easy-hyb Anti-digoxigenin AP | 400 µL |
| (Centrifuged at 1000 rpm for 10 minutes; 400 µL aliquot removed from surface) | |
| 1 x Blocking Solution | 20 mL |

1x Blocking Solution

| | |
|---------------------------------|-------|
| 10 x Easy-hyb blocking solution | 10 mL |
| Maleic acid buffer | 90 mL |

Colour Substrate Solution (per 100 cm² of nylon membrane used)

| | |
|----------------------------------|--------|
| Easy-hyb NBT/BCIP stock solution | 200 µL |
| Detection buffer | 10 mL |

Detection Buffer

Tris 6.057 g

Sodium chloride 2.922 g

Make up to 400 mL with distilled H₂O

Correct to pH 9.5 with 1 M Hydrochloric acid

Autoclave at 121°C for 20 minutes

Maleic Acid Buffer

Maleic acid 11.610 g

Sodium chloride 8.767 g

Make up to 1 L with distilled H₂O

Correct to pH 7.5 with Sodium hydroxide pellets

Autoclave at 121°C for 20 minutes

20x Salt Sodium Citrate (SSC)

Sodium chloride 87.660 g

Sodium citrate 44.115 g

Make up to 500 mL with distilled H₂O

Correct to pH 7.0 with 1 M Hydrochloric acid

Autoclave at 121°C for 20 minutes

Tween 20 (0.3% v/v)

Tween 20 30 µL

Distilled H₂O 10 mL

Washing Buffer

| | |
|---------------------|----------|
| Maleic acid buffer | 498.5 mL |
| Tween 20 (0.3% v/v) | 1.5 mL |

2.3.4 *Dictyostelium discoideum* culture solutions**SS Solution**

| | |
|----------------------------|---------|
| Sodium Chloride | 0.585 g |
| Potassium chloride | 0.750 g |
| Calcium chloride dihydrate | 0.400 g |

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

2.4 Culture Media

Unless otherwise stated, all culture media was allowed to cool to 50°C in a water bath prior to pouring 30 mL aliquots into sterile plastic petri dishes, the media was set at room temperature. One plate from each batch was incubated at 37°C overnight and examined for growth the following day as quality control. All media was stored at 4°C, and allowed to equilibrate to room temperature prior to use.

2.4.1 General culture and storage media

Glycerol Freezing Medium

| | |
|----------|-------|
| Glycerol | 25 mL |
|----------|-------|

| | |
|-----------------------------|-------|
| L37 Bacteriological peptone | 1.0 g |
|-----------------------------|-------|

| | |
|-----------------|-------|
| Sodium Chloride | 0.5 g |
|-----------------|-------|

Make up to 100 mL with distilled H₂O

Autoclave at 121°C for 20 minutes

Dispense into 1.5 mL cryogenic vials

Minimal Maintenance Media

| | |
|------------------------------------|-------|
| Agar bacteriological (No.1) LP0011 | 5.0 g |
|------------------------------------|-------|

| | |
|-----------------|-------|
| Sodium chloride | 5.0 g |
|-----------------|-------|

| | |
|-----------------------------|-------|
| Bacteriological peptone L37 | 3.5 g |
|-----------------------------|-------|

| | |
|---|-------|
| Neutralised bacteriological peptone L34 | 2.5 g |
|---|-------|

| | |
|--------------------------|-------|
| Sodium Phosphate dibasic | 2.9 g |
|--------------------------|-------|

| | |
|-------------------------------|-------|
| Potassium phosphate monobasic | 1.3 g |
|-------------------------------|-------|

Make up to 1 L with distilled H₂O

Heat until dissolved, correct to pH 6.7 with glacial acetic acid, distribute into 3 mL aliquots in sterile bijoux bottles. Autoclave at 121°C for 20 minutes.

MacConkey Agar

MacConkey agar CM7 53 g

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

Pseudomonas Selective Agar

Pseudomonas agar CM0559 48.4 g

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

Tryptone Soya Agar with Yeast Extract

Tryptone soya broth CM0129 60 g

Yeast extract LP0021 6 g

Agar bacteriological (No.1) LP0011 24 g

Make up to 1 L with distilled H₂O

Autoclave at 121°C for 20 minutes

Tryptone Soya Broth

Tryptone soya broth CM0129 30 g

Make up to 1 L with distilled H₂O

Ensure powder has completely dissolved into distilled H₂O

Dispense 10 mL aliquots into 50 mL glass universal screw cap containers

Autoclave at 121°C for 20 minutes

2.4.2 *Dictyostelium discoideum* culture media

SM Agar

| | |
|---|--------|
| Neutralised bacteriological peptone L34 | 5 g |
| Yeast extract LP0021 | 2.5 g |
| D-Glucose | 5 g |
| Agar bacteriological (No.1) LP0011 | 17 g |
| Sodium phosphate dibasic | 0.21 g |
| Potassium phosphate monobasic | 1.9 g |
| Potassium phosphate dibasic (anhydrous) | 1.3 g |
| Magnesium sulfate (dried) | 0.49 g |
| Correct to pH 6.5 with glacial acetic acid | |
| Make up to 1 L with distilled H ₂ O | |
| Autoclave at 121°C for 20 minutes | |
| Cool in a water bath to 50°C prior to pouring exactly 30 mL into plastic petri dishes | |

SM Agar With Azithromycin

| | |
|--------------|------|
| Azithromycin | 4 mg |
|--------------|------|

Add azithromycin powder to SM agar solution (cooled to 50°C) and invert agar bottle 20 times to dissolve powder prior to pouring exactly 30 mL into plastic petri dishes.

SM Agar With Ceftazidime

Ceftazidime hydrate 6.25 mg

Add ceftazidime hydrate powder to SM agar solution (cooled to 50°C) and invert agar bottle 20 times to dissolve powder prior to pouring exactly 30 mL into plastic petri dishes.

SM Agar With Tobramycin

Tobramycin sulfate 25 mg

Add tobramycin sulfate powder to SM agar solution (cooled to 50°C) and invert agar bottle 20 times to dissolve powder prior to pouring exactly 30 mL into plastic petri dishes.

CHAPTER THREE

Methods

3.1 Bacterial Isolation from CF Sputum by University of Tasmania

Culture, isolation and preservation of University of Tasmania CF library bacterial strains used in this study was primarily performed by Ms. Vicki Carroll or, in some cases the author, or Dr. Che O'May of the Cystic Fibrosis Research Group, Menzies Research Institute, University of Tasmania. Sputum samples were diluted 1:1 with 10% dithiothreitol (Sputolysin, Calbiotech) in phosphate buffered saline (PBS, materials 2.3.1), vortexed and allowed to mucolyse for 20 min at room temperature, agitating every 5 min. 100 μL of the mucolysed sputum was mixed with 900 μL of sterile PBS by vortexing, resulting in a 1:20 dilution. Further 1:10 serial dilutions were made until five specimens of diluted sputum were available for culture (5×10^{-1} , 5×10^{-2} , 5×10^{-3} , 5×10^{-4} , 5×10^{-5}). 100 μL of each dilution was inoculated onto the following media using a sterile pipette tip; 4 x Tryptone soya agar with 5% horse blood (Taslabs), MacConkey Agar (Taslabs), and Pseudomonas selective agar (PSA, materials 2.4.1). The inoculum was spread in a circular motion with an ethanol flamed glass spreader to form confluent lawn of growth. Two plates of Tryptone soya agar with 5% horse blood and incubated at 37°C sealed with an anaerobic gas generator (Oxoid). All other plates were incubated at 37°C in aerobic conditions.

Following 48 hours incubation, the plates were examined for growth. Colonies morphologically resembling *P. aeruginosa*, having a positive oxidase test (method 3.7), and growing on PSA agar were considered to be *P. aeruginosa*.

3.2 Bacterial Isolation of Royal Hobart Hospital Clinical Isolates

CF and non-CF clinical isolates of *P. aeruginosa* were cultured by staff of the Department of Microbiology and Infectious Diseases of the Royal Hobart Hospital, including the author, as part of

their routine laboratory practice. Isolates were cultured directly from clinical specimens onto MacConkey agar (Taslabs) in aerobic conditions or Tryptone soya agar with 5% horse blood (Taslabs) incubated in 5% CO₂ at 37°C. Colonies morphologically resembling *P. aeruginosa*, having a positive oxidase test (method 3.7), and showing resistance to the compound C390 (method 3.8) were considered to be *P. aeruginosa*. Purity plates on sensitest agar (Taslabs) were provided for isolate storage (method 3.5). Site of isolation, mucoid phenotype upon first isolation, patient sex, patient age, ward and in patient or out patient status were recorded by the author.

3.3 Bacterial Isolation from Hospital Environment

Sterile cotton swabs were collected from likely sources of *P. aeruginosa* in all wards of the Royal Hobart Hospital by the author with the assistance of a member of the Royal Hobart Hospital Infection Control cleaning team. Swabs were rolled over the surface of MacConkey agar plates and a C390 disc placed in the centre of the agar. Agar plates were incubated for 48 hours at 42°C under aerobic conditions. Mucoidy on first isolation was recorded. Oxidase (method 3.7) positive, colonies growing up to the C390 disc were selected and sub-cultured onto fresh MacConkey agar to obtain pure cultures. Purity plates were incubated at 37 °C in air overnight. Isolates from purity plates were stored in MMM at room temperature, protected from light.

3.4 Bacterial Isolation from Community Environment

Community Environmental isolates were cultured by staff of the Tasmanian Public Health laboratory (Hobart). Ten millilitres of water to be tested was filtered through a 1 µm filter with a vacuum apparatus. The filter paper was placed upon mPAC agar (Oxoid) and incubated at 42°C for 48 hours.

Colonies resembling *P. aeruginosa* (0.8 to 2.2 mm in diameter, flat with lighter outer rims and brownish to green-black centres) were selected as presumptive *P. aeruginosa*.

Quality Control: Positive: *Pseudomonas aeruginosa* ATCC 10145

Negative: *Escherichia coli* ATCC 11775

3.5 Isolate Storage

A single, well isolated colony of each isolate was inoculated into minimal maintenance media (MMM) and glycerol freezing media (materials 2.4.1) using a sterile bacteriological loop and stored at room temperature, protected from light.

3.6 Gram Stain Reaction

This test relies upon the capacity of bacterial cells to retain the stain crystal violet. Cells with a thick peptidoglycan wall with many teichoic acid cross linkages will retain crystal violet upon decolourisation with acetone or ethanol, while those with a thin peptidoglycan wall will not retain crystal violet.

A portion of a single, well isolated colony was removed from the agar plate with an sterile bacteriological loop and rubbed onto a glass microscope slide. The slide was allowed to dry and fixed with heat by passing it over a Bunsen burner flame. The slide was flooded with crystal violet (Oxoid) for thirty seconds, followed by washing in tap water. The slide was then flooded with Gram's iodine (Oxoid) for a further thirty seconds, followed by a second tap water wash. A 60% ethanol (Merck), 40% acetone (David Craig Galenicals) solution was then used to flood the slide until all soluble crystal violet was removed, followed by an immediate wash in tap water. Finally, the slide was flooded with dilute carbol fuchsin (Australian Biostain) for thirty seconds and then washed once more in tap water.

Slides were then blotted dry on blotting paper and examined under a microscope at x1000 magnification (with oil immersion). A Gram positive reaction was defined by the retention of crystal violet, demonstrated by a navy blue colour, whilst a negative reaction was defined by the removal of all crystal violet and subsequent counter-staining with carbol fuschin, as demonstrated by a bright pink colour. Morphology of bacterial cells (bacillus or coccus) was also recorded. Quality control of this test was carried out weekly by staff of the Department of Microbiology and Infectious Diseases of the Royal Hobart Hospital (including the author) as part of their routine laboratory practice.

Quality Control: Gram Positive Coccus: *Staphylococcus aureus* NCTC 6571

Gram Negative Bacillus: *Escherichia coli* NCTC 10418

3.7 Oxidase Testing

This test is used to determine the presence of the enzyme cytochrome oxidase in a given isolate. A portion of a single, well isolated colony was removed from the agar plate with an orange stick and rubbed onto commercial paper strips impregnated with the substrate tetramethyl-*p*-phenylenediamine dihydrochloride (Oxoid). Visible production of the purple coloured end product, indophenol within five seconds of inoculation was considered to be a positive result, no colour change within five seconds was considered to represent a negative result. Quality control of this test was carried out weekly by staff of the Department of Microbiology and Infectious Diseases of the Royal Hobart Hospital (including the author) as part of their routine laboratory practice.

Quality Control: Positive: *Pseudomonas aeruginosa* NCTC 11560

Negative: *Escherichia coli* NCTC 10418

3.8 C390 Reaction

The compound C390 (Rosco) has no inhibitory effect on the growth of *Pseudomonas aeruginosa*, but will inhibit the growth of all other *Pseudomonas spp.*

P. aeruginosa isolates were sub cultured from MMM onto MacConkey agar and incubated at 37°C in air for 48 hours. For non-mucoid isolates, a sterile straight wire was vertically stabbed into a single, isolated colony. For mucoid isolates, the wire loop was used to obtain an amount of colony approximately 2mm³ in size from a single, isolated colony. The material collected on the wire loop was then suspended in 2.5 mL of sterile 0.85% saline (Oxoid).

The saline suspension was poured onto a Sensitest agar (TasLabs), ensuring that the entire surface of the agar was covered. Excess saline suspension was removed from the agar with a sterile pastuer pipette. Sensitest plates were then allowed to dry in air for 30 minutes. A single disc containing the compound C390 was placed onto the surface of the agar plate, followed incubation overnight at 37°C. Resistance to C390 was defined as no zone of inhibition around the C390 disc, whilst sensitivity was defined as any zone of inhibition around the disc. Quality control of this test was carried out weekly by staff of the Department of Microbiology and Infectious Diseases of the Royal Hobart Hospital (including the author) as part of their routine laboratory practice.

Quality Control: Positive: *Pseudomonas aeruginosa* ATCC 10662
Negative: *Pseudomonas fluorescens* ATCC 13525

3.9 Vitek Identification of Bacterial Species

Bacterial Vitek identification was performed using Vitek GNI+ (Biomerièux) cards in a Vitek 1 analyser. Vitek GNI+ cards compose of a plastic card containing 30 microwells, each microwell being

coated with a single biochemical substrate. Oxidase reactions (method 3.7) are inputted manually by the operator during inoculation of the GNI+ card to complete the biochemical profile. Isolates were grown on MacConkey agar (Taslabs) at 37°C under aerobic conditions for 24 hours prior to analysis. Vitek GNI+ analysis was undertaken according to the manufacturer's instructions in a Vitek 1 analyser. Quality control was performed monthly by the staff of the Royal Hobart Hospital, according to the manufacturer's instructions and in accordance with National Australian Testing Authority (NATA) guidelines.

Quality Control: *Klebsiella pneumoniae* ATCC 13883

3.10 API 20NE Identification of Bacterial Species

The API 20NE (Biomerièux) non fermentative Gram negative bacillus identification system consists of a plastic strip containing 20 microwells (consisting of a tube and a cupule), each coated with a separate biochemical substrate. Isolates were grown on MacConkey agar (Taslabs) at 37°C under aerobic conditions for 24 hours prior to analysis. Oxidase reactions (method 3.7) were inputted manually following determination of other testing results. API 20NE testing was performed according to the manufacturer's instructions. The resultant biocode was entered into the Biomerièux on-line database (<https://apiweb.biomerieux.com>) and used to determine a percentage probability and statistical significance (t) of identification. Quality control was performed upon each new kit lot number by the staff of the Royal Hobart Hospital, according to the manufacturer's instructions and in accordance with the National Australian Testing Authority (NATA) guidelines.

Quality Control: *Sphingobacterium multivoran* ATCC 35656

Aeromonas hydrophila ATCC 35654

Pseudomonas aeruginosa ATCC 27853

Achromobacter faecalis ATCC 35655

3.11 CLSI Antimicrobial Susceptibility Testing

P. aeruginosa isolates were sub cultured from MMM onto MacConkey agar and incubated at 37°C in air for 48 hours. A 2.5 mL volume of 0.85% sterile saline in a glass test tube was placed in and used to zero a Vitek nephelometer. Following this, a 0.5 McFarland turbidity colorimeter control (Remel) was tested in the nephelometer to confirm reading accuracy. Sufficient amounts of test organism were emulsified in the saline to reach an absorbance in the red test zone (equivalent to a 0.5 Mcfarland standard). A sterile swab was immersed in the saline suspension, excess saline removed by swirling against the side of the tube. The swab was used to make two lawn plates on Mueller Hinton agar (Oxoid) by crossing the agar in three separate directions using the “cross-hatch method”. Inoculated agar plates were allowed to dry in air for 30 minutes. Up to six separate antimicrobial susceptibility discs (Oxoid) were placed on each agar plate using a commercial antimicrobial disc dispenser (Oxoid). Plates were incubated for 18-24 hours in air at 37°C and the diametres of zones of inhibition around the antimicrobial discs recorded to the nearest whole millimetre using a graduated ruler. In cases where sparse growth was noted, plates were re-incubated for a further 18-24 hours and zone diameters recorded on the second day of incubation. Results were reported as “sensitive”, “intermediate” or “resistant” based upon the cut-off values recommended by CLSI (Appendix A). All tests were performed in duplicate on separate occasions, using new batches of media. In cases where discrepant

results were observed, the test was repeated in triplicate and the average result of all three tests recorded.

Quality Control: *Pseudomonas aeruginosa* ATCC 27853

3.12 DNA Extraction

DNA extraction was performed using the Mo Bio Microbial DNA Isolation Kit according to the manufacturer's instructions. A culture of bacteria was incubated on MacConkey agar (materials 2.4.1) at 37°C for between 24 and 48 hours. Well isolated colonies were removed from the agar plate and emulsified into 1.8 mL of ddH₂O in an eppendorf tube until a turbid suspension was obtained. This suspension was then washed twice by centrifugation at 10,000 x g for 30 seconds in an eppendorf centrifuge, followed by addition of a further 1.8 mL of ddH₂O to the pellet and resuspension of the pellet into solution. This suspension centrifuged for a further 10,000 x g for 30 seconds, after which all remaining ddH₂O was removed from the pellet.

300 µL of MicroBead solution was added to the eppendorf tube and the pellet resuspended, followed by emulsification by vortexing the tube on a vortex mixer. The entire solution was then transferred into a MicroBead tube. 50 µL of solution MD1 was added to the MicroBead tube, followed by vortexing. MicroBead tubes were placed in a water bath at 65 °C for ten minutes, with removal from the water bath and vortexing every three minutes during this incubation period. MicroBead tubes were then centrifuged at 10,000 x g for 30 seconds, prior to the supernatant being transferred to a clean microcentrifuge tube.

A 100 μL of solution MD2 added to the microcentrifuge tube with an pipette. This solution was vortexed for five seconds and then incubated for five minutes at 4°C . Following incubation, the tube was then centrifuged in an eppendorf microcentrifuge at $10,000 \times g$ for one minute.

The supernatant of this solution was removed following centrifugation, taking care not to dislodge the pellet, and placed in a new 1.9 mL eppendorf tube. Following this, 900 μL of solution MD3 was added to the supernatant, and the solution vortexed for a further five seconds. A MoBio spin filter was loaded with 700 μL of the MD3/supernatant solution and then centrifuged at $10,000 \times g$ for 30 seconds, and the flow through discarded. The same filter was loaded with all remaining MD3/supernatant solution and centrifuged once more at $10,000 \times g$ for 30 seconds, followed by discarding the flow through liquid.

Following these purification steps, 300 μL of solution MD4 was added to the spin filter, which was then centrifuged at $10,000 \times g$ for 30 seconds, the flow through being discarded. The spin filter was centrifuged at $10,000 \times g$ for a further one minute to completely remove all flow through and dry the surface of the filter. This was followed by the addition of 50 μL of MD5 onto the centre of the dry filter using an pipette, without touching the sides of the spin filter or the surface of the filter itself. The spin filter was then centrifuged at $10,000 \times g$ for 30 seconds. The filter column was then discarded and the flow through kept in the 1.9 mL eppendorf tube that comprised part of the flow through apparatus. The flow through from this step contained 50 μL of purified bacterial DNA in solution MD5. A further 100 μL of sterile distilled water was added and the solution vortexed for five seconds to dilute the DNA and provide a working volume of extract. This DNA extract solution was frozen at -80°C for later use.

Quality Control: Positive: *Pseudomonas aeruginosa* PAO1

Negative: Sterile ddH₂O contamination control

3.13 *Pseudomonas aeruginosa* Specific PCR

The *P. aeruginosa* specific PCR described by Spilker, *et al* (Spilker, Coenye *et al.* 2004) was employed in this study. Primers sequences are described in appendix B. PCR protocol was initial DNA denaturation at 94°C for two minutes, followed by 30 cycles of 94 °C for 40 sec, 58 °C for 30 seconds, and extension at 72 °C for 60 seconds, followed by a final extension at 72 °C for 7 minutes. PCR products were electrophoresed at 100V for 75 minutes on a 2% agarose gel, stained with ethidium bromide and visualised under UV light.

Quality Control: Positive: *Pseudomonas aeruginosa* PAO1
 Negative: *Pseudomonas fluorescens* ATCC 13525
 Extracted sterile ddH₂O contamination control

3.14 Random Amplified Polymorphic DNA PCR

RAPD PCR was performed using the method described by Mahenthiralingam, *et al* (Mahenthiralingam, Campbell *et al.* 1996). DNA concentration of extracts was measured using a DNA spectrophotometer (Pharmacia LKB-Ultrospec III), and then corrected to a concentration of 2.4 ng/μL with dH₂O. Each RAPD PCR reaction solution consisted of 0.825 U Taq polymerase, 2μL of 10x PCR buffer, 1.2 μL of MgCl₂, 0.4 μL DMSO, 0.16 μL of dNTPs (Fisher biotech), 0.1 μL of a 100 uM solution of the single primer 272 (appendix B) and 2.4 ng of extracted DNA, this solution was then adjusted to a volume of 20 μL with ddH₂O. PCR was performed on a GeneAmp 9600 cyclor (Applied Biosystems). The PCR protocol consisted of an initial 4 cycles of 94°C for five minutes, 36 °C for five minutes and 72 °C for five minutes followed by 30 cycles of 94 °C for one minutes, 36 °C for one minute and 72 °C for 2 minutes, then a final extension n of 72 °C for 10 minutes. A 1 kb DNA ladder marker (Geneworks), the relevant PAO1 positive control and dH₂O negative control were incorporated

into each run of gels performed. Submarine electrophoresis was performed in 1x Tris Acetate EDTA buffer at 100V for three hours in a 2% agarose gel and stained with ethidium bromide, followed by visualization under UV light. Digital photographs of gels were taken using a Gel-Doc (Biorad).

Quality Control: Positive: *Pseudomonas aeruginosa* PAO1
 Negative: Sterile ddH₂O contamination control extract

3.15 Pulsed Field Gel Electrophoresis

PFGE was performed on all isolates demonstrating >90% similarity by RAPD PCR. Isolates were grown for 20 hours on Mueller Hinton agar (Excel laboratory products), suspended in 3 mLs cold CSB buffer (0.1 mol Tris (Merck), pH 8.0, 0.1 mol EDTA (Invitrogen), pH 8.0) to a 5 McFarland standard. 200 µL of cell suspension was added to 10 µL of 20 mg/mL proteinase K (Promega). 200 µL of this suspension was added to 200 µL of 1.1% molten agarose with 1% SDS and allowed to set. Plugs were placed in 2.5 mL lysis buffer (0.05 mol Tris, pH 8.0, 0.05 mol EDTA, pH 8.0, 1% Sarcosyl (Sigma)), 0.1 mg proteinase K and incubated at 54°C for 4 hours. Plugs were washed twice in 4 mL Hi pure water at 54°C for 15 min with shaking, then washed four times in 2.5 mL 1x Tris EDTA buffer for 15 min with shaking at 54°C. Plugs were then washed twice in 300 µL buffer A (Promega) with 1% BSA, followed by restriction in 300 µL buffer A (Promega) with 1% BSA and 30 U of *Spe*I restriction enzyme (Promega) at 37°C overnight. Following restriction, plugs were placed in a 1% SKG agarose gel in 0.5x Tris Borate EDTA buffer and electrophoresed on a Chef-DRIII (Biorad) apparatus at 14°C. Pulsed field parameters were; two linear ramps 0.5 to 2.5 seconds for twenty hours, followed by 30 to 60 second ramps for four hours at 6V/cm. The international standard ACTC BAA-664 (*S. enterica* ser Braenderup H9812) was included as a marker in all gels. Isolates were considered to be genotypically indistinguishable when their PFGE macrorestriction patterns did not differ by more than one to three

bands, representing minor genetic events such as DNA insertions, deletions or point mutations (Armstrong, Nixon *et al.* 2002).

Quality Control: Positive: International standard ACTC BAA-664
(*S. enterica* ser Braenderup H9812)

3.16 Dendrogram of Isolate Relatedness by RAPD PCR

Gel analysis of RAPD PCR patterns was carried out with Biorad Diversity Database software (Biorad) and a dendrogram of isolate relatedness was produced from RAPD PCR results using unweighted pair group matched analysis with arithmetic averages and a dice coefficient. Isolates representative of Australian CF clonal complex strains AES 1 and AES 2 were included as controls, as was *P. aeruginosa* PAO1. *P. fluorescens* ATCC 13525 was included as an outlier control.

Quality Control: Genotype: *Pseudomonas aeruginosa* PAO1
Pseudomonas aeruginosa AES 1
Pseudomonas aeruginosa AES 2
Outlier: *Pseudomonas fluorescens* ATCC 13525

3.17 Virulence factor PCRs

3.20.1 *apr*, *lasB*, *phzI*, *phzII*, *phzH*, *phzM*, *phzS*, *exoS* and *exoT*

Each PCR reaction contained 0.825 U Taq polymerase, 2 µL of 10x PCR buffer, 1.2 µL of MgCl₂, 0.4 µL DMSO, 0.16 µL of dNTPs (Fisher biotech) and 0.1 µL each of a 100 uM solution of appropriate primers (appendix B) and 2.4 ng of extracted DNA. This was adjusted to a volume of 20 uL with ddH₂O. PCR was performed on a Geneamp 9600 cycler (Applied Biosystems). The PCR protocol consisted of initial denaturation at 94°C for two minutes, followed by 30 cycles of denaturation at 94

°C for one minute, annealing at appropriate temperature (appendix B) for 30 seconds and extension at 72 °C for 105 seconds. This was followed by a final extension n of 72 °C for 7 minutes. A 1 kb DNA ladder marker (Geneworks), PAO1 DNA positive control and dH₂O negative were incorporated into each run of gels performed. Submarine electrophoresis was performed in 1x Tris Acetate EDTA buffer at 100V for one hour and 15 minutes in a 3% agarose gel and stained with ethidium bromide, followed by visualization under UV light. Polaroid photographs of gels were taken using a Polaroid camera (Kodak) and black and white 667 Polaroid film (Kodak). All negative results were performed in duplicate.

Quality control: Positive: *Pseudomonas aeruginosa* PAO1
 Negative: Sterile ddH₂O contamination control extract

3.17.2 *exoY* and *exoU*

Each PCR reaction contained 1.1 U Taq polymerase, 2μL of 10x PCR buffer, 1.2 μL of MgCl₂, 0.4 μL DMSO, 0.16 μL of dNTPs (Fisher biotech) and 0.1 μL each of a 100 uM solution of appropriate primers (appendix B) and 2.4 ng of extracted DNA. This was adjusted to a volume of 20 uL with ddH₂O. PCR was performed on a Geneamp 9600 cycler (Applied Biosystems). The PCR protocol consisted of initial denaturation at 94°C for two minutes, followed by 30 cycles of denaturation at 94 °C for one minutes, annealing at appropriate temperature (appendix B) for 30 seconds and extension at 72 °C for 105 seconds. This was followed by a final extension n of 72 °C for 7 minutes. A 1 kb DNA ladder marker (Geneworks), PAO1 (*exoY*) or PA103 (*exoU*) DNA positive control and dH₂O negative were incorporated into each run of gels performed. Submarine electrophoresis was performed in 1x Tris Acetate EDTA buffer at 100V for one hour and 15 minutes in a 3% agarose gel and stained with ethidium bromide, followed by visualization under UV light. Polaroid photographs of gels were taken

using a Polaroid camera (Kodak) and black and white 667 Polaroid film (Kodak). All negative results were performed in duplicate.

Quality control: Positive: *Pseudomonas aeruginosa* PAO1 (*exoT*)

Pseudomonas aeruginosa PA103 (*exoU*)

Negative: Sterile ddH₂O contamination control extract

3.18 DNA-DNA Hybridisation Blots

Nylon membrane was cut to an appropriate size for the number of blot hybridisations to be performed then soaked in 2% SSC for two minutes. The membrane was then transferred into 10% SSC and allowed to soak for a further two minutes. Following soaking, the membrane was placed on absorbent paper and allowed to dry completely at room temperature.

Extracted DNA was denatured at 100°C in a heating block for five minutes, then immediately placed in iced water and allowed to cool. Following this, 1 µL drops of positive control DNA (PA01 for all virulence factors except *exoU*, for which PA103 DNA was used), negative control DNA (*E. coli* NCTC 10418) and test DNA were placed on the nylon membrane using a pipette, the membrane was then placed in a 600 watt microwave and DNA melted by microwaving on the “high” setting for 90 seconds. 20 mL of Easy-hyb (Roche) was heated to 37 °C in an incubator, and following it reaching temperature, the nylon membrane was incubated at 37°C in this solution for 30 minutes.

Digoxigenin-dUTP (DIG) labelled DNA was diluted 1:100 with ddH₂O and denatured at 100 °C for 5 minutes on a heating block, probes were then placed on ice to cool. The DIG labelled DNA was then diluted 1:12 in diluted Easy-hyb. Nylon membranes were placed in plastic bags and 2.5 mL of DIG

3.19 Purification of DNA from Agarose Gel

DNA purification from agarose gel was performed using a QIAquick gel extraction kit (Qiagen) by Mr. Roger Latham of the Cardiorespiratory Research Group, Menzies Research Institute, University of Tasmania. The PCR product band to be tested was excised from the agarose gel using a sterile scalpel, cut into small fragments, and placed in a sterile eppendorf tube. A weight balance was tared using an empty eppendorf tube, and then the weight of the gel fragments measured. Three volumes of buffer CG (Qiagen) was added per 1 volume of gel ($100\text{ mg} = 100\text{ }\mu\text{L}$). The mixture colour was observed to ensure change to an orange-violet hue. Following this step, $300\text{ }\mu\text{L}$ of sodium acetate 5 M was added and the colour change to yellow observed. The eppendorf tube was then incubated in a heat block at 50°C for ten minutes, vortexing the solution every two to three minutes to ensure the gel was dissolved. Following this step, a volume of isopropanol equal to the weight of the gel fragments was added to the solution. $800\text{ }\mu\text{L}$ of the solution was transferred to a QIAquick column (Qiagen), which was placed in a 2 mL collection tube provided in the kit, then centrifuged for 1 minute at 9000 rpm in a microfuge. The flow through was discarded and the previous step repeated until all of the dissolved gel solution had been processed. Following this step, $500\text{ }\mu\text{L}$ of buffer QG (Qiagen) was added to the column and the column centrifuged for a further 1 minute at 9000 rpm . This was followed by the addition of $750\text{ }\mu\text{L}$ of buffer PE (Qiagen) to the column and centrifuged at $13,000\text{ rpm}$ for one minute. The QIAquick column was placed in a fresh sterile 1.8 mL eppendorf tube. A volume of $50\text{ }\mu\text{L}$ of buffer EB was dispensed into the centre of the column and the column centrifuged at 9000 rpm for one minute. The flow through, containing purified DNA was referred for DNA sequencing at the Micromon DNA sequencing facility of Monash University (Melbourne, Victoria)

3.20 Determination of urease production

This test determines the presence of the enzyme urease by culturing the test bacterial isolate on agar infused with the enzymatic substrate urea. Urease producing organisms will hydrolyse urea and release the enzymatic end product, ammonia. Increased levels of ammonia substantially raise the pH of the surrounding medium, which results in a colour change of the indicator phenol red.

Bacterial isolates were sub cultured from MM onto Mueller Hinton agar for 48 hours at 37°C in air. A single, well isolated colony was touched with a sterile wire loop, and this loop was then thoroughly spread over the surface of a Christensen's urea agar slope (Oxoid). Agar slopes were then incubated at 21°C under aerobic conditions with increased moisture and protected from light for seven days, in concordance with the conditions employed in the *D. discoideum* virulence assay (method 3.25). Following incubation, slopes were visually examined for a colour change. A strong pink colouration in the agar was considered to be a positive result (score 1), whilst no change (yellow) or a very weak alteration in colour was considered to be a negative result (score 0). All tests were performed in duplicate. In cases where discrepant results were observed, triplicate testing was performed and the median average result tests recorded as the final result.

Quality control:

| | |
|-----------|--|
| Positive: | <i>Proteus vulgaris</i> ATCC 3427 |
| | <i>Klebsiella pneumoniae</i> (MDR, clinical isolate) |
| Negative: | <i>Escherichia coli</i> NCTC 10418 |
| | <i>Klebsiella aerogenes</i> (clinical isolate) |

3.21 *Dictyostelium discoideum* virulence assay

3.21.1 *Dictyostelium discoideum* continuous culture

Dictyostelium discoideum Continuous Culture was performed by both the author and Dr. Alan Champion of the Cardiorespiratory Research Group, Menzies Research Institute, University of Tasmania. A single colony of an overnight culture of a clinical strain of *Klebsiella aerogenes* was spread using a bacteriological loop over the surface of an SM agar (materials 2.4.2) plate. Five to ten spores of *D. discoideum* AX2 or X22 were picked from a week old culture using a sterile bacteriological loop and inoculated onto one side of the agar plate. A separate plate was used for each *D. discoideum* strain. The plate was then incubated at 21°C with moisture for up to one week, or until luxuriant spore development was observed. This process was repeated weekly to maintain permanent cultures.

3.21.2 Preparation of *D. discoideum* Virulence assay discs

Spores from 7 day old *D. discoideum* AX2 and X22 continuous cultures were harvested with a sterile bacteriological loop and inoculated in 3.5 mL sterile SS solution (materials 2.3.4). The suspension was thoroughly mixed by inversion, a small amount placed into a Improved Neubauer haemocytometer, the solution was then allowed to settle for five minutes. The chamber was placed under a light microscope and the number of spore in five large squares counted. The concentration of spores per mL was obtained using the following equation:

$$\text{Spores/mL} = \frac{\text{Number of spores counted in 5 large squares}}{5} \times 25 \times 10^4 \text{ mL}$$

Further spores were added to the suspension and counted until a concentration of $10^7 \pm 10\%$ spores/mL was obtained.

3.21.2.1 Ethanol shock treatment

D. discoideum AX2 and X22 spore suspensions were diluted 1:1 in 100% ethanol and incubated at room temperature for five minutes. Following incubation, the suspension was centrifuged at 500 g for 5 minutes. The supernatant was removed and replaced with SS solution. One hundred microlitres of each suspension was inoculated onto SM agar plate *K. aerogenes* lawn plate and incubated at 21°C for seven days with increased moisture and protected from light. A further 100 µL of each suspension was then added to 5 mL of TSB and incubated at 37°C overnight. Following overnight incubation, the TSB (materials 2.4.1) suspension was sub-cultured onto TSAY (materials 2.4.1), incubated for 24 hours at 37°C in air overnight.

3.21.2.2 Heat shock treatment

D. discoideum AX2 and X22 spore suspensions in a 10 mL glass universal bottle were and incubated at 70°C in a water bath for five minutes. Following incubation, one hundred microlitres of each suspension was inoculated onto SM agar plate *K. aerogenes* lawn plate and incubated at 21°C for seven days with increased moisture and protected from light. A further 100 µL of each suspension was then added to 5 mL of TSB and incubated at 37°C overnight. Following overnight incubation, the TSB suspension was sub-cultured onto TSAY, incubated for 24 hours at 37°C in air overnight.

3.21.2.3 Desiccation

Twenty microlitres of each spore suspension was then added to blank 8 mm diameter filter paper discs (Oxoid) in a sterile plastic petri dish. Control discs were prepared by the addition of 20 µL of sterile SS solution to blank filter paper discs. Discs were placed in a petri dish and allowed to dry at room temperature for 10 minutes. Petri dishes containing inoculated discs were then placed open under

laminar flow (laminar flow cabinet exposed to UV light for at least 30 minutes prior to use) for one hour to dry. The lids were replaced and discs dried at room temperature for a further 24 hours. One disc from each batch was placed in 5 mL of TSB and incubated at 37°C for three days. Following this period of incubation, the TSB was examined for turbidity. Batches of discs showing no visible turbidity or bacterial growth following three days in TSB culture were considered to be appropriate for use.

3.21.3 *Dictyostelium discoideum* virulence assay

P. aeruginosa isolates were sub cultured from MMM onto TSAY agar (appendix) and incubated at 37°C in air for 48 hours. For non-mucoid isolates, a sufficient amount of bacterial growth was collected from isolated colonies on the a loop in 3.5 mL of sterile SS solution to reach a 0.5 Mcfarland turbidity, judged by comparison to a commercial 0.5 McFarland standard preparation (Biomérieux). The same process was performed for mucoid isolates, but these were suspended to a turbidity equivalent to a 1.0 Mcfarland.

The SS bacterial suspension was poured onto the surface of an SM Agar plate. Excess SS suspension was removed and the surface of the agar plates allowed to dry for 30 minutes at room temperature. Following drying, a single AX2, X22 and an uninoculated control disc was placed on the surface of each plate using sterile forceps, approximately seven mm apart. Agar plates were then incubated at 21°C under aerobic conditions with increased moisture and protected from light for seven days.

Following seven days incubation, the annular radius of any zones of inhibition around each disc was measured. Where development of the *D. discoideum* was evident, this was recorded and scored as 0, 1,

2 or 3 according to predetermined rules regarding the degree of development shown. All assays were performed in triplicate.

Quality control: Positive: *Pseudomonas aeruginosa* PAO1
 Negative: *Klebsiella aerogenes* (clinical isolate)
 Klebsiella pneumoniae (MDR clinical isolate)

3.22 Statistical Methods

χ^2 test, κ tests, student's t tests, correlation co-efficient tests and determination of mean averages were all performed in Microsoft® Office Excel 2003 version (11.8220.8202) SP3 by the author.

Statistical analysis of clinical data in chapter 6 was carried out by Dr. Leigh Blizzard of the Menzies Research Institute, University of Tasmania using Fisher's exact test (Freeman-Halton test) in the SPSS statistical package was used for all analyses (SPSS 12.0.1 for Windows, 2003). A p value of ≤ 0.05 was considered to be significant.

CHAPTER FOUR

Source and Identification of Isolates

4.1 Introduction

In order to carry out the work required in this thesis, a large number of *Pseudomonas aeruginosa* isolates from a variety of sources was required. Isolates were obtained from hospital, community and environmental sources in Tasmania. Sputum from cystic fibrosis patients was obtained at CF clinics and during hospital stays, whilst other clinical isolates were obtained from Department of Microbiology and Infectious Diseases at the Royal Hobart Hospital (RHH), with details of infection site provided. Hospital environment isolates were obtained by direct sampling of likely sites throughout the RHH. It should be noted that the RHH is the only tertiary care facility in Tasmania, and acts as a referral centre for patients from around the state. Community environment isolates used in this study were provided by the Tasmanian Public Health laboratory.

Prior to any investigations on these strains, identity as *P. aeruginosa* was confirmed in order to avoid incorrect or inappropriate results and conclusions due to the inclusion of non *P. aeruginosa* isolates in the studies performed in any capacity other than controls. A number of biochemical and phenotypic characteristics are accepted as being indicative of a bacterial isolate belonging to the species *P. aeruginosa*, and these are most often used in diagnostic Microbiology laboratories to confirm *P. aeruginosa* identity. A combination of these simple tests provides the basis for identification of *P. aeruginosa* in the majority of diagnostic Microbiology laboratories. Further confirmation of speciation may be performed by growing isolates on specific biochemical substrates or by the use of molecular techniques to identify the presence of specific genes or DNA sequences unique to *P. aeruginosa*.

4.2 Methods

4.2.1 Clinical and community environment isolates

Low passage strains of clinical and community environment isolates included in this study were provided from either the University of Tasmania cystic fibrosis isolate library, the Department of Microbiology and Infectious Diseases at the Royal Hobart Hospital or the Tasmanian Public Health Laboratory, and had previously been identified as belonging to the species *P. aeruginosa* by these laboratories. Isolates were cultured on MacConkey agar at 42°C, with a C390 disc placed on the agar plate. Following 48 hours growth, the isolates were subjected to an oxidase test, as well as their Gram stain reaction and their degree of sensitivity to the compound C390 being recorded (methods 3.6, 3.7, 3.8). Isolates found to be *P. aeruginosa* by these simple phenotypic methods were then stored in minimal maintenance media, protected from light (method 3.5).

4.2.2 Hospital environment isolates

Sterile cotton swabs were used to sample likely sources of *P. aeruginosa* (aqueous environments) in all wards and intensive therapy units of the Royal Hobart Hospital. Swabs were rolled over MacConkey agar and a C390 disc placed in the centre of the agar. Agar plates were incubated for 48 hours at 42°C under aerobic conditions. C390 resistant oxidase positive isolates were then sub-cultured onto fresh MacConkey agar to obtain pure cultures. Any mucoid morphology displayed by primary isolates was recorded, as was their Gram stain reaction. Purity plates were incubated at 37 °C in air overnight. Isolates from purity plates were stored in MMM at room temperature, protected from light.

4.2.3 Confirmation of identification by PCR

To further confirm the identity of all isolates to be studied, a *P. aeruginosa* specific PCR was performed. A sterile bacteriological loop was placed into the MMM culture of each isolate and the culture streaked onto MacConkey agar. The agar plate was then incubated at 37 °C for 48 hours to obtain isolated colonies. DNA was extracted from each culture by use of the MoBio DNA extraction kit (method 3.9) and the DNA extracts frozen at -80°C. These DNA extracts were thawed at room temperature prior to performance of the *P. aeruginosa* specific PCR (method 3.10). A *P. aeruginosa* PAO1 positive control, *P. fluorescens* ATCC 13525 negative control and sterile ddH₂O contamination control was incorporated into each PCR performed.

In cases where an isolate in the collection previously identified as *P. aeruginosa* was negative by *P. aeruginosa* specific PCR, further investigations of identity were performed using the array of biochemical tests available through the Vitek® GNI+ card system. Isolates were cultured from MMM onto MacConkey agar for 48 hours at 37°C under aerobic conditions, and these cultures used to inoculate Vitek® GNI+ cards. Inoculated cards were analysed using a Vitek® 1 analyser to produce a percentage probability of species identity (method 3.11). In cases where a poor identification resulted, the isolate was identified by use of the API 20NE test kit (method 3.12).

4.3 Results

4.3.1 Sample collection

A total of 191 isolates from 143 separate patients or environmental sites and four control strains (*P. aeruginosa* PAO1 and *P. fluorescens* ATCC 13525, AES1, AES2) were included in this analysis. Of the clinical isolates, 69 were respiratory isolates from chronically infected CF patients, 19 were

provided by the Department of Microbiology and Infectious Diseases of the Royal Hobart Hospital (RHH) and 50 were provided by the University of Tasmania CF isolate library. Clinical isolates from 80 non-CF patients were provided by the RHH Department of Microbiology and Infectious Diseases, comprising 25 respiratory isolates (including two from chronically infected COPD patients), 26 skin and soft tissue isolates, 24 urine isolates (including five catheter urine isolates) and five isolates from cases of otitis externa.

Both in-patient and out-patient CF and the non-CF clinical isolates were used in this study. Of the non-CF clinical isolates, four were from patients in the ICU, five were from the NSU and 54 were from a variety of medical and surgical wards. Due to the small population of Tasmania, and the patient data already provided, individual wards other than ICU and NSU have not been described in the results of this study to protect patient confidentiality, where necessary to explain results, exceptions to this may be made. Seventeen isolates were from patients who had attended Out-patient clinics at the RHH, and eight isolates were recovered from patients within the community, either upon attendance to the Department of Emergency Medicine, RHH or through samples provided for Microscopy, Culture and Sensitivity testing by General Practitioners in the greater Hobart area.

Of the ten isolates from the wider Hobart community environment provided by the Tasmanian Public Health Laboratory, three morphologically distinct isolates came from two separate swimming pools in the Hobart area, two isolates were from spa baths or whirlpools, one isolate was from a sewerage effluent inlet, two from sewerage effluent spillage and one from a stream in the Hobart area.

Sixty-five environmental swabs were collected from likely sources of *P. aeruginosa* within the RHH. Thirty-one of the recovered isolates were identified as *P. aeruginosa* by preliminary phenotypic characteristics (Gram negative bacillus, oxidase positive, growth at 42°C and C390 resistance). Seventeen of these isolates were from sink drains on medical and surgical wards other than ICU and NSU (other wards have not been identified in the interests of patient confidentiality).

4.3.2 Increased recovery of *P. aeruginosa* in hospital intensive therapy units

A significantly higher recovery rate was noted in both the ICU and NSU sites (table 4.3). Isolates displaying characteristics phenotypically concordant with *P. aeruginosa* were recovered from every environmental swab taken in these units. In total, nine *P. aeruginosa* were recovered from six sink drains and one wash tub in the ICU. ICU sink drains DF and DD yielded two isolates each, each showing differing colonial morphology to the other. The four sink drains sampled in the NSU all yielded isolates phenotypically identified as *P. aeruginosa*, although only one colonial morphology per sink was identified. One hospital environmental strain was recovered from a suction unit used on the burns ward (isolate 74). *P. aeruginosa* was not recovered from any of the four sinks in the rooms used as a weekly CF out-patient clinic.

4.3.3 Mucoidy

The mucoid colonial phenotype (fig. 4.1) was noted in 50 isolates included in this study. By far the majority of these isolates were recovered from CF patients (45 isolates), the remaining being cultured from an NSU patient's sputum trap (isolate 49), A urine isolate (isolate 61) from a patient on a general ward, A COPD respiratory specimen (isolate 97) and a sink in the ICU (isolate 114).

4.3.4 Multiple isolates from CF patients

Sixty-five different isolates of *P. aeruginosa* were recovered from only 38 CF patients included in this study. This discrepancy was due to 19 of the CF patients involved in the study yielding multiple different morphotypes of *P. aeruginosa* in one or more sputum samples. One CF patient (isolates 108a, 108b, 108c) had three distinct morphotypes of *P. aeruginosa* recovered from a single sputum isolate. A further two patients had three separate morphotypes identified in multiple sputum samples (isolates 17a, 17b and 29b in one and isolates 47a, 47b and 58 in the other). Sixteen CF patients produced sputum samples which were found to contain at least two distinct morphotypes of *P. aeruginosa* (table 4.1). In most cases, the most notable difference in morphotypes of isolates from the same specimen was the recovery of mucoid and non-mucoid colonies, but in some cases distinct morphological differences between isolates other than this required them to be treated as separate.

Five CF patients (AG, AH, AJ, AS and CH) had isolates provided for this study by the Department of Microbiology and Infectious Diseases, RHH which were morphologically identical to those also provided from the same patients by the UTAS CF library, but were collected at different times. These isolates were included to act as further internal controls during the course of the study.

4.3.5 Multiple isolates from non-CF patients

In a small number of cases, multiple isolates of *P. aeruginosa* from non CF patients and environmental sites were included in this study. Isolates 1 and 17 (patient AA) were recovered from sputum of a patient during a single admission, but were obtained at different timepoints and from different wards (patient transferred wards). Isolates 10 and 37 were recovered from the urine of the same patient (AE) five months apart. Isolates 20 and 24 were recovered from the urine of another patient (AQ), initially

when non-catheterised and later when a urinary catheter had been inserted. Isolates 23 was recovered from the ear of one patient (AT) at an out patient clinic visit, whilst isolate 72 was recovered from the same patient's respiratory tract four months later, following their admission to hospital with a respiratory illness.

Isolates 49 and 50 were recovered from the same sputum trap sample provided by patient BN, due to the differing morphology (mucoid and non-mucoid) of these isolates, they were both included for study. Patient BU provided four samples included in this study, each from a different site of infection. Isolate 63 was recovered from a mouth swab, 64 from a neck wound and 66 from the patient's urine during an in-patient stay in hospital. Isolate 96 was a urinary isolate from the same patient recovered two months later, following their discharge from the RHH. Isolates 57 and 76 were both recovered from patient BQ's urine 9 months apart, on both occasions patient BQ was an out patient. Patient CB provided an isolate from sputum during an acute respiratory infection (isolate 73) and subsequently isolate 75 was recovered from an infected wound on the foot.

4.3.6 Isolate identification

All isolates collected and stored for this study were found to be oxidase positive Gram negative bacilli. All isolates were capable of growth on MacConkey agar in aerobic conditions at 37 °C. All test isolates were found to grow at 42°C, and to show resistance to the compound C390. Results for PAO1, *P. aeruginosa* ATCC 37025 and *P. fluorescens* ATCC 13525 control strains were as expected (fig 4.2).

DNA extracts from isolates found to conform to the identification of *P. aeruginosa* based upon the above phenotypic tests were subjected to a *P. aeruginosa* specific PCR to confirm their identification.

Of the test DNA extracts, 183 isolates yielded PCR products of appropriate molecular weight to be considered positive for the *P. aeruginosa* PCR. The PAO1 positive control, *P. fluorescens* ATCC 13525 negative control and ddH₂O contamination control all yielded expected results, confirming the validity of the PCRs (fig. 4.3).

Seven isolates (104, 105, 106, 107, 109, 110 and 117), identified as *P. aeruginosa* by the phenotypic tests performed did not produce any bands by *P. aeruginosa* specific PCR. All of these isolates were recovered from the hospital environment, with isolates 104-107 being recovered from sinks in the day surgery rooms used as a CF out-patient clinic.

In order to further investigate the identity of these seven mistakenly identified isolates, cultures were subjected to an array of biochemical tests using the Vitek® GNI+ system in a Vitek 1 analyser (Biomérieux). All seven isolates were found not to be *P. aeruginosa* by Vitek® GNI+ card (table 4.2). Isolate 109 yielded only a 74% probability of being *Chryseobacterium indologenes* by Vitek GNI+. To confirm this identification, an API 20NE (Biomérieux) test strip was used. This isolate was found to have API 20NE biocode 2652205 after 24 hours incubation, conforming to an identification of *C. indologenes* (99% probability, T value 0.82). There were no changes in the assimilation profile after 48 hours incubation.



Figure 4.1: Mucoid (top) and non-mucoid (bottom) morphology of *P. aeruginosa* on MacConkey agar.



Figure 4.2: C390 reactions of control *P. aeruginosa* ATCC 10662 (top) and *P. fluorescens* ATCC 13525 (bottom).

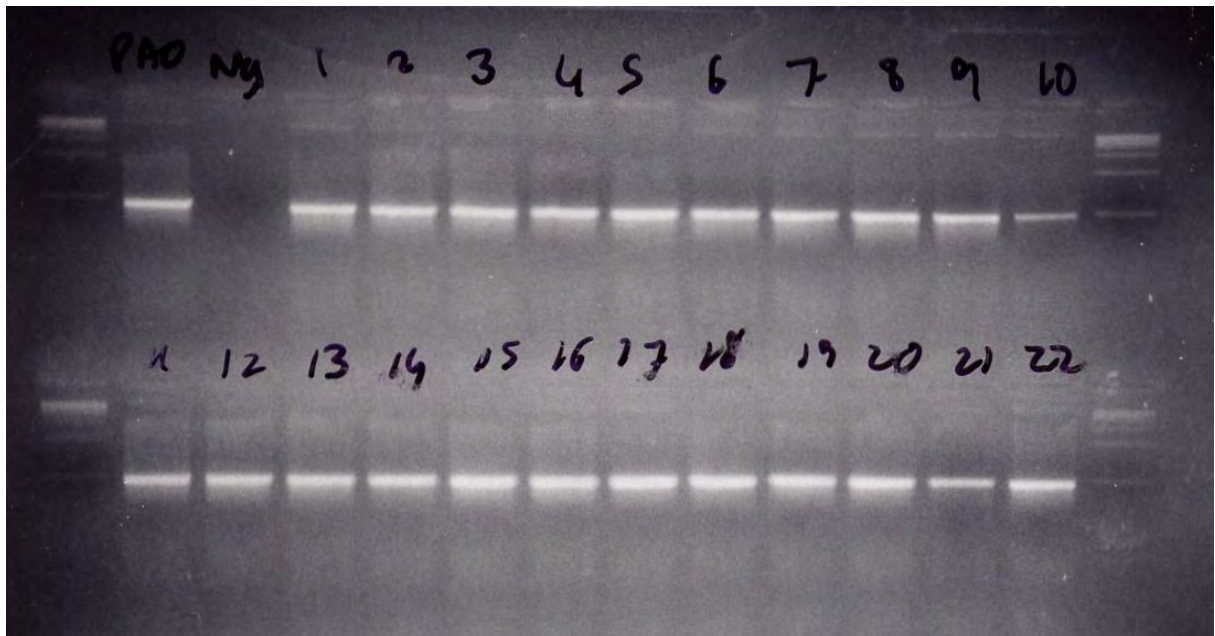


Figure 4.3: *P. aeruginosa* specific PCR gel
(L-R): Lane 1; 1 kb MW marker (Biorad), PAO1, *P. fluorescens* ATCC 30725, isolates 1-10, 1 kb MW marker; Lane 2; 1 kb MW marker, isolates 11-22, 1 kb MW marker.

| Isolate Number | Source | Site of Isolation | Mucoidy | Month of Isolation | Patient/ Site identifier | Patient Age | Source Lab | Growth on Mac Agar | Gram Reaction | Oxidase reaction | Growth at 42°C | <i>P. aeruginosa</i> Specific PCR |
|----------------|-------------|------------------------|---------|--------------------|--------------------------|-------------|------------|--------------------|---------------|------------------|----------------|-----------------------------------|
| 1 | Other Ward | Respiratory | non | Jan 04 | AA | 33 | RHH | POS | GNB | POS | POS | POS |
| 2 | Other Ward | Ear | non | Jan 04 | AB | 76 | RHH | POS | GNB | POS | POS | POS |
| 3 | Other Ward | Skin/soft tissue | non | Jan 04 | AC | 92 | RHH | POS | GNB | POS | POS | POS |
| 4 | Other Ward | Skin/soft tissue | non | Dec 03 | AD | 77 | RHH | POS | GNB | POS | POS | POS |
| 5 | Other Ward | Respiratory | non | Dec 03 | AE | 76 | RHH | POS | GNB | POS | POS | POS |
| 6 | Other Ward | Urine | non | Jan 04 | AF | 43 | RHH | POS | GNB | POS | POS | POS |
| 7 | Out-patient | CF Respiratory | muc | Dec 03 | AG | 23 | RHH | POS | GNB | POS | POS | POS |
| 8 | Out-patient | CF Respiratory | muc | Dec 03 | AH | 17 | RHH | POS | GNB | POS | POS | POS |
| 10 | Other Ward | Urine | non | Dec 03 | AE | 76 | RHH | POS | GNB | POS | POS | POS |
| 11 | Other Ward | Skin/soft tissue | non | Dec 03 | AI | 76 | RHH | POS | GNB | POS | POS | POS |
| 12 | Other Ward | CF Respiratory | muc | Dec 03 | AJ | 26 | RHH | POS | GNB | POS | POS | POS |
| 13 | Other Ward | Respiratory | non | Dec 03 | AK | 75 | RHH | POS | GNB | POS | POS | POS |
| 14 | Other Ward | Respiratory | non | Dec 03 | AL | 79 | RHH | POS | GNB | POS | POS | POS |
| 15 | Other Ward | Respiratory | non | Dec 03 | AM | 71 | RHH | POS | GNB | POS | POS | POS |
| 16 | Other Ward | Respiratory | non | Dec 03 | AN | 76 | RHH | POS | GNB | POS | POS | POS |
| 17 | Out-patient | Respiratory | non | Jan 04 | AA | 33 | RHH | POS | GNB | POS | POS | POS |
| 18 | Other Ward | Urine | non | Jan 04 | AO | 92 | RHH | POS | GNB | POS | POS | POS |
| 19 | Other Ward | Urine | non | Jan 04 | AP | 68 | RHH | POS | GNB | POS | POS | POS |
| 20 | Other Ward | Urine | non | Jan 04 | AQ | 83 | RHH | POS | GNB | POS | POS | POS |
| 21 | Out-patient | COPD Respiratory | muc | Jan 04 | AR | 60 | RHH | POS | GNB | POS | POS | POS |
| 22 | Other Ward | CF Respiratory | muc | Jan 04 | AS | 24 | RHH | POS | GNB | POS | POS | POS |
| 23 | Community | Ear | non | Jan 04 | AT | 84 | RHH | POS | GNB | POS | POS | POS |
| 24 | Other Ward | Urine | non | Jan 04 | AQ | 83 | RHH | POS | GNB | POS | POS | POS |
| 25 | Other Ward | Catheter Urine | non | Jan 04 | AU | 72 | RHH | POS | GNB | POS | POS | POS |
| 26 | Community | Urine | non | Jan 04 | AV | 73 | RHH | POS | GNB | POS | POS | POS |
| 28 | Other Ward | Skin/soft tissue | non | Feb 04 | AW | 78 | RHH | POS | GNB | POS | POS | POS |
| 29 | Other Ward | Skin/soft tissue | non | Mar 04 | AX | 73 | RHH | POS | GNB | POS | POS | POS |
| 30 | NSU | Respiratory | non | Mar 04 | AY | 43 | RHH | POS | GNB | POS | POS | POS |
| 31 | Other Ward | Respiratory | non | Mar 04 | AZ | 1 | RHH | POS | GNB | POS | POS | POS |
| 32 | Out-patient | Respiratory | non | Mar 04 | BA | 23 | RHH | POS | GNB | POS | POS | POS |
| 33 | Other Ward | Skin/soft tissue | non | Mar 04 | BB | 76 | RHH | POS | GNB | POS | POS | POS |
| 34 | Other Ward | Catheter Urine | non | Mar 04 | BC | 53 | RHH | POS | GNB | POS | POS | POS |
| 35 | Other Ward | Urine | non | Mar 04 | BD | 73 | RHH | POS | GNB | POS | POS | POS |
| 36 | Other Ward | Skin/soft tissue | non | Apr 04 | BE | 74 | RHH | POS | GNB | POS | POS | POS |
| 37 | Other Ward | Catheter Urine | non | Apr 04 | AE | 76 | RHH | POS | GNB | POS | POS | POS |
| 38 | Other Ward | Respiratory | non | Apr 04 | BF | 84 | RHH | POS | GNB | POS | POS | POS |
| 39 | Other Ward | Urine | non | Apr 04 | BG | 66 | RHH | POS | GNB | POS | POS | POS |
| 40 | Other Ward | Skin/soft tissue | non | Apr 04 | BH | 76 | RHH | POS | GNB | POS | POS | POS |
| 44 | Other Ward | Skin/soft tissue | non | Apr 04 | BI | 53 | RHH | POS | GNB | POS | POS | POS |
| 45 | Out-patient | Catheter Urine | non | Apr 04 | BJ | 54 | RHH | POS | GNB | POS | POS | POS |
| 46 | Out-patient | Skin/soft tissue | non | Apr 04 | BK | 76 | RHH | POS | GNB | POS | POS | POS |
| 47 | Community | Urine | non | May 04 | BL | 48 | RHH | POS | GNB | POS | POS | POS |
| 48 | Other Ward | Respiratory | non | Apr 04 | BM | 84 | RHH | POS | GNB | POS | POS | POS |
| 49 | NSU | Respiratory | muc | Apr 04 | BN | 75 | RHH | POS | GNB | POS | POS | POS |
| 50 | NSU | Respiratory | non | Apr 04 | BN | 75 | RHH | POS | GNB | POS | POS | POS |
| 51 | Community | Urine | non | May 04 | BO | 48 | RHH | POS | GNB | POS | POS | POS |
| 55 | Other Ward | Skin/soft tissue | non | Apr 04 | BP | 67 | RHH | POS | GNB | POS | POS | POS |
| 57 | Out-patient | Urine | non | Apr 04 | BQ | 80 | RHH | POS | GNB | POS | POS | POS |
| 60 | ICU | Respiratory | non | May 04 | BR | 78 | RHH | POS | GNB | POS | POS | POS |
| 61 | Other Ward | Urine | muc | May 04 | BS | 87 | RHH | POS | GNB | POS | POS | POS |
| 62 | ICU | Respiratory | non | May 04 | BT | 64 | RHH | POS | GNB | POS | POS | POS |
| 63 | Out-patient | Skin/soft tissue | non | May 04 | BU | 61 | RHH | POS | GNB | POS | POS | POS |
| 64 | Out-patient | (alternate site to 63) | non | May 04 | BU | 61 | RHH | POS | GNB | POS | POS | POS |
| 65 | Other Ward | Respiratory | non | May 04 | BV | 45 | RHH | POS | GNB | POS | POS | POS |
| 66 | Out-patient | Urine | non | May 04 | BU | 61 | RHH | POS | GNB | POS | POS | POS |
| 67 | Other Ward | Skin/soft tissue | non | May 04 | BW | 64 | RHH | POS | GNB | POS | POS | POS |
| 68 | Other Ward | Respiratory | non | May 04 | BX | 23 | RHH | POS | GNB | POS | POS | POS |
| 69 | Other Ward | Respiratory | non | May 04 | BY | 83 | RHH | POS | GNB | POS | POS | POS |
| 70 | Other Ward | Skin/soft tissue | non | May 04 | BZ | 65 | RHH | POS | GNB | POS | POS | POS |
| 71 | Other Ward | Respiratory | non | May 04 | CA | 50 | RHH | POS | GNB | POS | POS | POS |
| 72 | Other Ward | Respiratory | non | May 04 | AT | 84 | RHH | POS | GNB | POS | POS | POS |
| 73 | Other Ward | Respiratory | non | May 04 | CB | 68 | RHH | POS | GNB | POS | POS | POS |
| 74 | Other Ward | Suction Unit | non | Jun 04 | CC | na | RHH | POS | GNB | POS | POS | POS |
| 75 | Other Ward | Skin/soft tissue | non | Jun 04 | CB | 68 | RHH | POS | GNB | POS | POS | POS |
| 76 | Out-patient | Urine | non | Jan 05 | BQ | 81 | RHH | POS | GNB | POS | POS | POS |
| 77 | NSU | Catheter Urine | non | Jun 04 | CD | 77 | RHH | POS | GNB | POS | POS | POS |
| 78 | Other Ward | Skin/soft tissue | non | Jun 04 | BW | 64 | RHH | POS | GNB | POS | POS | POS |
| 79 | Other Ward | Skin/soft tissue | non | Jun 04 | CE | 77 | RHH | POS | GNB | POS | POS | POS |
| 80 | ICU | Skin/soft tissue | non | Jun 04 | CF | 72 | RHH | POS | GNB | POS | POS | POS |
| 81 | Out-patient | Ear | non | May 04 | CG | 10 | RHH | POS | GNB | POS | POS | POS |
| 82 | Out-patient | CF Respiratory | muc | May 04 | CH | 26 | RHH | POS | GNB | POS | POS | POS |
| 83 | Out-patient | CF Respiratory | non | May 04 | CH | 26 | RHH | POS | GNB | POS | POS | POS |
| 85 | Other Ward | Skin/soft tissue | non | May 04 | CI | 37 | RHH | POS | GNB | POS | POS | POS |
| 86 | Other Ward | Skin/soft tissue | non | Aug 04 | CJ | 49 | RHH | POS | GNB | POS | POS | POS |
| 87 | Community | Ear | non | Aug 04 | CK | 39 | RHH | POS | GNB | POS | POS | POS |
| 89 | Out-patient | Skin/soft tissue | non | Jul 04 | CL | 59 | RHH | POS | GNB | POS | POS | POS |
| 90 | Other Ward | Urine | non | Jul 04 | CM | 76 | RHH | POS | GNB | POS | POS | POS |
| 91 | NSU | Skin/soft tissue | non | Jul 04 | CN | 29 | RHH | POS | GNB | POS | POS | POS |
| 92 | ICU | Respiratory Trap | non | Jun 04 | CO | 59 | RHH | POS | GNB | POS | POS | POS |
| 93 | Other Ward | Skin/soft tissue | non | Jul 04 | CP | 78 | RHH | POS | GNB | POS | POS | POS |
| 94 | Other Ward | Skin/soft tissue | non | Jul 04 | CQ | 74 | RHH | POS | GNB | POS | POS | POS |

| Isolate Number | Source | Site of Isolation | Mucoidy | Month of Isolation | Patient/ Site identifier | Patient Age | Source Lab | Growth on Mac Agar | Gram Reaction | Oxidase reaction | Growth at 42°C | <i>P. aeruginosa</i> Specific PCR |
|----------------|-------------|----------------------|---------|--------------------|--------------------------|-------------|---------------|--------------------|---------------|------------------|----------------|-----------------------------------|
| 95 | Community | Ear | non | Jul 04 | CR | 0 | RHH | POS | GNB | POS | POS | POS |
| 96 | Community | Urine | non | Jul 04 | BU | 62 | RHH | POS | GNB | POS | POS | POS |
| 97 | Other Ward | COPD Respiratory | muc | Aug 04 | CS | 75 | RHH | POS | GNB | POS | POS | POS |
| 98 | Out-patient | Skin/soft tissue | non | Jul 04 | CT | 65 | RHH | POS | GNB | POS | POS | POS |
| 99 | Other Ward | Urine | non | Jan 05 | CU | 70 | RHH | POS | GNB | POS | POS | POS |
| 100 | Community | Urine | non | Jan 05 | CV | 58 | Cultured | POS | GNB | POS | POS | POS |
| 104 | Other Ward | SINK | non | Jun 05 | CW | na | Cultured | POS | GNB | POS | POS | NEG |
| 105 | Other Ward | SINK | non | Jun 05 | CX | na | Cultured | POS | GNB | POS | POS | NEG |
| 106 | Other Ward | SINK | non | Jun 05 | CY | na | Cultured | POS | GNB | POS | POS | NEG |
| 107 | Other Ward | SINK | non | Jun 05 | CZ | na | Cultured | POS | GNB | POS | POS | NEG |
| 108 | Other Ward | SINK | non | Jun 05 | DA | na | Cultured | POS | GNB | POS | POS | POS |
| 109 | Other Ward | SINK | non | Jun 05 | DB | na | Cultured | POS | GNB | POS | POS | NEG |
| 110 | Other Ward | SINK | non | Jun 05 | DC | na | Cultured | POS | GNB | POS | POS | NEG |
| 111 | ICU | SINK | non | Jun 05 | DD | na | Cultured | POS | GNB | POS | POS | POS |
| 112 | ICU | SINK | non | Jun 05 | DE | na | Cultured | POS | GNB | POS | POS | POS |
| 114 | ICU | SINK | muc | Jun 05 | DF | na | Cultured | POS | GNB | POS | POS | POS |
| 115 | ICU | SINK | non | Jun 05 | DF | na | Cultured | POS | GNB | POS | POS | POS |
| 116 | ICU | WASH TUB | non | Jun 05 | DG | na | Cultured | POS | GNB | POS | POS | POS |
| 117 | ICU | SINK | non | Jun 05 | DH | na | Cultured | POS | GNB | POS | POS | NEG |
| 118 | ICU | SINK | non | Jun 05 | DI | na | Cultured | POS | GNB | POS | POS | POS |
| 119 | ICU | SINK | non | Jun 05 | DD | na | Cultured | POS | GNB | POS | POS | POS |
| 120 | ICU | SINK | non | Jun 05 | DJ | na | Cultured | POS | GNB | POS | POS | POS |
| 121 | NSU | SINK | non | Jun 05 | DK | na | Cultured | POS | GNB | POS | POS | POS |
| 122 | NSU | SINK | non | Jun 05 | DL | na | Cultured | POS | GNB | POS | POS | POS |
| 123 | NSU | SINK | non | Jun 05 | DM | na | Cultured | POS | GNB | POS | POS | POS |
| 124 | NSU | SINK | non | Jun 05 | DN | na | Cultured | POS | GNB | POS | POS | POS |
| 127 | Other Ward | SINK | non | Jun 05 | DO | na | Cultured | POS | GNB | POS | POS | POS |
| 128 | Other Ward | SINK | non | Jun 05 | DP | na | Cultured | POS | GNB | POS | POS | POS |
| 130 | Other Ward | SINK | non | Jun 05 | DQ | na | Cultured | POS | GNB | POS | POS | POS |
| 134 | Other Ward | SINK | non | Jun 05 | DR | na | Cultured | POS | GNB | POS | POS | POS |
| 135 | Other Ward | SINK | non | Jun 05 | DS | na | Cultured | POS | GNB | POS | POS | POS |
| 136 | Other Ward | SINK | non | Jun 05 | DT | na | Cultured | POS | GNB | POS | POS | POS |
| 141 | Other Ward | SINK | non | Jun 05 | DU | na | Cultured | POS | GNB | POS | POS | POS |
| 142 | Other Ward | SINK | non | Jun 05 | DV | na | Cultured | POS | GNB | POS | POS | POS |
| 144 | Other Ward | SINK | non | Jun 05 | DW | na | Cultured | POS | GNB | POS | POS | POS |
| 145 | Other Ward | Shower | non | Jun 05 | DX | na | Cultured | POS | GNB | POS | POS | POS |
| 146 | Other Ward | Shower | non | Jun 05 | DX | na | Cultured | POS | GNB | POS | POS | POS |
| 147 | Community | Pool | non | Jul 05 | DY | na | Public Health | POS | GNB | POS | POS | POS |
| 149 | Community | Pool | non | Jul 05 | DZ | na | Public Health | POS | GNB | POS | POS | POS |
| 150 | Community | Spa | non | Jul 05 | DZ | na | Public Health | POS | GNB | POS | POS | POS |
| 151 | Community | Pool | non | Jul 05 | EA | na | Public Health | POS | GNB | POS | POS | POS |
| 153 | Community | Spa | non | Jul 05 | EB | na | Public Health | POS | GNB | POS | POS | POS |
| 154 | Community | Effluent inlet | non | Jul 05 | EC | na | Public Health | POS | GNB | POS | POS | POS |
| 156 | Community | Effluent spillage | non | Jul 05 | ED | na | Public Health | POS | GNB | POS | POS | POS |
| 157 | Community | Effluent spillage | non | Jul 05 | EE | na | Public Health | POS | GNB | POS | POS | POS |
| 158 | Community | Landfill Groundwater | non | Jul 05 | EE | na | Public Health | POS | GNB | POS | POS | POS |
| 160 | Community | Stream | non | Jul 05 | EF | na | Public Health | POS | GNB | POS | POS | POS |
| U1a | Out-patient | CF Respiratory | muc | Mar 03 | CH | 26 | UTAS | POS | GNB | POS | POS | POS |
| U1b | Out-patient | CF Respiratory | non | Mar 03 | CH | 26 | UTAS | POS | GNB | POS | POS | POS |
| U3a | Out-patient | CF Respiratory | muc | Mar 03 | EG | 19 | UTAS | POS | GNB | POS | POS | POS |
| U3b | Out-patient | CF Respiratory | non | Mar 03 | EG | 19 | UTAS | POS | GNB | POS | POS | POS |
| U5a | Out-patient | CF Respiratory | muc | Mar 03 | EH | 33 | UTAS | POS | GNB | POS | POS | POS |
| U5b | Out-patient | CF Respiratory | non | Mar 03 | EH | 33 | UTAS | POS | GNB | POS | POS | POS |
| R14a | Out-patient | CF Respiratory | muc | Apr 03 | EI | 26 | UTAS | POS | GNB | POS | POS | POS |
| R14b | Out-patient | CF Respiratory | non | Apr 03 | EI | 26 | RHH | POS | GNB | POS | POS | POS |
| U16a | Out-patient | CF Respiratory | muc | May 03 | EJ | 23 | RHH | POS | GNB | POS | POS | POS |
| U16b | Out-patient | CF Respiratory | non | May 03 | EJ | 23 | UTAS | POS | GNB | POS | POS | POS |
| U17a | Out-patient | CF Respiratory | muc | May 03 | EK | 22 | UTAS | POS | GNB | POS | POS | POS |
| U17b | Out-patient | CF Respiratory | non | May 03 | EK | 22 | UTAS | POS | GNB | POS | POS | POS |
| U19a | Out-patient | CF Respiratory | muc | Apr 03 | EL | 19 | UTAS | POS | GNB | POS | POS | POS |
| U19b | Out-patient | CF Respiratory | muc | Apr 03 | EL | 19 | UTAS | POS | GNB | POS | POS | POS |
| U20 | Out-patient | CF Respiratory | muc | Apr 03 | EM | 49 | UTAS | POS | GNB | POS | POS | POS |
| U21 | Out-patient | CF Respiratory | muc | May 03 | EN | 45 | UTAS | POS | GNB | POS | POS | POS |
| U26 | Out-patient | CF Respiratory | muc | May 03 | EO | 27 | UTAS | POS | GNB | POS | POS | POS |
| R29b | Out-patient | CF Respiratory | non | May 03 | EK | 22 | UTAS | POS | GNB | POS | POS | POS |
| U32a | Out-patient | CF Respiratory | muc | May 03 | AJ | 26 | UTAS | POS | GNB | POS | POS | POS |
| U32b | Out-patient | CF Respiratory | muc | May 03 | AJ | 26 | UTAS | POS | GNB | POS | POS | POS |
| U33a | Out-patient | CF Respiratory | muc | May 03 | AS | 24 | UTAS | POS | GNB | POS | POS | POS |
| U33b | Out-patient | CF Respiratory | non | May 03 | AS | 24 | UTAS | POS | GNB | POS | POS | POS |
| R43 | Out-patient | CF Respiratory | muc | Jun 03 | EP | 41 | RHH | POS | GNB | POS | POS | POS |
| R44a | Out-patient | CF Respiratory | muc | Jun 03 | EQ | 22 | RHH | POS | GNB | POS | POS | POS |
| R44b | Out-patient | CF Respiratory | non | Jun 03 | EQ | 22 | RHH | POS | GNB | POS | POS | POS |
| U47a | Out-patient | CF Respiratory | muc | Jun 03 | ER | 22 | UTAS | POS | GNB | POS | POS | POS |
| U47b | Out-patient | CF Respiratory | non | Jun 03 | ER | 22 | UTAS | POS | GNB | POS | POS | POS |
| U50 | Out-patient | CF Respiratory | muc | July 03 | ES | 33 | UTAS | POS | GNB | POS | POS | POS |
| U51 | Out-patient | CF Respiratory | muc | July 03 | ET | 34 | UTAS | POS | GNB | POS | POS | POS |

| Isolate Number | Source | Site of Isolation | Mucoidy | Month of Isolation | Patient/ Site identifier | Patient Age | Source Lab | Growth on Mac Agar | Gram Reaction | Oxidase reaction | Growth at 42°C | <i>P. aeruginosa</i> Specific PCR |
|----------------------------------|-------------|-------------------|---------|--------------------|--------------------------|-------------|------------|--------------------|---------------|------------------|----------------|-----------------------------------|
| R58 | Out-patient | CF Respiratory | muc | July 03 | ER | 22 | RHH | POS | GNB | POS | POS | POS |
| U61a | Out-patient | CF Respiratory | muc | Jul 03 | EU | 16 | UTAS | POS | GNB | POS | POS | POS |
| U61b | Out-patient | CF Respiratory | muc | Jul 03 | EU | 16 | UTAS | POS | GNB | POS | POS | POS |
| U66a | Out-patient | CF Respiratory | muc | Aug 03 | EV | 22 | UTAS | POS | GNB | POS | POS | POS |
| U66b | Out-patient | CF Respiratory | non | Aug 03 | EV | 22 | UTAS | POS | GNB | POS | POS | POS |
| U73 | Out-patient | CF Respiratory | muc | Sep 03 | EW | 26 | UTAS | POS | GNB | POS | POS | POS |
| U80a | Out-patient | CF Respiratory | muc | Sep 03 | EX | 29 | UTAS | POS | GNB | POS | POS | POS |
| U80b | Out-patient | CF Respiratory | muc | Sep 03 | EX | 29 | UTAS | POS | GNB | POS | POS | POS |
| U81a | Out-patient | CF Respiratory | muc | Sep 03 | EY | 10 | UTAS | POS | GNB | POS | POS | POS |
| U81b | Out-patient | CF Respiratory | non | Sep 03 | EY | 10 | UTAS | POS | GNB | POS | POS | POS |
| R86a | Out-patient | CF Respiratory | muc | Sep 03 | EZ | 47 | RHH | POS | GNB | POS | POS | POS |
| R86b | Out-patient | CF Respiratory | non | Sep 03 | EZ | 47 | RHH | POS | GNB | POS | POS | POS |
| U108a | Out-patient | CF Respiratory | non | Dec 03 | FA | 32 | UTAS | POS | GNB | POS | POS | POS |
| U108b | Out-patient | CF Respiratory | non | Dec 03 | FA | 32 | UTAS | POS | GNB | POS | POS | POS |
| U108c | Out-patient | CF Respiratory | muc | Dec 03 | FA | 32 | UTAS | POS | GNB | POS | POS | POS |
| U110 | Out-patient | CF Respiratory | non | Dec 03 | FB | 14 | UTAS | POS | GNB | POS | POS | POS |
| U111a | Out-patient | CF Respiratory | muc | Dec 03 | AH | 17 | UTAS | POS | GNB | POS | POS | POS |
| U111b | Out-patient | CF Respiratory | muc | Dec 03 | AH | 17 | UTAS | POS | GNB | POS | POS | POS |
| R112 | Out-patient | CF Respiratory | muc | Dec 03 | CH | 22 | RHH | POS | GNB | POS | POS | POS |
| R113a | Out-patient | CF Respiratory | muc | Dec 03 | AG | 23 | RHH | POS | GNB | POS | POS | POS |
| R113b | Out-patient | CF Respiratory | muc | Dec 03 | AG | 23 | RHH | POS | GNB | POS | POS | POS |
| U115 | Out-patient | CF Respiratory | non | Dec 03 | FC | 25 | UTAS | POS | GNB | POS | POS | POS |
| U126 | Out-patient | CF Respiratory | muc | Feb 04 | FD | 16 | UTAS | POS | GNB | POS | POS | POS |
| U156 | Out-patient | CF Respiratory | muc | Aug 04 | FE | 16 | UTAS | POS | GNB | POS | POS | POS |
| U162 | Out-patient | CF Respiratory | non | Dec 04 | FF | 29 | UTAS | POS | GNB | POS | POS | POS |
| U171 | Out-patient | CF Respiratory | non | Dec 04 | FG | 27 | UTAS | POS | GNB | POS | POS | POS |
| U175 | Out-patient | CF Respiratory | muc | Dec 04 | FH | 17 | UTAS | POS | GNB | POS | POS | POS |
| U184 | Out-patient | CF Respiratory | non | Feb 05 | FI | 8 | UTAS | POS | GNB | POS | POS | POS |
| U194a | Out-patient | CF Respiratory | muc | Feb 05 | FJ | 16 | UTAS | POS | GNB | POS | POS | POS |
| U194b | Out-patient | CF Respiratory | non | Feb 05 | FJ | 16 | UTAS | POS | GNB | POS | POS | POS |
| U195a | Out-patient | CF Respiratory | muc | Feb 05 | FK | 10 | UTAS | POS | GNB | POS | POS | POS |
| U195b | Out-patient | CF Respiratory | non | Feb 05 | FK | 10 | UTAS | POS | GNB | POS | POS | POS |
| U240 | Out-patient | CF Respiratory | muc | Jun 05 | FL | 17 | UTAS | POS | GNB | POS | POS | POS |
| RWE2 | Out-patient | CF Respiratory | muc | Aug 05 | FM | 37 | RHH | POS | GNB | POS | POS | POS |
| <i>P. aeruginosa</i> PA01 | | | | | | | | POS | GNB | POS | POS | POS |
| <i>P. fluorescens</i> ATCC 13525 | | | | | | | | POS | GNB | POS | NEG | NEG |

Table 4.1: Source, phenotypic characterisation and *P. aeruginosa* PCR results of isolates in this study.

muc: mucoid, **non:** non mucoid, **RHH:** Provided by RHH Department of Microbiology and Infectious Diseases, **Culture:** Cultured as a part of this study, **Public Health:** Provided by Tasmanian Public Health Laboratory, **UTAS:** Provided by University of Tasmania CF isolate library, **GNB:** Gram negative bacillus, **ND:** Not performed, * Known *P. fluorescens* strain.

| Isolate | Source | Site of infection / colonisation | Patient/ Site identifier | <i>Pseudomonas aeruginosa</i> specific PCR | Vitek® GNI+ ID Bionumber | Probability of correct Vitek® ID | Vitek® GNI+ Identification |
|---------|------------|----------------------------------|--------------------------|--|--------------------------|----------------------------------|--|
| 104 | Other Ward | SINK | CY | NEG | 7600344074 | 99% | <i>Aeromonas hydrophila/caviae</i> |
| 105 | Other Ward | SINK | CZ | NEG | 7600344074 | 99% | <i>Aeromonas hydrophila/caviae</i> |
| 106 | Other Ward | SINK | DA | NEG | 7600344274 | 99% | <i>Aeromonas hydrophila/caviae</i> |
| 107 | Other Ward | SINK | DB | NEG | 70200000440 | 99% | <i>Achromobacter xylosoxidans</i> ssp. <i>Xylosoxidans</i> |
| 109 | Other Ward | SINK | DC | NEG | 60021400040 | 74% | <i>Chryseobacterium indologenes</i> |
| 110 | Other Ward | SINK | DD | NEG | 40700000040 | 93% | <i>Bordetella bronchiseptica</i> |
| 117 | ICU | SINK | DJ | NEG | 70200000440 | 99% | <i>Achromobacter xylosoxidans</i> ssp. <i>Xylosoxidans</i> |

Table 4.2: Vitek® GNI+ identification of isolates conforming to identity of *P. aeruginosa* by Gram reaction, oxidase positivity, capacity for growth on MacConkey agar at 42°C and resistance to the compound C390, but yielding no product by *P. aeruginosa* specific PCR.

| Site of Isolation (no. of isolates) | Number of Sites Sampled | Number of Sites Isolates Recovered From | Number of sites with multiple isolates | Recovery of Isolates From Sites Sampled (%) |
|--|-------------------------------|---|---|---|
| ICU (n=8) | 8 | 7 | 1 | 88 |
| NSU (n=4) | 4 | 4 | 0 | 100 |
| Other Ward (n=13) | 52 | 13 | 0 | 25 |
| Total (n=25) | 65 | 24 | 1 | 37 |

Table 4.3: Recovery rates of *Pseudomonas aeruginosa* from differing aqueous environmental sites throughout the hospital.

4.4 Discussion

Of the 190 test isolates included in this study 183 were found to belong to the species *P. aeruginosa* by the test methods used. Only seven isolates were misidentified as *P. aeruginosa* using routine clinical laboratory methods and all of these were environmental strains. Further characterisation showed that these isolates belonged to other species of environmental, non-fermenting Gram negative bacilli.

4.4.1 Distribution of hospital *Pseudomonas aeruginosa* isolates

The prevalence of *P. aeruginosa* in specialist units dealing with very high dependency patients is of concern, but not entirely unexpected, given the frequent use of broad spectrum antimicrobials in these settings. *P. aeruginosa* is widely recognised as one of the most prevalent and important causes of infection in the ICU setting (Deplano, Denis *et al.* 2005). It is notable that in contrast, few other hospital environmental sites swabbed isolated *P. aeruginosa*. All aqueous environmental sites in the ICU and NSU contained at least one strain of *P. aeruginosa*, and in some cases multiple morphotypes were recovered.

Both the neurosurgical and intensive care units generally contain an increased proportion of patients on empirical broad spectrum antimicrobial therapy compared to other units in the hospital. Furthermore, there will be patients with indwelling medical devices at multiple anatomical sites. Such patients will

also be in poor clinical condition, and thus will have challenged or compromised immunity. All of these factors have been described previously as predisposing and selecting for infection with *P. aeruginosa* (Brisse, Milatovic *et al.* 2000; Dinesh, Grundmann *et al.* 2003; Stryjewski and Sexton 2003). Combined with these factors, such units often use much greater amounts of disinfectants in cleaning and infection control practices, and *P. aeruginosa* has a demonstrated capacity to develop resistance to multiple types of disinfectants (Learn, Brestel *et al.* 1987; Tabata, Nagamune *et al.* 2003), which may also act as a selective factor in such environments. The diverse range of sites and sources from which *P. aeruginosa* was cultured in this study demonstrates this organism's capacity to colonise multiple sites within the hospital and community environment and to cause many different types of disease.

Morphological differences in isolates obtained from a single biological specimen were uncommon, except in the case of CF sputum specimens. The presence of multiple morphotypes of *P. aeruginosa* in CF sputum is a well recognised occurrence and these differing morphotypes may or may not be genotypically indistinguishable (Anthony, Rose *et al.* 2002; Armstrong, Nixon *et al.* 2002; Nixon, Armstrong *et al.* 2002). For the purpose of this study, isolates displaying markedly different morphotypes were selected for analysis so as not to overlook differences or similarities between strains obtained from the same patient as well as the CF population overall.

In a few of the non-CF patients, *P. aeruginosa* was isolated from the same individual from different infection sites several months apart. Infection with *P. aeruginosa* is dependent upon a number of host factors, particularly impaired or compromised host immunity, and is often preceded by colonisation of the gastrointestinal tract (Speijer, Savelkoul *et al.* 1999). New infections may be acquired either from

the hospital environment and staff through poor infection control, or from the patient's own bowel flora. Thus, it is unsurprising that colonised patients may be infected in multiple sites or re-infected with the same or a new strain of *P. aeruginosa*. Genotyping and antimicrobial susceptibility testing of the isolates concerned will provide greater insight into the mode of acquisition and persistence of *P. aeruginosa* isolates from these patients.

4.4.2 Muroid morphology

Muroid morphology was particularly associated with CF isolates in this study, but was also observed in some urine, sink and non-CF respiratory isolates. The muroid morphotype is closely associated with biofilm development (Nivens, Ohman *et al.* 2001). This combined with the protection against the antimicrobial agents commonly used in CF prophylaxis that is generated by biofilm growth (Høiby, Krogh Johansen *et al.* 2001; Fux, Stoodley *et al.* 2003) explains the presence of mucoidy in these samples. Similarly, the muroid morphology of isolates 21 and 97 from a COPD respiratory specimen is not unexpected, due to the association of chronic *P. aeruginosa* COPD infection with muroid phenotype (Pujana, Gallego *et al.* 1999), due to selective pressures and biofilm formation in the COPD lung very similar to CF infections (Maciá, Blanquer *et al.* 2005).

Both muroid and non-muroid isolates were recovered from an intubated patient in the ICU (isolates 49 and 50). A muroid isolate (isolate 61) was recovered from the urine of a patient on a general ward and a further muroid isolate from an ICU sink (isolate 114). The phenotypic variability in isolates 49 and 50 from the same respiratory specimen is reminiscent of CF respiratory infection, and has probably been induced by similar environmental effectors, such as exposure to antimicrobials and biofilm mode of growth. The predilection of *P. aeruginosa* isolates to form biofilms along tracheal tubes and other

plastic indwelling medical devices is a well described (Engel 2003). Although an intubated patient in ICU is unlikely to develop chronic *P. aeruginosa* pulmonary infection, as seen in CF and occasionally in COPD, the growth of *P. aeruginosa* in biofilm and selective pressures of antibiotic prophylaxis may easily lead to mutation and the development of a mucoid morphotype. Similarly, a pipeline with fluid flowing through it, such as a sink drain provides an excellent substrate for biofilm growth (den Aantrekker 2003), which will be further enhanced by the selective pressure of disinfectants used in cleaning (Høiby, Krogh Johansen *et al.* 2001; Fux, Stoodley *et al.* 2003). Similarly, mucoidy and biofilm growth have been associated with urinary isolates, particularly those from urinary catheters (Engel 2003), which is consistent with the findings in isolate 61.

4.5 Conclusion

In summary, this study incorporated 190 isolates from a diverse range of clinical and environmental sources, 183 of which were found by phenotypic and genotypic methods to belong to the species *P. aeruginosa*. Of the seven isolates found not to be *P. aeruginosa*, and seven were hospital environment isolates belonging to a number of genera other than *Pseudomonas*.

Multiple morphotypes and mucoid isolates were recovered from both environmental sites and patients, particularly those with CF. In a number of cases, patients had *P. aeruginosa* collected from multiple sites or from the same site many months apart. Further investigation into the antimicrobial resistance patterns and genotypic similarity of these isolates is warranted to gain a greater understanding of the epidemiology of *P. aeruginosa* infection.

CHAPTER FIVE

Antimicrobial Resistance

5.1 Introduction

P. aeruginosa is known to be capable of the acquisition of many different antimicrobial resistance genes. In particular, CF and nosocomial isolates are associated with much higher levels of antimicrobial resistance and may express an MDR phenotype. Antimicrobial susceptibility testing was performed on all isolates of *P. aeruginosa* in this study in order to elucidate the distribution and prevalence of antimicrobial resistance in Tasmanian *P. aeruginosa* isolates and to identify any clustering of antimicrobial resistance patterns within the studied isolates. Such information may be linked to epidemiological and clinical outcomes in patients. Similarly, this information may assist in identifying causes of the increased prevalence of *P. aeruginosa* in the environments of the intensive therapy wards of the hospital.

5.2 Methods

The CLSI method (method 3.13) was employed to test the sensitivity of all isolates to comprehensive range of antimicrobial agents (carbenicillin, ticarcillin, timentin, ceftazidime, cefepime, aztreonam, imipenem, amikacin, gentamicin, tobramycin and ciprofloxacin). This method is internationally accepted, simple and allows ease of use when screening a large number of isolates. This allowed the production of a large antibiogram, including multiple classes of antibiotic. Only true resistant rather than intermediate results were considered in the interpretation of this study.

The US CF foundation consensus guidelines definition of multi-drug resistance was employed; defining multi-drug resistance as being resistance to all agents in two or more of the following classes: β -lactams (including cephalosporins, monobactams and carbapenems), aminoglycosides and

fluoroquinolones (Armstrong, Nixon *et al.* 2002). The statistical validity of results was determined by χ^2 squared analysis.

5.3 Results

Forty-nine (27%) of isolates in this study showed true resistance to two or more of the antimicrobials tested. Most resistance was seen in the β -lactam class drugs, specifically the anti-pseudomonal penicillins and the monobactam, aztreonam (table 5.1). Lower rates of resistance were seen in the aminoglycosides, cephalosporins and fluoroquinolones (ciprofloxacin). Isolates recovered from the CF lung demonstrated significantly ($p < 0.001$) increased resistance to all classes antimicrobials. Out-patient isolates appeared to showed significantly ($p < 0.001$) increased resistance (table 5.3). Due to the disproportionately high of antimicrobial resistance demonstrated in the CF isolates, separate statistical analyses was carried out with these isolates excluded. This action demonstrated that the increased resistance noted in out-patient isolates was due to statistical skewing by the large number of highly resistant CF isolates in this group (table 5.4). These analyses showed that ICU and NSU isolates showed significantly ($p < 0.01$) increased resistance to all β -lactam derived agents when compared to other strains recovered in this study (table 5.4). This was reflected in the significantly ($p < 0.01$) increased resistance level in hospital environment isolates overall (table 5.2), many of which were isolates obtained from these two intensive therapy wards. Isolates from wards other than these two intensive therapy wards showed significantly lower resistance to antimicrobial until the analysis was repeated with CF isolates excluded. Whilst many isolates showed increased resistance to a number of antimicrobials, true multi-drug resistance by the strict definition employed was observed only in two CF isolates and two neurosurgical unit environmental isolates.

CLSI Antibigram

| Isolate | Identifier | Age | Place of | Site of Isolation | Mucoidy | Carbenicillin | Ticarcillin | Timentin | Ceftazidime | Cefepime | Aztreonam | Imipenem | Amikacin | Gentamicin | Tobramycin | Ciprofloxacin |
|---------|------------|-----|-------------|------------------------|---------|---------------|-------------|----------|-------------|----------|-----------|----------|----------|------------|------------|---------------|
| 1 | AA | 33 | Other Ward | Respiratory | non | 17 | 22 | 22 | 26 | 26 | 21 | 24 | 21 | 17 | 22 | 30 |
| 2 | AB | 76 | Other Ward | Ear | non | 21 | 25 | 25 | 28 | 30 | 25 | 24 | 24 | 20 | 23 | 35 |
| 3 | AC | 92 | Other Ward | Skin/soft tissue | non | 20 | 24 | 25 | 28 | 27 | 25 | 26 | 23 | 18 | 22 | 32 |
| 4 | AD | 77 | Other Ward | Skin/soft tissue | non | 21 | 23 | 20 | 23 | 21 | 21 | 26 | 21 | 16 | 21 | 27 |
| 5 | AE | 76 | Other Ward | Respiratory | non | 20 | 24 | 24 | 29 | 32 | 23 | 30 | 24 | 21 | 26 | 34 |
| 6 | AF | 43 | Other Ward | Urine | non | 0 | 10 | 11 | 26 | 23 | 13 | 15 | 21 | 12 | 20 | 16 |
| 7 | AG | 23 | Out-patient | CF Respiratory | muc | 33 | 35 | 34 | 38 | 32 | 36 | 19 | 26 | 20 | 27 | 27 |
| 8 | AH | 17 | Out-patient | CF Respiratory | muc | 25 | 30 | 30 | 33 | 38 | 28 | 20 | 30 | 24 | 30 | 30 |
| 10 | AE | 76 | Other Ward | Urine | non | 22 | 26 | 26 | 29 | 29 | 26 | 27 | 24 | 22 | 25 | 35 |
| 11 | AI | 76 | Other Ward | Skin/soft tissue | non | 17 | 22 | 22 | 30 | 29 | 26 | 27 | 22 | 19 | 24 | 33 |
| 12 | AJ | 26 | Other Ward | CF Respiratory | muc | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 12 | 18 | 16 |
| 13 | AK | 75 | Other Ward | Respiratory | non | 20 | 25 | 22 | 25 | 29 | 23 | 22 | 22 | 19 | 24 | 36 |
| 14 | AL | 79 | Other Ward | Respiratory | non | 19 | 24 | 23 | 28 | 30 | 24 | 24 | 22 | 21 | 25 | 33 |
| 15 | AM | 71 | Other Ward | Respiratory | non | 21 | 26 | 25 | 30 | 33 | 27 | 22 | 26 | 22 | 26 | 35 |
| 16 | AN | 76 | Other Ward | Respiratory | non | 34 | 34 | 34 | 31 | 37 | 36 | 32 | 34 | 31 | 31 | 36 |
| 17 | AA | 33 | Out-patient | Respiratory | non | 17 | 22 | 22 | 28 | 28 | 22 | 23 | 20 | 18 | 22 | 31 |
| 18 | AO | 92 | Other Ward | Urine | non | 18 | 24 | 24 | 30 | 31 | 23 | 29 | 26 | 23 | 26 | 36 |
| 19 | AP | 68 | Other Ward | Urine | non | 20 | 25 | 23 | 28 | 28 | 23 | 18 | 20 | 17 | 22 | 33 |
| 20 | AQ | 83 | Other Ward | Urine | non | 11 | 21 | 8 | 13 | 21 | 18 | 28 | 24 | 22 | 26 | 33 |
| 21 | AR | 60 | Out-patient | COPD Respiratory | muc | 14 | 19 | 19 | 26 | 24 | 19 | 34 | 26 | 20 | 25 | 26 |
| 22 | AS | 24 | Other Ward | CF Respiratory | muc | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 10 | 19 | 16 |
| 23 | AT | 84 | Community | Ear | non | 21 | 24 | 24 | 33 | 33 | 26 | 26 | 24 | 24 | 27 | 33 |
| 24 | AG | 83 | Other Ward | Urine | non | 18 | 23 | 23 | 28 | 31 | 23 | 29 | 25 | 21 | 26 | 34 |
| 25 | AU | 72 | Other Ward | Catheter Urine | non | 22 | 26 | 25 | 29 | 32 | 27 | 24 | 23 | 20 | 23 | 33 |
| 26 | AV | 73 | Community | Urine | non | 17 | 21 | 21 | 27 | 26 | 19 | 27 | 20 | 16 | 20 | 30 |
| 28 | AW | 78 | Other Ward | Skin/soft tissue | non | 0 | 18 | 16 | 24 | 18 | 16 | 21 | 26 | 18 | 27 | 30 |
| 29 | AX | 73 | Other Ward | Skin/soft tissue | non | 24 | 26 | 26 | 30 | 30 | 27 | 24 | 25 | 21 | 25 | 32 |
| 30 | AY | 43 | NSU | Respiratory | non | 0 | 10 | 13 | 20 | 28 | 29 | 23 | 22 | 20 | 0 | 13 |
| 31 | AZ | 1 | Other Ward | Respiratory | non | 21 | 24 | 23 | 25 | 30 | 24 | 27 | 22 | 0 | 21 | 31 |
| 32 | BA | 23 | Out-patient | Respiratory | non | 36 | 36 | 36 | 30 | 22 | 35 | 0 | 20 | 18 | 22 | 26 |
| 33 | BB | 76 | Other Ward | Skin/soft tissue | non | 19 | 26 | 26 | 31 | 32 | 24 | 26 | 23 | 16 | 24 | 34 |
| 34 | BC | 53 | Other Ward | Catheter Urine | non | 16 | 17 | 16 | 20 | 23 | 26 | 23 | 22 | 21 | 23 | 40 |
| 35 | BD | 73 | Other Ward | Urine | non | 19 | 24 | 24 | 28 | 28 | 23 | 24 | 22 | 19 | 22 | 36 |
| 36 | BE | 74 | Other Ward | Skin/soft tissue | non | 23 | 24 | 24 | 20 | 32 | 26 | 23 | 20 | 20 | 22 | 33 |
| 37 | AE | 76 | Other Ward | Catheter Urine | non | 23 | 26 | 26 | 28 | 34 | 26 | 23 | 24 | 17 | 25 | 34 |
| 38 | BF | 84 | Other Ward | Respiratory | non | 14 | 19 | 20 | 31 | 22 | 21 | 26 | 17 | 20 | 20 | 13 |
| 39 | BG | 66 | Other Ward | Urine | non | 22 | 27 | 27 | 29 | 33 | 26 | 23 | 23 | 12 | 24 | 35 |
| 40 | BH | 76 | Other Ward | Skin/soft tissue | non | 19 | 23 | 23 | 27 | 30 | 22 | 29 | 25 | 20 | 24 | 31 |
| 44 | BI | 53 | Other Ward | Skin/soft tissue | non | 19 | 22 | 22 | 22 | 30 | 23 | 24 | 24 | 19 | 23 | 28 |
| 45 | BJ | 54 | Out-patient | Catheter Urine | non | 18 | 25 | 22 | 30 | 30 | 23 | 23 | 19 | 17 | 22 | 33 |
| 46 | BK | 76 | Out-patient | Skin/soft tissue | non | 22 | 28 | 28 | 31 | 32 | 30 | 27 | 28 | 22 | 27 | 32 |
| 47 | BL | 48 | Community | Urine | non | 23 | 26 | 26 | 34 | 30 | 26 | 29 | 25 | 21 | 25 | 25 |
| 48 | BM | 84 | Other Ward | Respiratory | non | 34 | 32 | 36 | 30 | 34 | 32 | 36 | 31 | 18 | 21 | 6 |
| 49 | BN | 75 | NSU | Respiratory | muc | 22 | 25 | 25 | 32 | 34 | 26 | 33 | 25 | 21 | 25 | 30 |
| 50 | BN | 75 | NSU | Respiratory | non | 22 | 27 | 25 | 28 | 31 | 26 | 26 | 25 | 18 | 25 | 31 |
| 51 | BO | 48 | Community | Urine | non | 10 | 13 | 17 | 30 | 29 | 19 | 23 | 23 | 18 | 23 | 32 |
| 55 | BP | 67 | Other Ward | Skin/soft tissue | non | 0 | 15 | 17 | 27 | 26 | 19 | 23 | 24 | 20 | 24 | 31 |
| 57 | BQ | 80 | Out-patient | Urine | non | 0 | 9 | 5 | 24 | 21 | 14 | 21 | 20 | 16 | 22 | 17 |
| 60 | BR | 78 | ICU | Respiratory | non | 9 | 12 | 25 | 28 | 30 | 31 | 23 | 23 | 19 | 22 | 35 |
| 61 | BS | 87 | Other Ward | Urine | muc | 23 | 30 | 32 | 32 | 33 | 29 | 36 | 25 | 20 | 27 | 6 |
| 62 | BT | 64 | ICU | Respiratory | non | 15 | 22 | 19 | 32 | 32 | 25 | 15 | 25 | 18 | 25 | 45 |
| 63 | BU | 61 | Out-patient | Skin/soft tissue | non | 20 | 24 | 24 | 30 | 29 | 26 | 22 | 23 | 17 | 23 | 34 |
| 64 | BU | 61 | Out-patient | (alternate site to 63) | non | 0 | 14 | 14 | 24 | 23 | 18 | 25 | 23 | 18 | 23 | 28 |
| 65 | BV | 45 | Other Ward | Respiratory | non | 21 | 26 | 22 | 27 | 33 | 25 | 23 | 21 | 17 | 22 | 32 |
| 66 | BU | 61 | Out-patient | Urine | non | 21 | 24 | 23 | 29 | 23 | 24 | 23 | 22 | 18 | 23 | 31 |
| 67 | BW | 64 | Other Ward | Skin/soft tissue | non | 0 | 0 | 0 | 13 | 14 | 16 | 24 | 22 | 20 | 25 | 35 |
| 68 | BX | 23 | Other Ward | Respiratory | non | 10 | 12 | 9 | 13 | 15 | 20 | 5 | 20 | 18 | 20 | 24 |
| 69 | BY | 83 | Other Ward | Respiratory | non | 0 | 12 | 12 | 25 | 24 | 27 | 26 | 24 | 19 | 24 | 28 |
| 70 | BZ | 65 | Other Ward | Skin/soft tissue | non | 25 | 29 | 26 | 27 | 25 | 28 | 29 | 26 | 21 | 26 | 8 |
| 71 | CA | 50 | Other Ward | Respiratory | non | 20 | 23 | 24 | 29 | 30 | 24 | 25 | 25 | 21 | 25 | 35 |
| 72 | AT | 84 | Other Ward | Respiratory | non | 36 | 37 | 36 | 30 | 22 | 31 | 23 | 20 | 16 | 21 | 31 |
| 73 | CB | 68 | Other Ward | Respiratory | non | 17 | 23 | 23 | 27 | 22 | 23 | 20 | 21 | 18 | 22 | 29 |
| 74 | CC | na | Other Ward | Suction Unit | non | 0 | 0 | 0 | 32 | 19 | 31 | 24 | 23 | 0 | 9 | 13 |
| 75 | CB | 68 | Other Ward | Skin/soft tissue | non | 20 | 23 | 22 | 27 | 27 | 26 | 23 | 25 | 20 | 24 | 33 |
| 76 | BQ | 81 | Out-patient | Urine | non | 15 | 21 | 19 | 25 | 17 | 21 | 18 | 17 | 12 | 18 | 22 |
| 77 | CD | 77 | NSU | Catheter Urine | non | 16 | 25 | 20 | 27 | 20 | 21 | 5 | 21 | 16 | 21 | 29 |
| 78 | BW | 64 | Other Ward | Skin/soft tissue | non | 20 | 24 | 23 | 29 | 32 | 26 | 25 | 22 | 19 | 22 | 36 |
| 79 | CE | 77 | Other Ward | Skin/soft tissue | non | 19 | 24 | 22 | 26 | 28 | 24 | 25 | 21 | 18 | 22 | 30 |
| 80 | CF | 72 | ICU | Skin/soft tissue | non | 19 | 25 | 21 | 27 | 26 | 25 | 25 | 22 | 19 | 22 | 30 |
| 81 | CG | 10 | Out-patient | Ear | non | 20 | 23 | 20 | 24 | 28 | 26 | 23 | 19 | 16 | 23 | 34 |
| 82 | CH | 26 | Out-patient | CF Respiratory | muc | 25 | 30 | 26 | 28 | 26 | 26 | 30 | 20 | 16 | 20 | 30 |
| 83 | CH | 26 | Out-patient | CF Respiratory | non | 16 | 22 | 20 | 28 | 27 | 22 | 26 | 21 | 18 | 23 | 16 |
| 85 | CI | 37 | Other Ward | Skin/soft tissue | non | 19 | 22 | 21 | 27 | 26 | 25 | 19 | 20 | 17 | 21 | 0 |
| 86 | CJ | 49 | Other Ward | Skin/soft tissue | non | 23 | 28 | 24 | 28 | 31 | 28 | 20 | 25 | 19 | 24 | 32 |
| 87 | CK | 39 | Community | Ear | non | 25 | 27 | 26 | 28 | 30 | 28 | 16 | 24 | 19 | 25 | 29 |
| 89 | CL | 59 | Out-patient | Skin/soft tissue | non | 10 | 16 | 16 | 24 | 34 | 17 | 26 | 21 | 18 | 22 | 26 |
| 90 | CM | 76 | Other Ward | Urine | non | 21 | 26 | 25 | 32 | 17 | 28 | 26 | 22 | 19 | 26 | 22 |
| 91 | CN | 29 | NSU | Skin/soft tissue | non | 20 | 26 | 25 | 26 | 27 | 26 | 23 | 24 | 20 | 24 | 38 |
| 92 | CO | 59 | ICU | Respiratory Trap | non | 18 | 23 | 23 | 25 | 27 | 24 | 25 | 23 | 19 | 24 | 32 |
| 93 | CP | 78 | Other Ward | Skin/soft tissue | non | 20 | 25 | 25 | 28 | 25 | 27 | 23 | 30 | 30 | 29 | 32 |
| 94 | CQ | 74 | Other Ward | Skin/soft tissue | non | 0 | 12 | 13 | 23 | 25 | 18 | 25 | 23 | 20 | 23 | 34 |
| 95 | CR | 0 | Community | Ear | non | 23 | 23 | 23 | 28 | 30 | 25 | 23 | 23 | 20 | 25 | 26 |
| 96 | BU | 62 | Community | Urine | non | 30 | 25 | 23 | 28 | 25 | 27 | 23 | 23 | 18 | 24 | 34 |
| 97 | CS | 75 | Other Ward | COPD Respiratory | muc | 32 | 32 | 30 | 27 | 32 | 30 | 28 | 22 | 19 | 22 | 32 |
| 98 | CT | 65 | Out-patient | Skin/soft tissue | non | 15 | 19 | 19 | 26 | 24 | 23 | 23 | 24 | 20 | 25 | 36 |
| 99 | CU | 70 | Other Ward | Urine | non | 21 | 24 | 25 | 27 | 31 | 26 | 23 | 24 | 20 | 25 | 27 |
| 100 | CV | 58 | Community | Urine | non | 16 | 21 | 21 | 22 | 21 | 19 | 27 | 19 | 18 | 21 | 35 |

| Isolate | Identifier | Age | Place of | Site of Isolation | Mucoidy | Carbenicillin | Ticarcillin | Timentin | Ceftazidime | Cefepime | Aztreonam | Imipenem | Amikacin | Gentamicin | Tobramycin | Ciprofloxacin |
|--------------------|------------|-----|-------------|----------------------|---------|---------------|-------------|----------|-------------|----------|-----------|----------|----------|------------|------------|---------------|
| 108 | DA | na | Other Ward | SINK | non | 19 | 21 | 20 | 26 | 23 | 23 | 19 | 20 | 16 | 21 | 31 |
| 111 | DD | na | ICU | SINK | non | 13 | 15 | 16 | 23 | 20 | 17 | 20 | 19 | 17 | 23 | 27 |
| 112 | DE | na | ICU | SINK | non | 0 | 0 | 0 | 28 | 18 | 19 | 25 | 22 | 0 | 8 | 33 |
| 114 | DF | na | ICU | SINK | muc | 0 | 14 | 13 | 27 | 19 | 18 | 11 | 21 | 18 | 22 | 27 |
| 115 | DF | na | ICU | SINK | non | 0 | 11 | 12 | 26 | 18 | 16 | 10 | 23 | 18 | 23 | 30 |
| 116 | DG | na | ICU | WASH TUB | non | 23 | 24 | 24 | 30 | 24 | 27 | 26 | 24 | 19 | 25 | 31 |
| 118 | DI | na | ICU | SINK | non | 0 | 13 | 13 | 26 | 24 | 16 | 11 | 22 | 17 | 21 | 31 |
| 119 | DD | na | ICU | SINK | non | 18 | 17 | 16 | 29 | 23 | 21 | 27 | 23 | 20 | 25 | 26 |
| 120 | DJ | na | ICU | SINK | non | 11 | 16 | 21 | 26 | 20 | 19 | 26 | 22 | 18 | 22 | 26 |
| 121 | DK | na | NSU | SINK | non | 22 | 21 | 18 | 24 | 23 | 22 | 23 | 18 | 15 | 20 | 29 |
| 122 | DL | na | NSU | SINK | non | 17 | 18 | 19 | 26 | 21 | 19 | 27 | 21 | 17 | 21 | 26 |
| 123 | DM | na | NSU | SINK | non | 0 | 0 | 19 | 17 | 17 | 0 | 21 | 10 | 0 | 0 | 9 |
| 124 | DN | na | NSU | SINK | non | 0 | 0 | 16 | 17 | 18 | 0 | 19 | 10 | 0 | 0 | 0 |
| 127 | DO | na | Other Ward | SINK | non | 13 | 16 | 18 | 26 | 22 | 18 | 29 | 22 | 18 | 23 | 26 |
| 128 | DP | na | Other Ward | SINK | non | 14 | 16 | 21 | 25 | 18 | 18 | 28 | 19 | 16 | 22 | 28 |
| 130 | DQ | na | Other Ward | SINK | non | 19 | 20 | 23 | 25 | 23 | 15 | 18 | 19 | 16 | 21 | 27 |
| 134 | DR | na | Other Ward | SINK | non | 22 | 23 | 21 | 25 | 21 | 24 | 20 | 18 | 15 | 21 | 27 |
| 135 | DS | na | Other Ward | SINK | non | 19 | 21 | 21 | 26 | 21 | 19 | 16 | 19 | 15 | 20 | 29 |
| 136 | DT | na | Other Ward | SINK | non | 23 | 24 | 26 | 27 | 21 | 26 | 26 | 19 | 15 | 20 | 29 |
| 141 | DU | na | Other Ward | SINK | non | 18 | 23 | 24 | 26 | 25 | 25 | 23 | 23 | 19 | 23 | 34 |
| 142 | DV | na | Other Ward | SINK | non | 21 | 24 | 23 | 26 | 21 | 24 | 26 | 21 | 17 | 22 | 29 |
| 144 | DW | na | Other Ward | SINK | non | 20 | 21 | 22 | 24 | 20 | 23 | 24 | 20 | 16 | 22 | 29 |
| 145 | DX | na | Other Ward | Shower | non | 22 | 23 | 24 | 27 | 28 | 24 | 26 | 20 | 17 | 23 | 35 |
| 146 | DX | na | Other Ward | Shower | non | 20 | 22 | 21 | 24 | 28 | 21 | 26 | 22 | 17 | 22 | 32 |
| 147 | DY | na | Community | Pool | non | 20 | 24 | 22 | 29 | 24 | 26 | 33 | 21 | 16 | 22 | 31 |
| 149 | DZ | na | Community | Pool | non | 22 | 24 | 23 | 27 | 30 | 23 | 26 | 23 | 14 | 23 | 32 |
| 150 | DZ | na | Community | Spa | non | 22 | 23 | 24 | 26 | 28 | 24 | 28 | 22 | 17 | 22 | 33 |
| 151 | EA | na | Community | Pool | non | 26 | 29 | 25 | 34 | 33 | 28 | 31 | 24 | 19 | 24 | 34 |
| 153 | EB | na | Community | Spa | non | 18 | 17 | 17 | 24 | 26 | 18 | 11 | 23 | 18 | 24 | 34 |
| 154 | EC | na | Community | Effluent inlet | non | 20 | 24 | 20 | 28 | 31 | 23 | 29 | 31 | 26 | 31 | 36 |
| 156 | ED | na | Community | Effluent spillage | non | 22 | 24 | 21 | 28 | 28 | 25 | 26 | 24 | 18 | 23 | 32 |
| 157 | EE | na | Community | Effluent spillage | non | 22 | 22 | 22 | 26 | 26 | 23 | 25 | 22 | 18 | 23 | 33 |
| 158 | EE | na | Community | Landfill Groundwater | non | 19 | 21 | 21 | 28 | 27 | 24 | 23 | 22 | 16 | 22 | 33 |
| 160 | EF | na | Community | Stream | non | 21 | 21 | 19 | 25 | 23 | 23 | 24 | 19 | 16 | 21 | 32 |
| U1a | CH | 26 | Out-patient | CF Respiratory | muc | 23 | 23 | 21 | 29 | 24 | 22 | 30 | 24 | 20 | 24 | 28 |
| U1b | CH | 26 | Out-patient | CF Respiratory | non | 24 | 16 | 30 | 29 | 34 | 17 | 17 | 28 | 36 | 36 | 37 |
| U3a | EG | 19 | Out-patient | CF Respiratory | muc | 26 | 25 | 24 | 25 | 21 | 26 | 30 | 15 | 13 | 19 | 30 |
| U3b | EG | 19 | Out-patient | CF Respiratory | non | 34 | 34 | 30 | 30 | 28 | 30 | 30 | 20 | 17 | 23 | 34 |
| U5a | EH | 33 | Out-patient | CF Respiratory | muc | 25 | 27 | 25 | 32 | 28 | 28 | 30 | 18 | 20 | 25 | 30 |
| U5b | EH | 33 | Out-patient | CF Respiratory | non | 26 | 25 | 25 | 28 | 32 | 30 | 30 | 20 | 20 | 23 | 27 |
| R14a | EI | 26 | Out-patient | CF Respiratory | muc | 30 | 30 | 32 | 26 | 25 | 30 | 28 | 22 | 17 | 26 | 32 |
| R14b | EI | 26 | Out-patient | CF Respiratory | non | 17 | 21 | 19 | 25 | 19 | 28 | 27 | 16 | 14 | 21 | 23 |
| U16a | EJ | 23 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 15 | 16 | 0 | 0 | 18 | 0 | 0 | 18 |
| U16b | EJ | 23 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 16 | 0 | 0 | 15 | 14 | 0 | 13 |
| U17a | EK | 22 | Out-patient | CF Respiratory | muc | 24 | 25 | 25 | 30 | 30 | 26 | 27 | 23 | 20 | 25 | 12 |
| U17b | EK | 22 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 8 | 0 | 10 | 13 | 13 | 20 | 19 |
| U19a | EL | 19 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 24 | 9 | 32 | 0 | 12 | 0 | 18 | 18 |
| U19b | EL | 19 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 6 | 13 | 19 |
| U20 | EM | 49 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 9 | 9 | 14 | 8 | 12 | 15 | 20 | 16 |
| U21 | EN | 45 | Out-patient | CF Respiratory | muc | 25 | 28 | 26 | 30 | 30 | 28 | 25 | 28 | 21 | 26 | 32 |
| U26 | EO | 27 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 8 | 0 | 10 | 15 | 12 | 20 | 20 |
| R22b | EK | 22 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 11 | 8 | 20 | 15 |
| U32a | AJ | 26 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 15 | 9 | 20 | 0 | 9 | 0 | 19 | 20 |
| U32b | AJ | 26 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 24 | 22 |
| U33a | AS | 24 | Out-patient | CF Respiratory | muc | 20 | 22 | 24 | 25 | 26 | 28 | 13 | 24 | 20 | 25 | 35 |
| U33b | AS | 24 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| R43 | EP | 41 | Out-patient | CF Respiratory | muc | 26 | 26 | 30 | 30 | 22 | 30 | 35 | 17 | 13 | 22 | 28 |
| R44a | EQ | 22 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 13 | 0 | 9 | 11 | 9 | 17 | 17 |
| R44b | EQ | 22 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |
| U47a | ER | 22 | Out-patient | CF Respiratory | muc | 25 | 25 | 20 | 26 | 20 | 27 | 30 | 21 | 19 | 21 | 24 |
| U47b | ER | 22 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 11 | 19 | 18 | 18 |
| U50 | ES | 33 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 12 | 12 | 20 | 20 |
| U51 | ET | 34 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 9 | 0 | 11 | 14 | 14 | 21 | 20 |
| R59 | ER | 22 | Out-patient | CF Respiratory | muc | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 10 | 19 | 15 |
| U81a | EU | 16 | Out-patient | CF Respiratory | muc | 20 | 22 | 24 | 30 | 25 | 29 | 25 | 22 | 17 | 20 | 25 |
| U81b | EU | 16 | Out-patient | CF Respiratory | muc | 16 | 19 | 20 | 26 | 30 | 23 | 28 | 22 | 19 | 23 | 28 |
| U86a | EV | 22 | Out-patient | CF Respiratory | muc | 13 | 12 | 16 | 22 | 22 | 26 | 30 | 24 | 20 | 26 | 12 |
| U86b | EV | 22 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 10 | 16 | 0 | 0 | 21 | 19 | 25 | 14 |
| U73 | EW | 26 | Out-patient | CF Respiratory | muc | 30 | 33 | 21 | 31 | 24 | 25 | 30 | 23 | 21 | 24 | 5 |
| U80a | EX | 29 | Out-patient | CF Respiratory | muc | 19 | 20 | 22 | 28 | 19 | 23 | 30 | 21 | 18 | 23 | 26 |
| U80b | EX | 29 | Out-patient | CF Respiratory | muc | 22 | 26 | 27 | 29 | 21 | 28 | 28 | 20 | 16 | 22 | 25 |
| U81a | EY | 10 | Out-patient | CF Respiratory | muc | 44 | 35 | 41 | 34 | 26 | 41 | 33 | 18 | 17 | 21 | 30 |
| U81b | EY | 10 | Out-patient | CF Respiratory | non | 30 | 29 | 30 | 30 | 27 | 31 | 29 | 21 | 15 | 20 | 28 |
| R86a | EZ | 47 | Out-patient | CF Respiratory | muc | 34 | 36 | 30 | 28 | 26 | 28 | 29 | 23 | 21 | 27 | 11 |
| R86b | EZ | 47 | Out-patient | CF Respiratory | non | 30 | 34 | 30 | 25 | 23 | 28 | 23 | 20 | 18 | 23 | 8 |
| U108a | FA | 32 | Out-patient | CF Respiratory | non | 40 | 40 | 38 | 34 | 32 | 38 | 28 | 19 | 15 | 20 | 27 |
| U108b | FA | 32 | Out-patient | CF Respiratory | non | 10 | 16 | 16 | 28 | 34 | 18 | 30 | 25 | 23 | 25 | 30 |
| U108c | FA | 32 | Out-patient | CF Respiratory | muc | 41 | 41 | 42 | 38 | 26 | 30 | 26 | 15 | 13 | 26 | 25 |
| U110 | FB | 14 | Out-patient | CF Respiratory | non | 0 | 0 | 0 | 27 | 0 | 16 | 11 | 10 | 0 | 17 | 14 |
| U111a | AH | 17 | Out-patient | CF Respiratory | muc | 21 | 27 | 23 | 31 | 32 | 28 | 24 | 30 | 25 | 30 | 25 |
| U111b | AH | 17 | Out-patient | CF Respiratory | muc | 11 | 19 | 17 | 28 | 27 | 18 | 30 | 26 | 23 | 26 | 29 |
| R112 | CH | 22 | Out-patient | CF Respiratory | muc | 40 | 38 | 40 | 38 | 30 | 40 | 27 | 21 | 22 | 21 | 26 |
| R113a | AG | 23 | Out-patient | CF Respiratory | muc | 28 | 28 | 28 | 32 | 30 | 30 | 28 | 24 | 22 | 28 | 30 |
| R113b | AG | 23 | Out-patient | CF Respiratory | muc | 18 | 18 | 18 | 26 | 22 | 20 | 14 | 20 | 19 | 24 | 24 |
| U115 | FC | 25 | Out-patient | CF Respiratory | non | 0 | 0 | 12 | 25 | 12 | 30 | 11 | 15 | 12 | 19 | 16 |
| U126 | FD | 16 | Out-patient | CF Respiratory | muc | 16 | 0 | 0 | 16 | 17 | 24 | 20 | 11 | 0 | 13 | 27 |
| U156 | FE | 16 | Out-patient | CF Respiratory | muc | 44 | 37 | 44 | 37 | 26 | 44 | 34 | 18 | 19 | 22 | 32 |
| U162 | FF | 29 | Out-patient | CF Respiratory | non | 37 | 34 | 36 | 28 | 26 | 36 | 28 | 20 | 17 | 21 | 24 |
| U171 | FG | 27 | Out-patient | CF Respiratory | non | 35 | 37 | 38 | 30 | 38 | 35 | 37 | 31 | 31 | 31 | 40 |
| U175 | FH | 17 | Out-patient | CF Respiratory | muc | 30 | 28 | 34 | 26 | 22 | 26 | 32 | 16 | 14 | 20 | 27 |
| U184 | FI | 8 | Out-patient | CF Respiratory | non | 27 | 30 | 31 | 27 | 23 | 27 | 28 | 19 | 17 | 22 | 23 |
| U194a | FJ | 16 | Out-patient | CF Respiratory | muc | 40 | 40 | 5 | 36 | 38 | 40 | 47 | 0 | 0 | 0 | 19 |
| U194b | FJ | 16 | Out-patient | CF Respiratory | non | 48 | 36 | 46 | 44 | 32 | 40 | 44 | 0 | 0 | 10 | 18 |
| U195a | FK | 10 | Out-patient | CF Respiratory | muc | 36 | 13 | 19 | 22 | 15 | 28 | 36 | 13 | 34 | 18 | 30 |
| U195b | FK | 10 | Out-patient | CF Respiratory | non | 24 | 11 | 16 | 20 | 12 | 0 | 31 | 16 | 25 | 20 | 22 |
| U240 | FL | 17 | Out-patient | CF Respiratory | muc | 15 | 17 | 16 | 22 | 19 | 22 | 0 | 20 | 17 | 26 | 18 |
| RWE2 | FM | 37 | Out-patient | CF Respiratory | muc | 39 | 38 | 34 | 20 | 30 | 35 | 19 | 20 | 18 | 23 | 18 |
| AES1 | | | | | | 30 | 30 | 24 | 30 | 21 | 30 | 12 | 0 | 0 | 12 | 25 |
| AES2 | | | | | | 33 | 28 | 24 | 26 | 22 | 30 | 30 | 13 | 10 | 20 | 28 |
| P. aeruginosa PA01 | | | | | | 23 | 25 | 26 | 30 | 31 | 27 | 26 | 25 | | | |

| Type of Infection | Two Or More Antibiotics (%) | All Penicillins (Including timentin) (%) | Cephalosporins (One or More) (%) | Aztreonam (%) | Imipenem (%) | Aminoglycosides (Two or More) (%) | Ciprofloxacin (%) | Multi-Drug Resistant (%) | x ² test value | p value (CF excluded) |
|-------------------------|-----------------------------|--|----------------------------------|---------------|--------------|-----------------------------------|-------------------|--------------------------|---------------------------|-----------------------|
| Respiratory (n=24) | 29 | 13 | 4 | 17 | 13 | 0 | 13 | 0 | 0.186 | 0.686 |
| CF Respiratory (n=75) | 23 | 31 | 33 | 20 | 32 | 32 | 40 | 3 | <0.001 | nd |
| Skin/soft tissue (n=26) | 23 | 12 | 8 | 27 | 0 | 0 | 8 | 0 | 0.043 | 0.645 |
| Urine (n=19) | 37 | 16 | 16 | 42 | 11 | 0 | 16 | 0 | 0.458 | 0.804 |
| Ear (n=5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.376 | 0.498 |
| Hospital Env (n=25) | 48 | 20 | 20 | 60 | 16 | 16 | 12 | 0 | 0.027 | <0.001 |
| Community Env (n=10) | 0 | 0 | 0 | 10 | 10 | 0 | 0 | 0 | 0.102 | 0.228 |
| Total (n=184) | 27 | 20 | 20 | 27 | 18 | 15 | 22 | 1 | | |

Table 5.2: Percentage resistance by the CLSI method of *Pseudomonas aeruginosa* isolated from different types of infection or environmental sites. Nd = not done

| Place of Isolation | Two Or More Antibiotics (%) | All Penicillins (Including timentin) (%) | Cephalosporins (One or More) (%) | Aztreonam (%) | Imipenem (%) | Aminoglycosides (Two or More) (%) | Ciprofloxacin (%) | Multi-Drug Resistant (%) | x ² test value | p |
|----------------------|-----------------------------|--|----------------------------------|---------------|--------------|-----------------------------------|-------------------|--------------------------|---------------------------|---|
| ICU (n=12) | 5 | 4 | 2 | 7 | 4 | 1 | 0 | 0 | 0.292 | |
| NSU (n=9) | 3 | 1 | 2 | 4 | 1 | 2 | 3 | 0 | 0.964 | |
| Other Ward (n=65) | 9 | 9 | 8 | 17 | 5 | 3 | 9 | 0 | 0.002 | |
| Out-patient (n=80) | 41 | 23 | 24 | 28 | 23 | 22 | 29 | 2 | <0.001 | |
| Community (n=18) | 1 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 0.005 | |
| Total (n=184) | 32 | 20 | 20 | 33 | 18 | 15 | 22 | 1 | | |

Table 5.3: Percentage resistance by the CLSI method of *Pseudomonas aeruginosa* isolated from strains in this study from different places of isolation both within and outside of the hospital.

| Place of Isolation | Two Or More Antibiotics (%) | All Penicillins (Including timentin) (%) | Cephalosporins (One or More) (%) | Aztreonam (%) | Imipenem (%) | Aminoglycosides (Two or More) (%) | Ciprofloxacin (%) | Multi-Drug Resistant (%) | x ² test p value |
|----------------------|-----------------------------|--|----------------------------------|---------------|--------------|-----------------------------------|-------------------|--------------------------|-----------------------------|
| ICU (n=12) | 42 | 33 | 17 | 58 | 33 | 8 | 0 | 0 | 0.002 |
| NSU (n=9) | 33 | 11 | 22 | 44 | 11 | 22 | 33 | 0 | 0.008 |
| Other Ward (n=63) | 21 | 11 | 10 | 24 | 5 | 2 | 11 | 0 | 0.822 |
| Out-patient (n=13) | 15 | 15 | 8 | 38 | 8 | 0 | 8 | 0 | 0.980 |
| Community (n=18) | 6 | 0 | 0 | 22 | 6 | 0 | 0 | 0 | 0.180 |
| Total (n=115) | 21 | 12 | 10 | 30 | 9 | 3 | 10 | 0 | |

Table 5.4: Percentage resistance by the CLSI method of *Pseudomonas aeruginosa* isolated from strains in this study from different places of isolation both within and outside of the hospital (CF isolates excluded).

5.4 Discussion

The work presented in this chapter consists of a comprehensive survey of the antimicrobial resistance of *P. aeruginosa* isolates from clinical and environmental sources in Tasmania. Whilst the majority of strains tested were fully sensitive to all antibiotics tested, patterns of increased antimicrobial resistance were identified in isolates originating in the CF lung and the hospital environment.

5.4.1 Distribution of antimicrobial resistance

In the previous chapter, far greater percentage recovery of *P. aeruginosa* from environmental sites in the ICU and NSU was noted. These isolates were shown in this study to exhibit significantly greater patterns of antimicrobial resistance. ICU and NSU environmental sites are subjected to far greater disinfection practices than other wards of the hospital, and patients in these units are often on a stringent prophylactic antibiotic regimen. These regimes will positively select for resistant strains of *P. aeruginosa* (Sidhu, Heir, *et al.* 2002, Walsh, Toleman *et al.* 2005), explaining the increased rates of recovery and antimicrobial resistance of environmental isolates from these units. Of the non-CF isolates in this study, only intensive therapy ward environmental isolates showed resistance to two or more of the aminoglycosides tested. This specific pattern of resistance is often associated with *aac* class enzymes (Poole 2005), and it is possible that such an enzyme is present on transmissible genetic element (plasmid or transposon) in some strains within the hospital environment. Isolates of *P. aeruginosa* containing these enzymes have been reported as causing a nosocomial infection outbreak in a neurosurgical unit in Japan (Sekiguchi, Asagi, *et al.* 2005). That such isolates may have colonized the environment of the NSU in this hospital is of concern.

True resistance or intermediate resistance to a large number of β -lactam agents was also observed in the hospital environment isolates. Two of these isolates (123 and 124) have been shown in unpublished data from a Belgian research group to possess the horizontally acquired SHV ESBL gene (Pimay 2008, pers. com.), and it is reasonable to assume that at least some of the increased β -lactam resistance in other hospital isolates is due to these mobile elements. Without comprehensive DNA analysis and typing of antimicrobial resistance genes, however, inferences regarding the genetic basis of resistance in these isolates can only be speculative.

The markedly increased propensity towards a resistant phenotype observed in CF isolates may be explained by the increased long term exposure and selective pressure of antimicrobial agents upon these bacteria. Unlike other patients, who are acutely infected with *P. aeruginosa*, chronically infected CF patients are routinely prescribed prophylactic antibiotic regimens, augmented when they are treated for exacerbations of disease.

5.5 Conclusion

Nosocomial and CF isolates of *P. aeruginosa* have been shown to have greater resistance to antimicrobials than those isolated in community settings (Kiska and Gilligan 2003). Outbreaks of multi-drug resistant *P. aeruginosa* infections have previously been reported in ICUs (Deplano, Denis *et al.* 2005) and CF clonal complexes of *P. aeruginosa* have shown a propensity towards expression of a resistant phenotype (Pederson, Koch *et al.* 1986; Cheng, Smyth *et al.* 1996; Jones, Govan *et al.* 2001). Therefore, the increased resistance profiles of CF and intensive therapy unit isolates observed in this study is of concern. However, antibiogram alone is insufficient to identify the presence of a resistant clonal complex of *P. aeruginosa* in a population (Williams 1997). Epidemiological analysis of these

isolates using molecular techniques is warranted to monitor strains within the Tasmanian CF populations, and to be vigilant against the possibility of a single antimicrobial resistant strain of *P. aeruginosa* genotype colonising the RHH intensive therapy units.

CHAPTER SIX

Molecular Epidemiology of *Pseudomonas aeruginosa*

Infections from Cystic Fibrosis Patients:

A Whole Population Study

6.1 Introduction

There are increasing reports of CF clonal complexes (often referred to as “clonal” or “epidemic”) strains of *Pseudomonas aeruginosa* amongst cystic fibrosis (CF) patient populations attending large CF centres (Anthony, Rose *et al.* 2002; Scott and Pitt 2004). Whether cross-infection because of increased transmissibility is the explanation for their widespread distribution or whether these isolates are more virulent remains unclear (Smith, Gumery *et al.* 1993; Al-Aloul, Crawley *et al.* 2004; Salunkhe, Smart *et al.* 2005). Two such genotypically identical strains, referred to as Australian Epidemic Strain I (AES1) and AES2, have been found in three large CF centres located in major cities on the eastern seaboard of Australia (O'Carroll, Syrmis *et al.* 2004; Syrmis, O'Carroll *et al.* 2004). It has been suggested that infection with AES1 results in poorer clinical outcome, but this remains to be confirmed (Armstrong, Nixon *et al.* 2002).

The institution of cohort segregation at the Melbourne CF clinics resulted in significantly less acquisition of AES1 in uninfected patients than was the case prior to the introduction of these measures, suggesting that transmission of AES1 must occur at least in some cases during attendance to the CF clinics (Griffiths, Jansen *et al.* 2005). In most parts of Australia, CF patients receive treatment at dedicated and centralized clinics. Globally, this model of care has resulted in improved outcomes in CF, but there are concerns that attendance of large numbers of patients at CF centres may increase the risk of cross-infection. Tasmania is a large island state situated south of mainland Australia, with a population of approximately 480,000 people. The island may be divided into three distinct geographic regions (South, North and North West) with historically little or no movement of the resident populations between regions.

The Tasmanian population is more dispersed geographically than the rest of Australia, where most people live in highly urbanized cities. Tasmanians are mainly of Caucasian and especially Anglo-Celtic descent, and there is a particularly high birth incidence of CF (1 in 1,600 births *versus* 1 in 2,500 on mainland Australia). Few Tasmanian CF patients travel to other states on the mainland to receive treatment and most choose to receive treatment in their immediate locale.

At the time that isolates for this study were collected (2003), Tasmania had no dedicated centralised CF clinic. Patients attended one of three regional hospitals in their local area, where they were managed either by general paediatricians or adult respiratory physicians. An out-reach team from the paediatric CF service at the Royal Children's Hospital in Melbourne visited the northwest and north of the state every six months. There were 92 individuals with CF living in Tasmania in 2003, and 50 of these were aged 15 years and above. The relative isolation of adults with CF in Tasmania and the absence of centralized care provided us with a unique opportunity to study the epidemiology of *P. aeruginosa* infection in this disease in the absence of centralized care facilities, which may act as sources of CF clonal complexes.

6.2 Methods

6.2.1 Isolate source

Isolates of *P. aeruginosa* from the CF lung used in this thesis were obtained from the University of Tasmania CF isolate library (prefix "U") and the Royal Hobart Hospital (prefix "R"). Collection of clinical samples and all clinical data referred to in this study was performed by Dr. David Reid of the Cystic Fibrosis Research Group, Menzies Research Institute, University of Tasmania. Sputum samples were collected from patients in all regions of the state during 2003 at monthly outreach clinics for CF

adults instituted by an adult respiratory physician from the South of Tasmania. Details of age, gender, place of residence, clinical history and FEV₁ were recorded. The Statewide Ethics Committee approved the study and all patients gave written informed consent prior to providing a sputum sample. Of the 50 adult (≥ 15 years) cystic fibrosis patients residing in Tasmania at the time of analysis, 41 (82%) were involved in this study. Four of these patients had previously received lung transplants. Multiple samples were collected from each participant over the two years following recruitment. Details on hospital admissions and length of stay were obtained for all CF patients living in the state for the years 2001-2003. In addition, hospital admission dates were reviewed to determine whether there was any association between CF patients being in-patients concurrently and the presence of clonal complex strains.

Isolates were recovered from MMM by culture on MacConkey agar at 37°C in aerobic conditions for 48 hours. *P. aeruginosa* isolate DNA extracts were subjected to RAPD PCR with primer 272 (method 3.18). Isolates showing greater than 90% similarity by RAPD PCR were then subjected to *Spe*I PFGE (method 3.19) to confirm genetic relatedness. CLSI antibiograms of all isolates were examined to determine any relationship between the wide array of antimicrobial susceptibility patterns and genotype. Multi-drug resistance was defined using criteria of absolute resistance to all agents tested belonging to two or more of the following classes: β -lactams, aminoglycosides and fluoroquinolones, as defined in the US CF Foundation Consensus Guidelines (Armstrong, Nixon *et al.* 2002).

6.2.2 Statistical analysis

Statistical analysis of clinical data was carried out with the assistance of Assoc. Prof. Leigh Blizzard of the Menzies Research Institute, University of Tasmania. Age, FEV₁ and the treatment requirements of

patients harbouring different bacterial strains (see below) were compared using the Kruskal-Wallis test for non-parametric data. An extension of Fisher's exact test (Freeman-Halton test) was used to determine the statistical significance of gender, region of residence (North, South or North West) and number of differing *P. aeruginosa* genotypes recovered from patients harbouring a clonal strain, as well as to examine differences between strains in terms of mucoidy and antimicrobial resistance profiles. Intermediate CLSI results were included in the resistant category for the purposes of statistical analysis, except for the definition of multi-resistance, in which only absolute resistance was considered in order to conform to the CF Foundation Consensus Guidelines. $P \leq 0.05$ was considered significant. The SPSS (SPSS 12.0.1 for Windows, 2003) statistical package was used for all analyses, except for χ^2 analysis of antimicrobial resistance data, which was carried out by the author, using in Microsoft Excel 2003 (11.8220.8202) SP3 for Windows.

6.3 Results

6.3.1 Sample collection

Sputum samples were collected from 40 patients around the state, including from two individuals who had previously received lung transplants. A nasopharyngeal swab was collected from one further post-transplant patient. Sputum samples could not be obtained in nine patients, including one of the four transplant recipients; seven of these patients were difficult to access and two patients, who were unable to spontaneously expectorate sputum, refused to undergo sputum induction. *P. aeruginosa* was recovered from 34 of the 41 patients (83%) who provided a respiratory sample and was not cultured in repeated samples from the remaining seven patients. Seventeen of the patients colonised with *P. aeruginosa* resided in the southern geographic region (population ~238, 000), 10 were from the north (population ~136, 000) and seven were from the northwest (population ~108, 000) (table 6.1, figure

6.1). Four patients who had provided sputum samples were subsequently excluded from aspects of the analysis; one patient (unique strain infected) developed severe lung function impairment following a non-pulmonary complication that required major abdominal surgery and was awaiting lung transplantation at the time that the study was undertaken. Three other patients were excluded from the longitudinal analysis because they had received lung transplants prior to 2001, well before this study was conducted.

6.3.2 RAPD PCR patterns

Of the 57 isolates collected, 33 distinct *P. aeruginosa* RAPD types were identified. The resultant dendrogram (figure 6.4) showed *P. aeruginosa* isolate similarities of between 25 and 100%. Isolates found to be identical by RAPD and PFGE displayed marked phenotypic differences from each other in terms of mucoidy and antimicrobial susceptibility.

A genotypically identical strain (named Australian Epidemic Strain 3) was found in 11 isolates from nine individual patients (26% of those typed) who lived in different regions of the state (table 6.1 & figure 6.4). Two of these patients were siblings. A further small cluster of identical strains was found in four patients (11%), this was referred to as the “Tasmanian CF cluster”. One patient was infected with three different strains including the mainland AES1, AES3 and one of the small cluster strains.

The presence of mucoidy on first isolation was demonstrated by 30 (73%) of unique isolates, seven (64%) of AES3 isolates and three (60%) of the Tasmanian CF cluster isolates. Mucoid isolates were not found to show increased antibiotic resistance when compared to non-mucoid isolates (table 6.4).

6.3.3 Epidemiological links between AES3 patients

Two potential links between eight of the nine patients infected with AES3 were identified. The first was attendance at CF family camps as either children or young teenagers in the early 1990's. Review of camp attendance lists and group photographs confirmed that five of the nine patients with AES3 had attended the camps at the same time (three from the southern region and two from the northwest). At the camps, children slept in dormitories, often in the same bed, shared washing and toileting facilities and ate meals together. Tasmanian patients did not generally travel to attend CF camps on mainland Australia. The second potential link related to hospital admissions. A review of hospital admission records found that between 1991 and 1994, the three individuals with AES3 from the southern region who had attended the summer camps were also sometimes in-patients on the paediatric ward at the same time as the other three patients from the southern region now identified as being infected with AES3. The two patients from the northwest with AES3 have had no identifiable contact since the summer camps ceased, either as in-patients or out-patients. The solitary subject from the north of the state infected with AES3 had not attended summer camps and has no identifiable contact with any of the other patients and at present there is no explanation as to how this individual acquired AES3. It remains possible that AES3 represents a clonal complex found at low levels in the Tasmanian environment, analogous to clone C in Europe. There was no obvious identifiable link between any of the patients infected with the Tasmanian CF cluster isolates.

Only one Tasmanian patient possessed a mainland epidemic strain (AES1), but this subject had no history of visits to Melbourne where this strain predominates in the paediatric population. However, this individual had previously attended hospitals in Queensland and was also one of the very few Tasmanians to attend summer camps in Queensland. Although AES1 remains uncommon, it has still

been isolated from almost 10% of the Queensland CF population screened thus far (Syrmis, O'Carroll *et al.* 2004).

6.3.4 Clinical status of patients infected with AES3

There was no statistical difference in terms of gender, age or FEV₁ between patients infected with AES3, the Tasmanian CF cluster or unique strains (Table 6.2). During the two years prior to this study, those patients infected with AES3 (n=9) had experienced significantly greater treatment requirements than patients harbouring unique strains and two of these patients have subsequently gone on to receive lung transplants and one further patient has died from respiratory failure. By comparison, two of the 25 patients with unique strains have been subsequently transplanted.

Patients harbouring genotypically indistinguishable strains appeared more likely to be concurrently infected with other strains of *P. aeruginosa* than those infected with unique strains, although this observation did not reach statistical significance ($p = 0.18$). AES3 did not display a greater degree of mucoidy compared to other isolates, but it was significantly more likely to display antibiotic multi-resistance (Table 6.3). Two of the 57 (18%) clinical isolates tested were found to be multi-resistant and these were all AES3 strains. Despite their overall increased antimicrobial resistance, two of the AES3 strains remained fully sensitive to all of the antimicrobials tested.

| Region | *AES 3 (n=8) | Tasmanian CF Cluster (n=3) | Unique (n=22) | Total (n=33*) |
|--------------------|--------------|-------------------------------|---------------|------------------|
| South | 5 | 1 | 11 | 17 |
| North | 2 | 1 | 7 | 10 |
| North West | 1 | 1 | 4 | 7 |
| Median Age (years) | 24 | 26 | 27 | 26 |

Table 6.1: RAPD PCR genotypes of CF *Pseudomonas aeruginosa* isolates by the geographic area of residence and age of infected patients (n=33)

| Clinical and Demographic Characteristics | *AES 3 (n=8) | *Tasmanian CF Cluster (n=3) | Unique (n=22) | Kruskall-Wallis test p value |
|--|---------------------|-----------------------------|---------------------|------------------------------|
| Age (years) (range) | 24 (19-34) | 26 (16-49) | 26 (16-49) | ns |
| Gender | 5F/3M | 1F/2M | 9F/16M | ns |
| BMI (range) | 19.8 (17-22.2) | 20.7 (18.4-21) | 19.7 (16-23) | ns |
| **FEV ₁ , L (range) | 2.14 (0.95-3.20) | 2.9 (0.61-3.88) | 2.06 (0.61-3.88) | ns |
| FEV ₁ (% predicted) (range) | 59 (29-85) | 67 (21-99) | 56 (21-109) | ns |
| FVC, L (range) | 3.16 (1.44-4.43) | 3.36 (1.25-4.01) | 3.54 (1.25-5.20) | ns |
| FVC (% predicted) (range) | 69 (42-110) | 79 (39-95) | 79 (39-114) | ns |
| **Number of admissions (range) | 4 (2-7) | 3 (3-4) | 2 (0-6) | 0.009 |
| **Bed days, days (range) | 62 (10-124) | 35 (14-68) | 19 (0-79) | 0.01 |

Table 6.2: Comparison of the clinical and demographic characteristics of patients infected with Australian Epidemic Strain (AES3) (n=8) and those with other strains (n=28).

* One patient harboured both AESIII and a strain belonging to the Tasmanian CF cluster and has been excluded from the statistical analysis.

AESIII was compared to the Tasmanian CF cluster and unique strains using the Kruskal-Wallis test for non-parametric data

** Lung function and admission data from three patients who had already received lung transplants and from one patient (unique strain) with a non-CF complication impacting on clinical disease severity are not included in these cross-sectional analyses.

AESIII compared to non-clonal (Tasmanian CF cluster and unique strains considered separately).

BMI: Body mass index; Number of admissions and total bed days were for the two years 2001-2003

Data expressed as median and range

| Antibiotic | AES 3 (n=11)* | Clonal cluster (n=5)* | Unique (n=41)* | Total (n=57) | χ^2 test p value |
|---------------|------------------|--------------------------|-------------------|-----------------|--------------------------|
| Carbenicillin | 11 (100%) | 1 (20%) | 14 (34%) | 26 (46%) | 0.011 |
| Ticarcillin | 11 (100%) | 1 (20%) | 11 (27%) | 23 (40%) | 0.002 |
| Timentin | 11 (100%) | 1 (20%) | 8 (20%) | 20 (35%) | 0.000 |
| Ceftazidime | 9 (82%) | 1 (20%) | 8 (20%) | 18 (32%) | 0.004 |
| Cefepime | 11 (100%) | 1 (20%) | 8 (20%) | 20 (35%) | 0.000 |
| Aztreonam | 9 (82%) | 1 (20%) | 11 (27%) | 21 (37%) | 0.023 |
| Imipenem | 10 (91%) | 1 (20%) | 9 (22%) | 20 (35%) | 0.002 |
| Gentamicin | 9 (82%) | 2 (40%) | 11 (27%) | 22 (39%) | 0.033 |
| Tobramycin | 2 (18%) | 0 (0%) | 4 (10%) | 6 (11%) | 0.559 |
| Amikacin | 10 (91%) | 1 (20%) | 8 (20%) | 19 (33%) | 0.001 |
| Ciprofloxacin | 10 (91%) | 1 (20%) | 15 (37%) | 26 (46%) | 0.041 |
| MDR | 2 (18%) | 0 (0%) | 0 (0%) | 2 (4%) | 0.015 |

Table 6.3. Rates of resistance to specific antibiotics of CF *P. aeruginosa* isolates in this study by the CLSI method.

| Antibiotic | Mucoid (n=39) | Non-Mucoid (n=18) | Total (n=57) | χ^2 test p value |
|---------------|------------------|----------------------|-----------------|--------------------------|
| Carbenicillin | 17 (44%) | 9 (50%) | 26 (46%) | 0.558 |
| Ticarcillin | 15 (38%) | 8 (44%) | 23 (40%) | 0.731 |
| Timentin | 12 (31%) | 8 (44%) | 20 (35%) | 0.083 |
| Ceftazidime | 11 (28%) | 7 (39%) | 18 (32%) | 0.498 |
| Cefepime | 12 (31%) | 8 (44%) | 20 (35%) | 0.412 |
| Aztreonam | 13 (33%) | 8 (44%) | 21 (37%) | 0.514 |
| Imipenem | 12 (31%) | 8 (44%) | 20 (35%) | 0.412 |
| Gentamicin | 15 (38%) | 7 (39%) | 22 (39%) | 0.932 |
| Tobramycin | 4 (10%) | 2 (11%) | 6 (11%) | 0.309 |
| Amikacin | 12 (31%) | 7 (39%) | 19 (33%) | 0.273 |
| Ciprofloxacin | 16 (41%) | 10 (55%) | 26 (46%) | 0.443 |
| MDR | 2 (5%) | 0 (0%) | 2 (4%) | 0.332 |

Table 6.4. Rates of *P. aeruginosa* resistance to specific antimicrobials by the CLSI method compared to mucoidy on first isolation.



Figure 6.1: Map of Tasmania showing the three geographic regions defined in this study

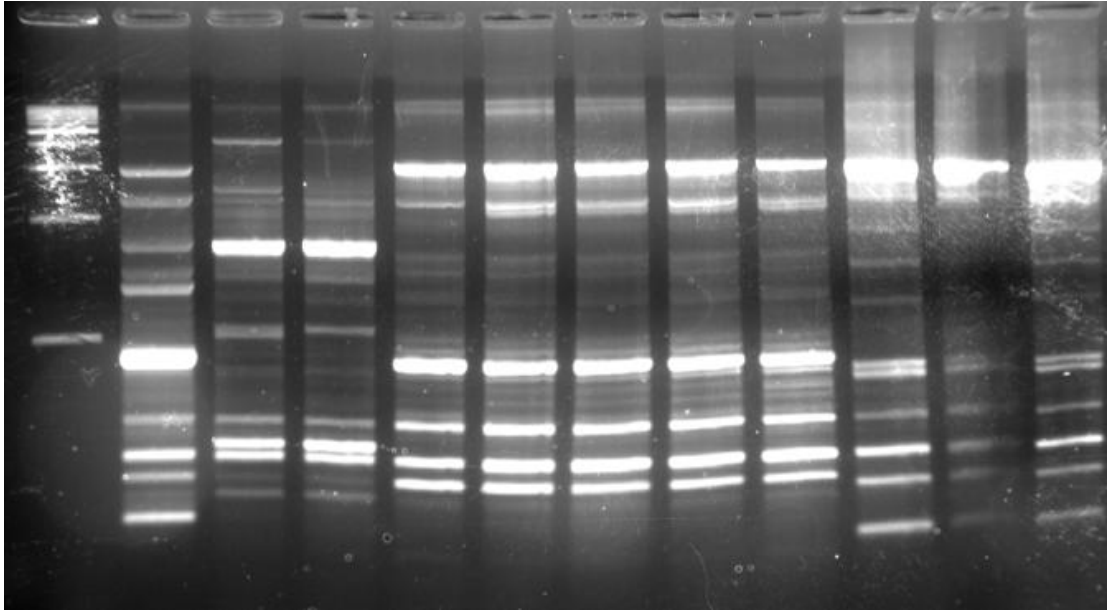


Figure 6.2: RAPD PCR Products using primer 272.

(L-R): 1kb MW Maker (Biorad), AES2, AES1, U17a, U3a, U3b, U43, U29b, U14a, U50, U44b, U44a.

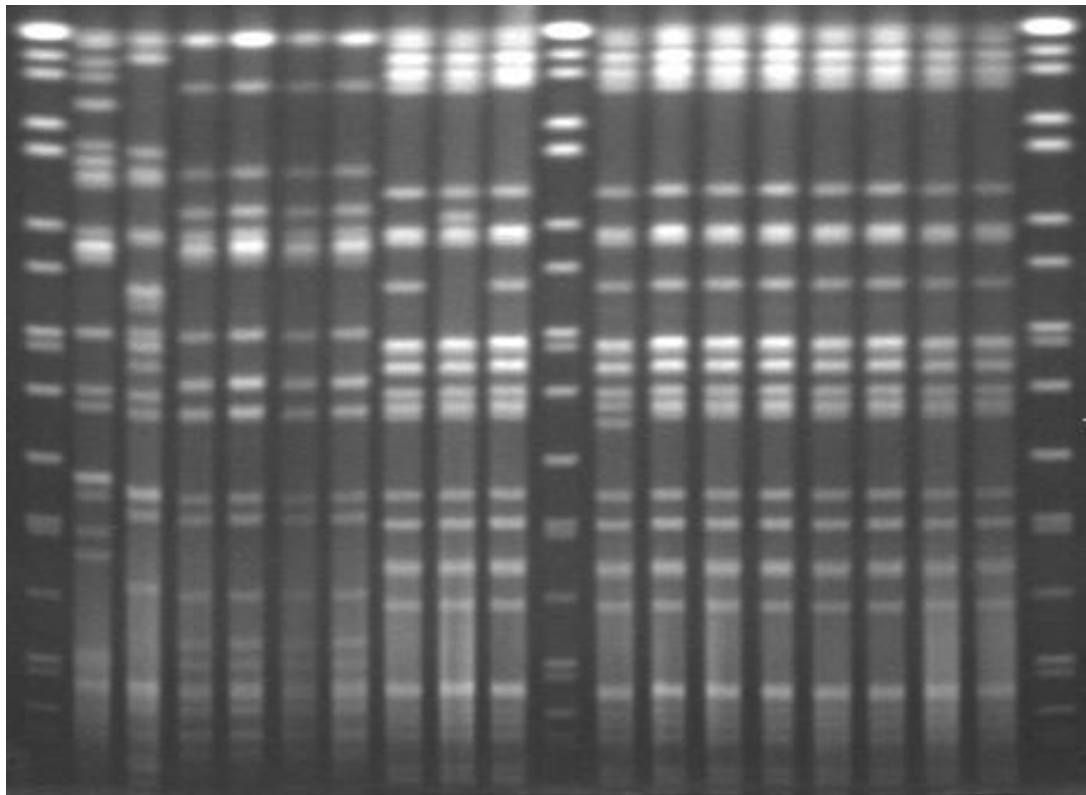


Figure 6.3: *Spel* PFGE Macrorestriction pattern of *P. aeruginosa* isolates.

(L-R): International standard ACTC BAA-664 (*S. enterica* ser Braenderup H9812), AES2, AES1, 3a, 3b, 43, 14a, U50, U44b, U44a, International standard ACTC BAA-664, 33b, U51, U115, U58, U32b, U19b, U19a, U17b, International standard ACTC BAA-664.

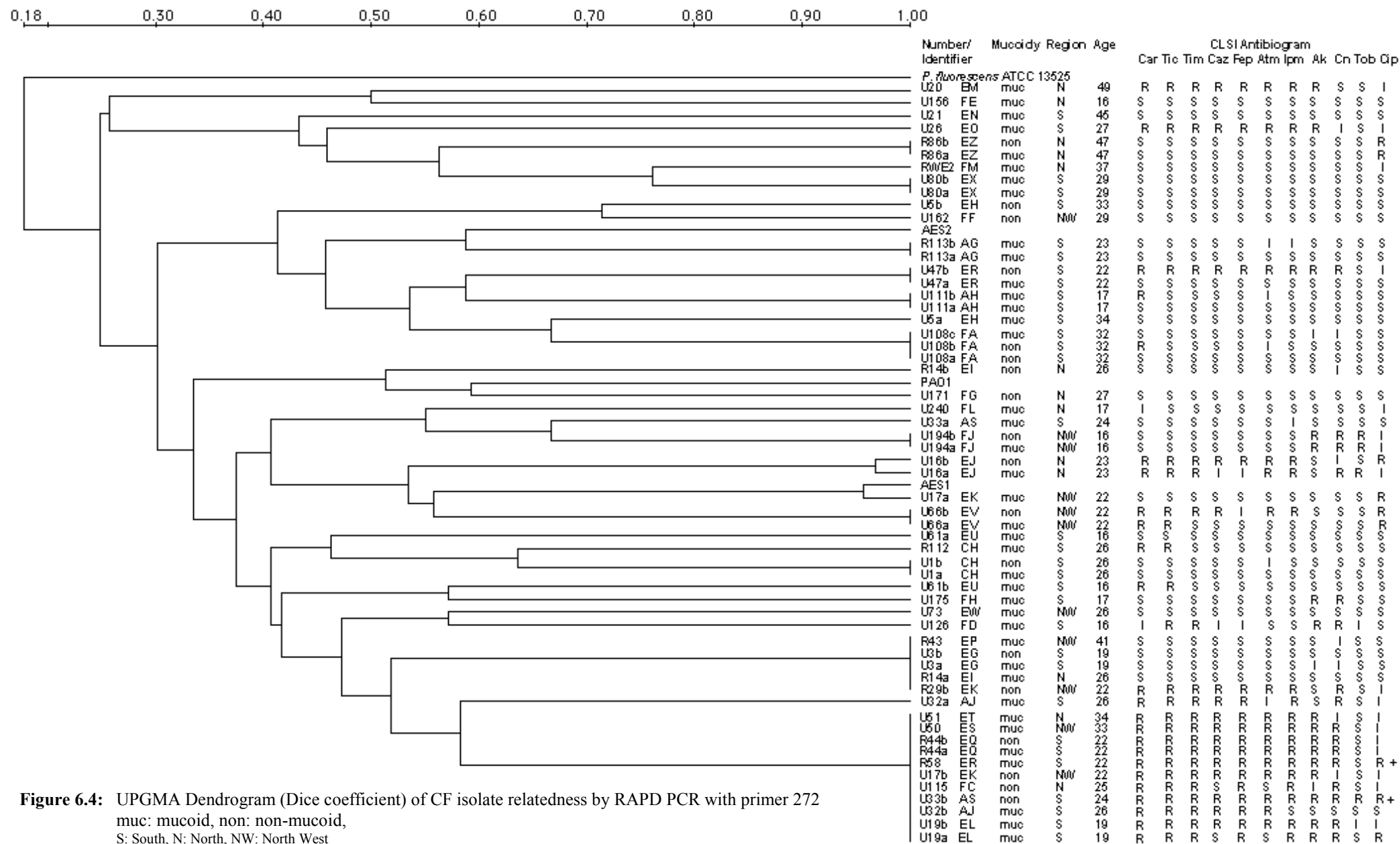


Figure 6.4: UPGMA Dendrogram (Dice coefficient) of CF isolate relatedness by RAPD PCR with primer 272

muc: mucoid, non: non-mucoid,

S: South, N: North, NW: North West

Age (years): 1: 16-19, 2: 20-24, 3: 25-30, 4: 31-34, 5: 35-39, 6: 40-44, 7: 45-49

+: Multi-drug resistant isolate

6.4 Discussion

The present study identified a genotypically identical strain of *P. aeruginosa* (AES3) present in just over a quarter of adults with CF in Tasmania and a further minor cluster of identical strains in four patients. Both Tasmanian clonal complexes were distinct from AES1 and AES2 prevalent on mainland Australia. AES3 exhibited significantly increased antibiotic resistance and patients infected with this strain had increased treatment requirements. AES3 was isolated from adult CF patients living in all regions of the state. Sampling of potential sources of infection in the State tertiary referral hospital, attended by most CF patients in the state, did not identify environmental contamination with any of the CF strains. Attendance at CF family camps in the early 1990's and in-patient stays on a paediatric ward at around the same time appeared to be the common historical link between most of the patients infected with AES3.

The identification of clonal complexes of *P. aeruginosa* in large CF centres around the world has raised concerns regarding cross-infection and increased virulence (Pedersen, Koch *et al.* 1986; Al-Aloul, Crawley *et al.* 2004; O'Carroll, Syrmis *et al.* 2004; Syrmis, O'Carroll *et al.* 2004). In some Australian centres, almost half of the paediatric and adult populations are infected with one of two dominant genotypically indistinguishable strains (AES1 or AES2) (Armstrong, Nixon *et al.* 2002; O'Carroll, Syrmis *et al.* 2004; Syrmis, O'Carroll *et al.* 2004). LES in the UK and both AES1 & AES2 in Australia, have been shown to exhibit phenotypic alterations when studied *ex vivo* that suggest increased virulence such as increased production of proteases, but whether these observations translate to clinical outcomes has not been established (Salunkhe, Smart *et al.* 2005; Smith, Rose *et al.* 2006; Tingpej, Smith *et al.* 2007). Previous studies to date have not demonstrated that CF clonal complexes are associated with enhanced airway or systemic inflammation in patients, although patients infected

with such strains do appear to have increased treatment requirements. It remains unclear whether this relates to bacterial virulence or whether patients with frequent admissions are simply more likely to be exposed to these organisms (Jones, Martin *et al.* 2003).

6.4.1 AES1 infection in a Tasmanian CF patient

This study identified only one individual infected with the mainland AES1. This patient had a history of attendance at a CF centre, as well as summer camps in Queensland and this is most probably where AES1 was acquired. Interestingly, this patient was also infected with both AES3 and a Tasmanian CF cluster strain, demonstrating that multiple CF clonal complex isolates can co-infect the same CF host. AES1 has not been identified in any of the other Tasmanian CF patients and in this respect Tasmania's isolation from mainland Australia may be advantageous. Similarly, the geographical dispersal of the CF population may explain the lower prevalence of CF clonal complex infection compared to mainland Australia.

These results suggest that AES3 may not have been acquired recently, as the most likely point of contact between most of the patients around the state was attendance at CF family camps many years previously. At around this time, patients with AES3 who had attended the summer camps were in-patients at the same time as the other patients in the Southern region now known to have AES3, raising the possibility that cross-infection may have occurred in hospital. Cross-infection at summer camps has been demonstrated in previous studies in Europe, but the relevance of this finding to the Australian experience has not been addressed (Fluge, Ojienyi *et al.* 2001). In Tasmania, CF families from all regions of the state attended these camps where there was very close and prolonged social contact across a spectrum of age groups. Patients in the North and North West infrequently attend clinic at the

same time and review of admission records did not identify times when these particular patients were admitted at the same time. In the south, it is possible that infection with AES3 occurred through an environmental source in the hospital setting, although we did not identify AES3 in hospital clinic areas or wards. There is also the possibility of an environmental source at the summer camps, but we have not explored this possibility by sampling the camp locations.

6.4.2 Poor clinical outcomes in AES3 infected patients

Although nutritional status and lung function at the time of the study were similar in patients infected with clonal complex and unique strains, individuals harbouring AES3 having approximately twice as many admissions with exacerbations and more than three times as many days in hospital over the preceding two years compared to patients not infected with this strain. Furthermore, of the nine patients infected with AES3, one has subsequently died of respiratory failure and two have received lung transplants (33%) compared to two transplants (9.1%) and no deaths in the patients infected with unique strains. Although the numbers are small, these findings suggest that infection with AES3 may be associated with a worse clinical outcome.

6.4.3 Increased antimicrobial resistance in AES3 isolates

AES3 was significantly more resistant to antibiotics than the other strains. This may have been influenced by the increased antibiotic use in these patients, although resistance to tobramycin was uncommon, despite its frequent use in the CF population. Increased antimicrobial resistance occurring in AES3 is consistent with similar findings in other CF clonal complexes (Scott and Pitt 2004; Jones, Govan *et al.* 2001; Armstrong, Nixon *et al.* 2002; O'Carroll, Syrmis *et al.* 2004). No differences were found in mucoidy on first isolation between clonal complex and unique strains, in contrast to the

increased mucoidy reported in AES1 (Armstrong, Nixon *et al.* 2002), but consistent with findings in AES2 (O'Carroll, Syrmis *et al.* 2004).

6.4.4 The Tasmanian CF Cluster Strain

Tasmanian CF cluster isolates were recovered from four patients. Two of these individuals lived in the North West, one in the North and one in the South of Tasmania. Three of these patients were between the ages of 20 and 25, whilst one was aged between 40 and 50 years. None of these patients had any history of significant contact, although the two patients from the northwest may on occasion have attended the same clinic on the same day, but not at the same time. These strains were not multi-resistant and the infected patients remained relatively well. The presence of this strain cannot be explained on the basis of cross-infection and there remains the possibility of an environmental source, but this will be difficult to establish. Due to the small number of patients infected by this strain, it has been referred to as cluster of isolates rather than a fourth Australian CF clonal complex. The possibility remains that this genotype does indeed represent another CF clonal complex of *P. aeruginosa* emerging in the Tasmanian CF population.

6.5 Conclusion

In summary, the present study identified a genotypically indistinguishable strain occurring in just over a quarter of *P. aeruginosa* infected CF adults in Tasmania, despite the geographic dispersal of the CF population. Historically, there is the suggestion that the original point of acquisition of AES3 may have been summer camps with subsequent spread to other individuals during hospital admissions, but this cannot be substantiated beyond doubt. AES3 isolates display a greater propensity for antimicrobial resistance and are associated with poorer clinical outcomes. Although the prevalence of CF clonal

complex strains in Tasmania was much lower than that reported on mainland Australia, the present study suggests that these organisms may arise even in small and isolated CF populations.

CHAPTER SEVEN

Molecular Epidemiology of *Pseudomonas aeruginosa* in the Hospitalised Population and the Wider Community

7.1 Introduction

In response to the finding of a significant number of Tasmanian CF patients infected with two separate genotypically indistinguishable strains of *P. aeruginosa*, further investigation into the source and distribution of these strains was carried out. Thus far, it has been shown that 26% of adult Tasmanian CF patients are colonised with AES3, whilst 11% carry the Tasmanian CF cluster strain. Two likely sources of AES3 were postulated in the previous chapter, these being a CF family camp in the 1990's; and the major tertiary referral hospital for the state of Tasmania, where many AES4 carriers have been in-patients, and where a CF clinic was established in 2003. However, until the distribution of these strains in the environment and non-CF infections is established, it remains possible that other foci of infection may be involved. It is possible that AES3 and the CF cluster strain are represented in high percentages in Tasmanian *P. aeruginosa* isolates both in human infection and the environment. Were this to be the case, the rates of CF infection would simply be representative of a higher rate of exposure to these strains, rather than any greater propensity of these strains to establish infection in the CF lung specifically. It also has not been determined if these strains may cause respiratory or other types of infection in patients without CF.

In most cases where CF clonal complexes of *P. aeruginosa* have been identified, extensive environmental sampling has occurred to define a source of infection. As discussed in the first chapter of this thesis, "clone" C has been recovered from numerous aqueous environmental sources throughout Europe, however, this strain is unusual in being so easily recovered from the environment, as the source of other clonal complex strains has proved elusive. LES has not been recovered from the hospital or community environment, and comprehensive air and equipment sampling found strains only in the air, on spirometry equipment and a chair in a room occupied by an infected patient (Panagea,

Winstanley *et al.* 2005). Sampling of the hospital and community environment in the area of Manchester first associated with MES found the strain to be present only in the air of a room occupied by an infected patient (notably it was not found on spirometry equipment or the hands of staff (Jones, Govan *et al.* 2003). While cross infection measures to reduce the possibility of cross infection with AES1 have been instituted in Melbourne (Griffiths, Armstrong *et al.* 2004), the strain was not found upon extensive sampling in the hospital environment (Armstrong, Nixon *et al.* 2002). Some researchers have suggested that the source of clonal complexes may not be hospital-based (Scott and Pitt 2004). The widespread presence of clone C throughout Europe, LES and midlands 1 in the UK and AES1 in Australia may represent patient to patient spread over time, but may also be due to the presence of these strains, which appear to have a greater capacity to establish infection in patients, at low levels in the environment.

Comparison of AES3 and the CF cluster strain with other CF clonal complexes of *P. aeruginosa* worldwide may provide some insight into the probability of these strains being acquired from the community or hospital environment, and their capacity to cause other forms of infection. One such strain, European “clone C” has been reported in 15 CF patients from 8 separate CF centres in the United Kingdom. European “clone C” has been shown to present over a wide geographic range and to be capable of infecting a variety of habitats other than the CF lung, including the human ear, as well as two strains with >75% similarity by PFGE infecting urine and peritoneal fluid (Dinesh, Grundmann *et al.* 2003). The Liverpool epidemic strain has also been shown to be capable of causing respiratory infection non-CF patients (McCallum, Gallagher *et al.* 2002) and has crossed species to cause respiratory infection in a domestic cat (Mohan, Fothergill *et al.* 2008). The carriage of genotypically indistinguishable strains in epidemiologically unrelated CF patients has been reported from Canada and

Denmark (Speert, Campbell *et al.* 2002; Jalsbak, Kroeg Johansen *et al.* 2007), and respiratory infection with such strains in non-CF patients has occurred (McCallum, Gallagher *et al.* 2002; Jalsbak, Kroeg Johansen *et al.* 2007). Very recently published work in the Netherlands examined a large number of CF and non-CF isolates from diverse infection types in paediatric patients, and did not find the presence of any CF clonal complex strains infecting non-CF patients. This work did however find that the CF chronic group was less genotypically diverse than isolates from other types of infections (Tramper-Stranders, van der Ent *et al.* 2008).

The two Australian multi-drug resistant genotypically indistinguishable strains described prior to this investigation (AES1 and AES 2) have been identified as infecting a significant number of CF patients on the Eastern seaboard of the country (Anthony, Rose *et al.* 2002; Armstrong, Nixon *et al.* 2002; O'Carroll, Syrmis *et al.* 2004). In many cases, patients colonised with these strains have exhibited poorer clinical outcomes than those infected with unique strains (Armstrong, Nixon *et al.* 2002; Al-Aloul, Crawley *et al.* 2004; O'Carroll, Syrmis *et al.* 2004). The emergence of CF clonal complexes has significant implications for infection control within the hospital and CF clinic setting. The primary purpose of this study was to determine if AES 3 or the Tasmanian CF cluster is present within the environment or patient infections at the major Tasmanian tertiary referral teaching hospital, or the community surrounding it. A secondary aim was to gain insight into the epidemiology of *P. aeruginosa* infections within that hospital and the community which it serves.

7.2 Methods

P. aeruginosa isolates collected in the work described in chapter 4 of this thesis were included in this study. In order to avoid error due to skewing of dendrogram results by the inclusion of multiple strains

of the same isolate, where multiples isolates were collected from the same site in the same patient or environmental location, and no variation in phenotypic properties were observed between these isolates, only one representative strain was included in the study. Results of genotyping of CF isolates studied in chapter 6 of this thesis were not included in this study. The CF isolates in chapter 6 were collected over a two year period from patients throughout the state. This study concerns a specific group of isolates chosen for their being collected from the major Tasmanian tertiary referral hospital, or the community directly surrounding it over a specific time period. Inclusion of CF isolates from the former study would be comparing two different bacterial populations, collected under different temporal and geographic constraints. Comparing two such populations of isolates could potentially skew the results of this study by distorting the relationships in the UPGMA dendrogram and decreasing the potential to identify significant relationships between the isolates studied herein, which were directly related to the epidemiology of *P. aeruginosa* infection in the hospital concerned, and its surrounding community.

P. aeruginosa DNA extracts were subjected to RAPD PCR using primer 272 (method 3.18). Any isolates showing >90% similarity by RAPD PCR had this genotypic similarity confirmed by PFGE with restriction enzyme *Spe*I (method 3.19). A computer generated dendrogram of isolate relatedness was produced from RAPD PCR results using unweighted pair group matched analysis with arithmetic averages and a dice coefficient using the Diversity Database program (Biorad).

Results of the CLSI method were chosen as this method allowed the determination of the most comprehensive antibiogram possible, allowing any relationship between a wide array of antimicrobial susceptibility patterns and genotype to be determined. Multi-drug resistance was defined using criteria

of absolute resistance to all agents tested belonging to two or more of the following classes: β -lactams, aminoglycosides and fluoroquinolones, as defined in the US CF Foundation Consensus Guidelines (Armstrong, Nixon *et al.* 2002). Results of patterns of antimicrobial resistance by site and place of isolation were compared statistically using the χ^2 test function of Microsoft® Office Excel. As an adjunct to this study, two alternative methods were compared with the CLSI results. This comparison is reported elsewhere in the thesis.

7.3 Results

RAPD analysis was carried out on a total of 120 clinical and environmental isolates. Sixty-one *P. aeruginosa* isolates from fifty-three different in-patients were included, as were thirty-five environmental isolates (table 5.1). CF AES1, AES2 and the Tasmanian CF cluster were not found in any of the clinical or environmental isolates tested. AES 3 was found in two (33%) of 6 CF patients (figure 7.3), all of whom were known to be infected with this strain.

7.3.1 Genotypic analysis

A number of Hospital environment isolates from the same ward yielded RAPD PCR patterns of $\geq 85\%$ similarity. When tested by *SpeI* PFGE, these isolates showed no more than two bands difference in macro-restriction pattern. Similarly, a number of isolates showing $\geq 85\%$ similarity by RAPD PCR were recovered from one patient, sometimes months apart and from different sites on the body. These isolates often displayed differing antibiograms or differed in phenotypic appearance (figure 7.3). Isolate 1 was isolated from a patient AA whilst admitted to the hospital, seventeen days later isolate 17 was isolated from the same patient following their discharge. Isolate 1 showed resistance to aztreonam, whilst isolate 17 was fully sensitive to all antibiotics tested. Isolates 20 and 24 were recovered from

the urine of the same patient only two days apart. Whilst isolate 24 was fully sensitive, isolate 20 was resistant to a number of β -lactam antibiotics. Four separate *P. aeruginosa* isolates were recovered from one individual (isolates 63, 64, 66 and 96). Two of these (66 and 96) were urine isolates collected two months apart. Isolates 63 and 64 were soft tissue isolates from separate sites on the body. The patient was an out-patient at the time that all of these specimens were collected. Isolates 63, 64 and 66 (collected at the same time) were identical by RAPD PCR and *Spe*1 PFGE, whilst isolate 96 showed 91% similarity to these strains by RAPD PCR (not tested by PFGE). Three of the isolates in question were fully sensitive, whilst two showed varying degrees of resistance to different classes of antibiotics (figure 7.3). Isolates 95 and 76 showed $\geq 95\%$ similarity by RAPD PCR and were identical by PFGE, despite their being recovered from different out-patients six months apart. These two patients resided in country towns more than 50 km apart.

7.3.2 Phenotypic properties

Mucoidy on primary isolation was noted in eleven isolates (9%), many having been isolated from CF (n=5; 45%) or COPD sputum (n=2; 18%). A urine isolate (isolate 61) an NSU sputum trap isolate (isolate 49), and an isolate cultured from a sink in the ICU. No significant RAPD pattern clustering of mucoid isolates was noted.

| Infection type | Site of Isolation | | | | |
|-------------------------|-------------------|----------|------------|-------------|-----------|
| | ICU | NSU | Other Ward | Out-patient | Community |
| Respiratory (n=23) | 2 | 3 | 15 | 2 | 0 |
| CF Respiratory (n=6) | 0 | 0 | 2 | 4 | 0 |
| COPD Respiratory (n=2) | 0 | 0 | 1 | 1 | 0 |
| Skin/soft tissue (n=26) | 1 | 1 | 19 | 5 | 0 |
| Urine (n=19) | 0 | 0 | 11 | 3 | 5 |
| Catheter Urine (n=5) | 0 | 1 | 3 | 1 | 0 |
| Ear (n=5) | 0 | 0 | 1 | 1 | 3 |
| Environment (n=35) | 8 | 4 | 13 | 0 | 10 |
| Total (n=120) | 11 | 9 | 65 | 17 | 18 |

Table 7.1: Isolation site and infection type of *Pseudomonas aeruginosa* strains used in this study.

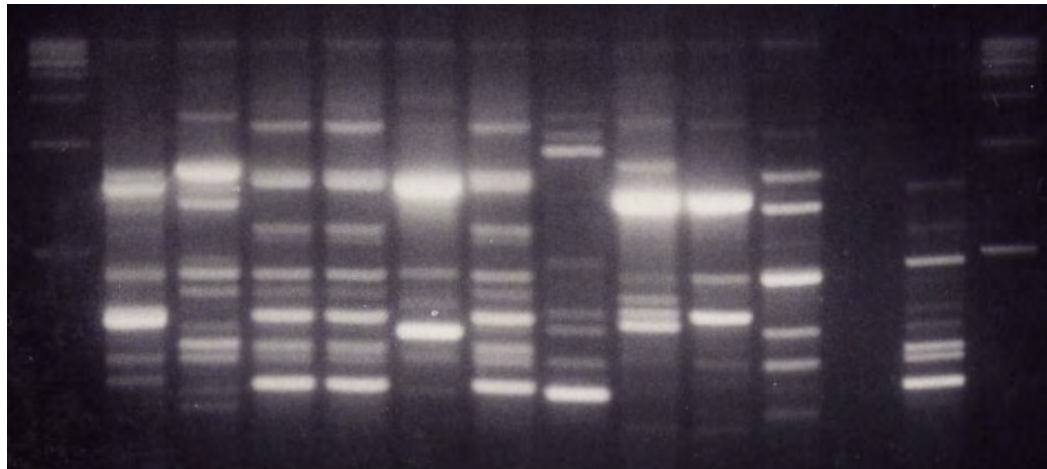


Figure 7.1: RAPD PCR Products using primer 272.
(L-R): 1kb MW Maker (Biorad), Isolates 61-70, ddH₂O negative control, PAO1, 1kb MW Maker.

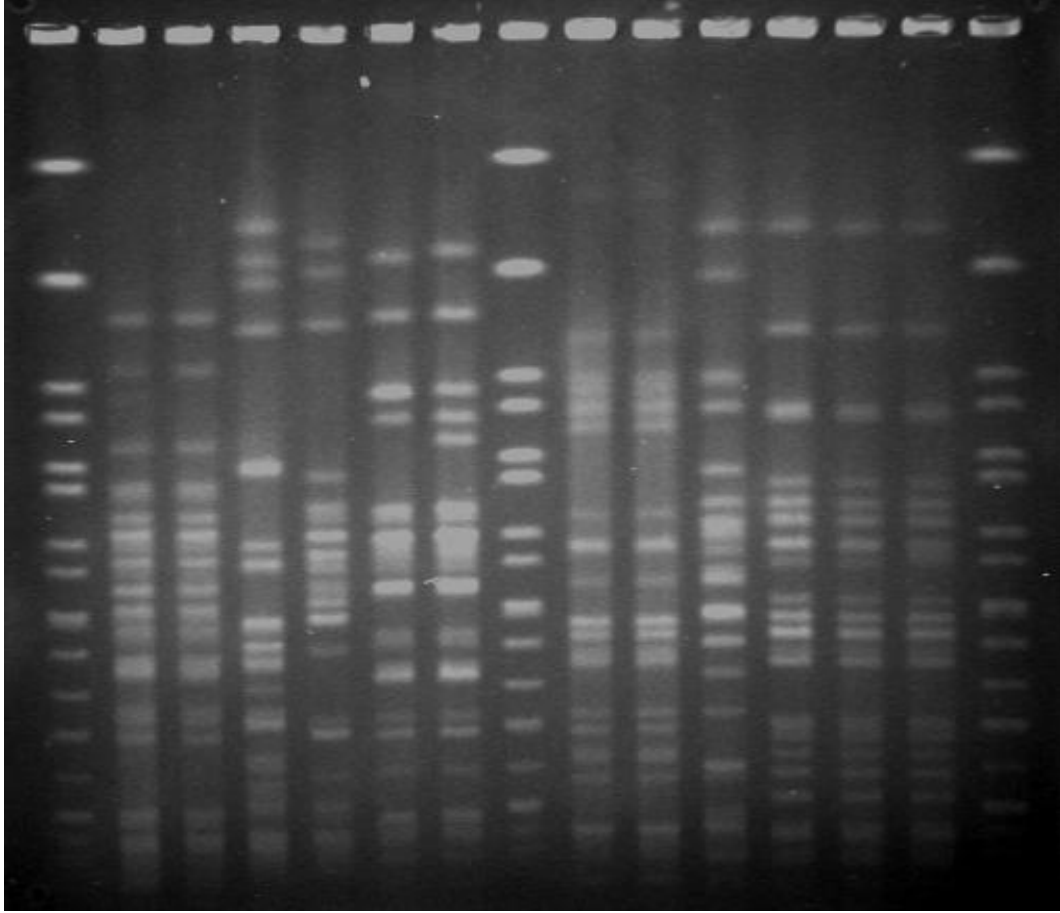
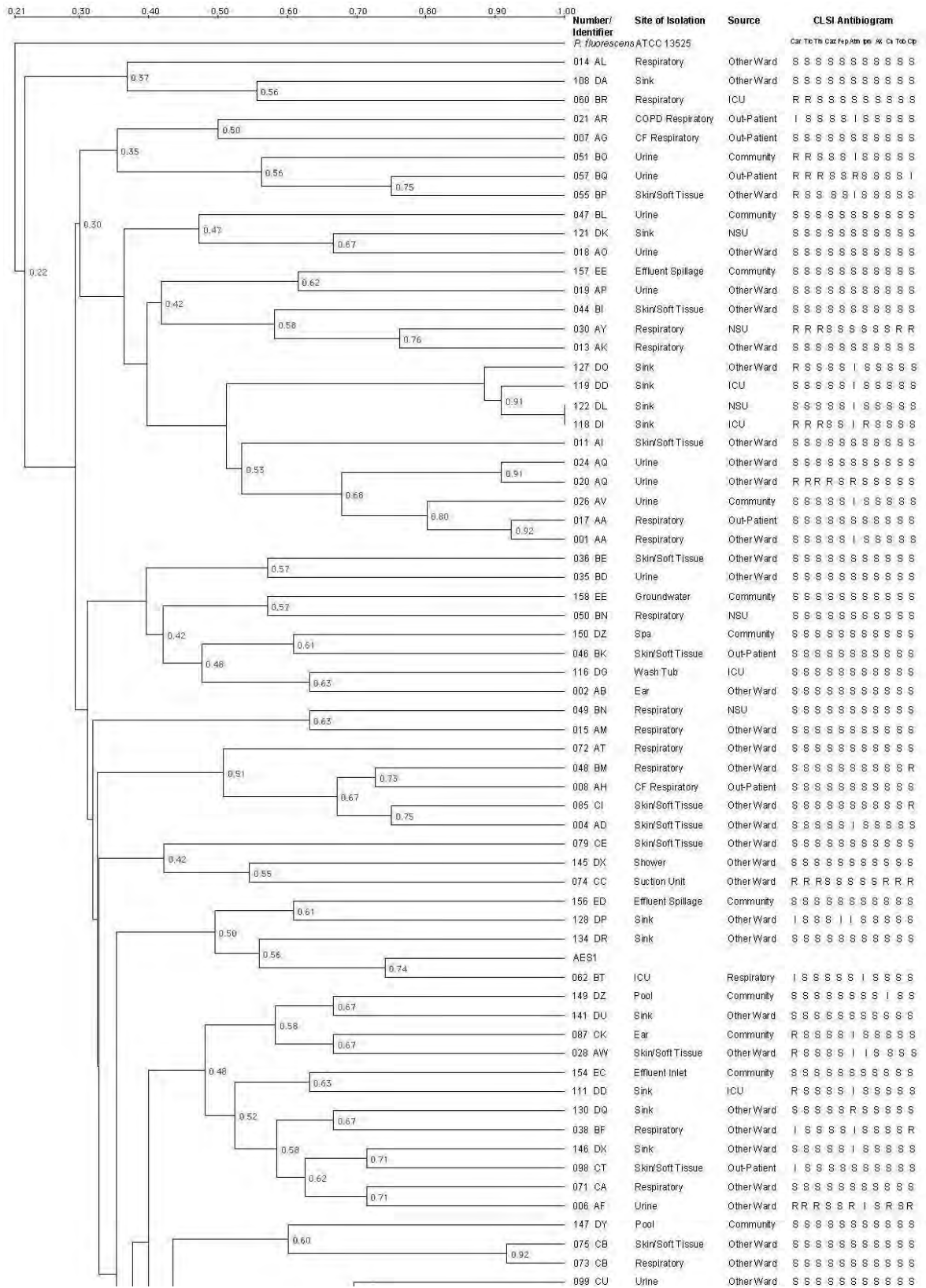
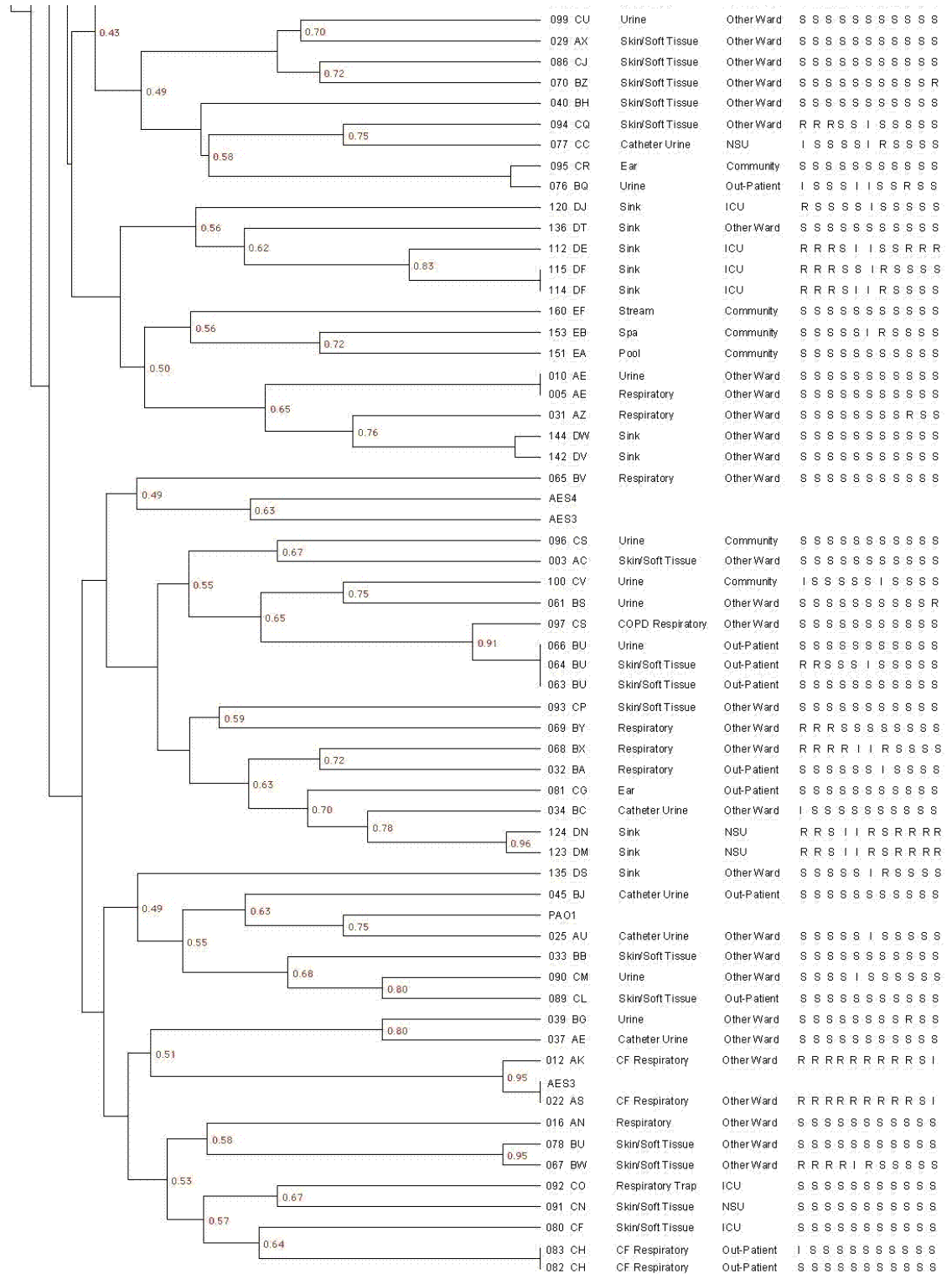


Figure 7.2: *SpeI* PFGE Macrorestriction pattern of *P. aeruginosa* isolates.
(L-R): International standard ACTC BAA-664 (*S. enterica* ser Braenderup H9812), AES123, 124, 149, 55, 95, 76, International standard ACTC BAA-664, 22, AES3, 82, 63, 64, 66, International standard ACTC BAA-664.



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**Figure 7.3** UPGMA Dendrogram (Dice coefficient) of isolate relatedness by RAPD PCR with primer 272.

(Tic: Ticarcillin, Tim: Timentin, Car: Carbenicillin, Fep: Cefepime, Caz: Ceftazidime, Atm: Aztreonam, Ipm: Imipenem, Ak: Amikacin, Cn: Gentamicin, Tob: Tobramycin, Cip: Ciprofloxacin. S=Sensitive, I= Intermediate, R= Resistant)

7.4 Discussion

In this study, the RAPD PCR pattern and CLSI antibiograms of 120 *P. aeruginosa* isolates from in-patients, out-patients and the environment of both the tertiary care facility in Hobart, Tasmania and the surrounding community were tested. No isolates of any CF *P. aeruginosa* clonal complexes were recovered from sources or sites other than the sputum of CF patients in the course of this study. This suggests that AES3 and the Tasmanian CF cluster strain do not have a reservoir in the environment of the major tertiary referral hospital in Tasmania. The lack of these strains in non-CF infections and the wider community environment strongly indicates that such strains are not common in the environment, and may be specific to CF respiratory infection.

7.4.1 Cystic fibrosis isolates

CF isolates recovered from patients attending the hospital during the period of this study were distributed throughout the dendrogram. No less diversity in the CF isolates compared to other clinical isolates was observed when the genotypically indistinguishable AES3 isolates were excluded from analysis. It was also noted that the isolates from non-CF infections and environmental samples were all genotypically distinct from the CF isolates included in this study.

7.4.2 Absence of cross-infection

No cross-infection was observed amongst in-patient isolates from the Royal Hobart Hospital and there were no significant similarities between the RAPD PCR patterns of isolates recovered from the hospital environment and those found in any patients involved in this study. These findings are in concordance with a previous study which suggested that patients become intestinally colonised with *P. aeruginosa*

outside of hospital, and that infection whilst hospitalized is by autogenous isolates (Speijer, Savelkoul *et al.* 1999).

The similarity by RAPD PCR of isolates 95 and 76 requires further discussion. These isolates were tested further by *Spe1* PFGE and found to have only one band difference in macrorestriction profile. The recovery of such similar *P. aeruginosa* isolates in two different out-patients, residing in different geographical locales far apart from each other is difficult to explain. Insufficient clinical data was available to exclude cross-infection, social contact between the two patients or a common reservoir of infection. It may be that these two isolates are representative of a strain that is present at low levels in the background of the Southern Tasmanian environment (analogous to “clone C” in Europe). However, it is most likely that these patients have contracted this strain through exposure to the same environmental source in a shared community facility such as a spa or swimming pool.

A number of patients were found to harbour genotypically similar or indistinguishable isolates in different sites of infection, or at differing times. These results were not considered unusual, as colonisation of one site of infection could easily lead to colonisation of other sites on the body, particularly under the selective pressure of antibiotic treatment. The differences in antibiogram associated with such isolates may be explained by the acquisition of antimicrobial resistance genes or chromosomal mutation. Phenotypic differences, such as those observed in the mucoidy of isolates 82 and 83 may also be explained in this way. Some patients were infected with different genotypes of *P. aeruginosa* at different times or different sites during their hospital treatment. In one case (isolates 49 and 50), differing genotypes were recovered from the same respiratory sample. These results prove that separate genotypes of *P. aeruginosa* are not mutually exclusive, even in non-CF infection.

One cluster containing four hospital environment strains with $\geq 85\%$ similarity by RAPD PCR was identified. That a genetically close community of environmental *P. aeruginosa* isolates should be present within a limited geographic space under selective pressure from the use of disinfectants and other infection control measures is not an unexpected finding.

7.5 Conclusion

In summary, this study has shown that the CF clonal complexes previously identified within Australia, including AES3, do not cause infections in non-CF patients at detectable levels. These strains were also absent in samples taken from the environment of the statewide tertiary referral hospital and the wider community environment within Hobart. No significant cross-infection was found to occur in hospitalised patients, and non-CF *P. aeruginosa* infection within the hospital and the wider Hobart community was found to be essentially panmictic.

CHAPTER EIGHT

Virulence Gene Distribution

8.1 Introduction

Thus far in the course of this study, the wide variation in antimicrobial resistance patterns and genotype of individual *P. aeruginosa* strains from numerous different types of infection and sites of isolation has been demonstrated. Whether these differences affect the capacity of specific strains to cause disease deserves attention. The virulence factors of *P. aeruginosa* by definition play a significant role in the type and severity of infections caused by this organism. This concept, and the action and activity of specific virulence factors of *P. aeruginosa* have been thoroughly discussed in the introduction to this thesis, and will not be re-examined here. To further understand why specific strains colonise and cause infection in specific sites, investigation of the prevalence and significance of specific virulence factor genes in alternate types of infections was undertaken.

Previous work has been performed on the presence and absence of virulence genes in differing *P. aeruginosa* isolates. A number of conclusions have been drawn from these studies regarding the prevalence of specific virulence factor genes and the type of infection that may be caused by *P. aeruginosa*, as well as the degree of global conservation of virulence genes in the *P. aeruginosa* genome. A number of these studies have investigated potential associations between the presence of known virulence factor genes and differing types of infection. Other studies have identified a specific link between possession of *exoU* and resistance to the fluoroquinolone antimicrobial agent, ciprofloxacin (Wong-Beringer, Wiener-Kronish *et al.* 2007).

One such study investigated all four known T3SS exo-enzyme genes in 95 clinical and 20 environmental isolates. This study found *exoT* to be universally present, with variable prevalence of other T3SS exo-enzyme genes. It also identified a decreased prevalence of *exoY* in urinary isolates and

exoU in CF isolates. CF isolates of *P. aeruginosa* were found to have a higher prevalence of *exoS* in comparison to isolates from other sources. No other variations in T3SS *exo*-enzyme gene prevalence were noted (Feltman, Schulert *et al.* 2001).

Another comprehensive study focused on ocular infections and employed 145 isolates, 69 of which were isolated from the eye. Virulence genes investigated in this study were *lasA*, *lasB*, *apr*, *exoS*, *exoT*, *exoU*, but not *exoY*. This study identified a number of *exoT* negative isolates, and found far greater *exoU* prevalence in ocular infection strains than in those from other sources. The large number of ocular isolates employed in this study allowed powerful statistical correlations to be made regarding the prevalence of the virulence genes investigated in regard to ocular infection (Lomholt, Poulson *et al.* 2001).

Work by other investigators involved PCRs for *lasB*, *toxA*, *exoS*, *nan1* and *nan2* on 162 genotypically distinct strains from a wide variety of clinical and environmental sources. This study found universal possession of *toxA*, *lasB* and *nan2*, with variable prevalence of *nan1* and *exoS*. It was concluded that the prevalence of *exoS* was highest in plant and CF isolates when compared to other human disease isolate (from urine, wounds and non-CF respiratory specimens). No CF clonal complex strains were included (Lanotte, Watt *et al.* 2004). A study of *exoS* and *exoU* prevalence alone in 45 *P. aeruginosa* isolates including 11 resistant to levofloxacin, found a proportionally greater prevalence of *exoU* in both wound isolates and those resistant to the fluoroquinolones, suggesting co-selection of *exoU* and fluoroquinolone resistance mechanisms (Wong-Beringer, Wiener-Kronish *et al.* 2007). A very comprehensive study of virulence and housekeeping genes was carried out on only 16 isolates taken from CF patients and the hospital and community environment. This found that all eleven CF isolates

tested possessed *exoS*, but also possessed *exoU*, conflicting with the results of all other studies (Finnan, Morrissey *et al.* 2004). Unfortunately, the small number of isolates employed in this study adds difficulties in determining how representative these results are of the species as a whole.

It has previously been asserted by a number of authors that *exoS* and *exoU* are mutually exclusive genes (Lee, Smith *et al.* 2005; Vance, Rietsch *et al.* 2005), despite their being widely separated in the *P. aeruginosa* genome (Feltman, Schulert *et al.* 2001). A number of isolates possessing both genes have since been identified, but this is considered a rare occurrence. It is accepted that *exoS* is far more prevalent than *exoU* in strains of *P. aeruginosa* (Feltman, Schulert *et al.* 2001; Lomholt, Poulson *et al.* 2001; Kulasekara, Kulasekara *et al.* 2006; Wong-Beringer, Wiener-Kronish *et al.* 2007).

As can be seen from the above, debate still exists about the role that individual virulence genes play in determining the type of infection caused by strains of *P. aeruginosa*. While a substantial amount of work has been performed on T3SS exo-enzyme genes in a large number of isolates, results have often been contradictory and many studies have not included all known T3SS exo-enzyme genes. Whilst the prevalence of some other virulence genes has been determined over large number of isolates in a number of studies, the genes involved in phenazine production have often been ignored. In addition, all previous studies relied on isolates from widely varying geographic and temporal sources. To clarify and consolidate the often conflicting results of the studies performed thus far requires the examination of a large number of virulence genes in statistically significant number of *P. aeruginosa* strains, taken from disparate sources and collected from a limited geographic space over a limited period of time.

8.2 Methods

A sub-set of secreted *P. aeruginosa* virulence factor genes was chosen based upon their variable presence in the genomes of *P. aeruginosa* isolates previously studied. These were; *apr*, coding for a type I secreted alkaline protease enzyme; *lasB* and the phenazine gene complex; *phzI*, *phzII*, *phzM*, *phzS* and *phzH*, coding for the type II secreted virulence factors elastase and phenazine. Genes coding for all known effector exo-enzymes of the type III secretion system, *exoS*, *exoT*, *exoU* and *exoY* were included in the study. Thus, the selected genes were representative of all three types of secretion system employed by the organism. The gene coding for the major virulence factor, exotoxin A (*toxA*) was not included, as a previous large studies have found this gene to be universally present in *P. aeruginosa* (Lanotte, Watt *et al.* 2004). While the same study found *lasB* to be present in all isolates investigated (Lanotte, Watt *et al.* 2004), a second smaller study identified a number of *lasB* negative *P. aeruginosa* isolates (Finnan, Morrissey *et al.* 2004), and thus *lasB* was included this analysis.

PCR primers designed by Finnan, *et al* (Finnan, Morrissey *et al.* 2004) were employed in this study, with the exception of the *exoU* forward primer (method 3.21). The binding site of each primer in the PAO1 genome (NCBI Database; <http://www.ncbi.nlm.nih.gov>) was determined, and it was ensured that these primers did not bind to other genomes in the database with any significant stringency.

For the *exoU* PCR, the forward primer previously described by Lanotte was used as a reverse primer, whilst a novel *exoU* forward primer, designed by Dr. Louise Roddam of the Cardiorespiratory Research Group, Menzies Research Institute, University of Tasmania, Hobart, Tasmania, was employed. As *exoU* is not present in the PAO1 genome, the binding site of the *exoU* PCR primers was confirmed using the PA14 genome (NCBI database).

PCR was performed on extracted bacterial DNA. Each specific assay incorporated an appropriate positive control (PAO1 DNA extract for all but the *exoU* PCR). Each performance of the *exoU* PCR included a PA103 DNA extract positive control. Every Set of PCRs performed included ddH₂O negative control. All negative test results were repeated. A 1 kb molecular weight ladder (Geneworks) was included in one well of each lane of gels in which products were visualised.

To confirm the newly designed *exoU* PCR was truly amplifying the *exoU* gene, a number of *exoU* PCR products were cut out of an agarose gel, purified using a QIAquick kit (Qiagen, method 3.22) and referred to the Micromon DNA sequencing facility of Monash University (Melbourne, Victoria), where the DNA sequence of these products were determined. The resultant product sequences were entered into a BLAST search on the NCBI database to confirm their identity as belonging to the *exoU* gene of *P. aeruginosa*.

A digoxigenin labelled probe for each virulence factor gene yielding at least one negative PCR result was prepared using a PAO1 PCR product (with the exception of the *exoU* PCR, for which a PA103 PCR product was employed) using the Roche® easy-hyb kit. These probes were used to confirm all negative results by DNA hybridisation against extracted bacterial DNA (method 3.22).

8.3 Results

8.3.1 *exoU* PCR primer design and validation

The priming sites of virulence factor gene PCRs described by Finnan, *et al* and employed in this study were compared with the genomes of *P. aeruginosa* strains held in the NCBI database. Whilst the

majority of these PCR primers bound to points in the *P. aeruginosa* genome consistent with the gene they were intended to amplify, discrepancies were identified for the described *exoU* PCR primers. Firstly, inversion of the forward and reverse primers published was identified. More so, the published reverse primer (actually a forward primer) was found to bind to a site outside of the gene.

In order to ensure accurate amplification of only *exoU* in this study, a new forward primer was designed (5'ATGCATATCCAATCGTTGG'3) by Dr. Louise Roddam of the Cardiorespiratory Research Group, Menzies Research Institute, University of Tasmania. This new forward primer was combined with the reverse primer developed by Finnan, *et al* (nominally a forward primer) resulting in a novel *exoU* PCR. This new PCR was shown to amplify an appropriate region within *exoU* in the *P. aeruginosa* UCBPP-PA14 complete genome, and not amplify any other genes from *P. aeruginosa* genomes or sequences held in the NCBI BLAST. The resultant product was estimated to have a size of 2415 bp by NCBI BLAST. Performance of this PCR on DNA extracts from *P. aeruginosa* strain PA103 yielded a product approximately of the expected size (2.4 kb) when compared to the molecular weight ladder. When performed on PAO1 DNA extracts, no product was resultant.

The *exoU* PCR products of the control strain PA103 and two hospital environment strains (isolates 123 and 124) were sequenced, resulting in partial sequences of between 145 and 519 bp in length. The returned sequences all provided between 91 and 98% similarity to the *P. aeruginosa* UCBPP-PA14 *exoU* in NCBI BLAST searches (table 8.1), confirming that the newly designed PCR does indeed amplify the *exoU* gene.

8.3.2 Virulence Factor PCR Results

The vast majority of *P. aeruginosa* strains tested in this study were shown to possess all virulence factor genes, with the exception of *exoU* (table 8.2). Thus, the presence of these virulence genes was found to be highly conserved across the genome of *P. aeruginosa*, regardless of the secretion system to which their products belonged. While a small number of individual isolates did show deletions of some of the specific virulence factor genes tested (table 8.2), no pattern was observed with regard to the source or site of infection that these isolates were recovered from (tables 8.3-8.6). All negative PCR results were confirmed by dot blot hybridisation, and no discrepancies between PCR results and DNA hybridisation results were observed.

One exception to the conserved nature of *P. aeruginosa* virulence factor genes was found in the T3SS, specifically in *exoU* and its relationship to the *exoS* gene. Only 23 of 184 isolates tested (12.5%) were of genotype *exoS-exoU*⁺, whilst 170 isolates (92%) were *exoS+exoU*⁻. Just ten isolates (5%) of isolates tested were found to have the *exoU+exoS*⁺ genotype (table 8.7, figure 8.7). Four of the latter isolates were recovered from different sites of infection in the same patient (BU) and were shown in chapter 7 to be genotypically indistinguishable by RAPD PCR and PFGE. When the relative prevalence of these T3SS exo-enzyme genes was compared, it was noted that loss of *exoS* was significantly ($p < 0.001$) more prevalent in isolates harbouring *exoU* when compared to the rate of *exoS* loss overall (table 8.7). Strains resistant to the fluoroquinolone antibiotic ciprofloxacin were no more likely to carry *exoU* than other isolates in this study (table 8.8).

When the relative prevalence of the *exoU*⁺ strains recovered in this study was investigated by site and source of isolation, a number of significant patterns were observed (appendix D). Firstly, isolates from

the environment were found to be significantly more likely to carry *exoU* than other isolates ($p=0.003$). Further analysis of this finding showed that this was due to a relative increase in *exoU* prevalence in ICU and NSU ward environmental isolates specifically ($p < 0.001$ and $p = 0.032$, respectively). Whilst no significant difference in *exoU* positivity was noted in isolates recovered from urine compared to all other tested, when compared only to other clinical isolates, urinary isolates were significantly ($p = 0.028$) more likely to harbour *exoU* than other clinical isolates. Two *exoU* positive urinary isolates (66 and 96) were recovered from patient BU. Following the exclusion of one of these isolates from the analysis to exclude duplication bias, the significance of these results was lost.

CF isolates were found to be significantly ($p = 0.001$) less likely to carry *exoU* when compared to other isolates in this study. Following exclusion of environmental isolates from the analysis, this trend was maintained ($p = 0.005$). Whilst out-patient isolates were found to also show propensity towards *exoU* negativity with further analysis excluding the large number of CF isolates in this group, the significance of this result was lost.

| Source Isolate | DNA Sequence | Size (bp) | <i>P. aeruginosa</i> UCBPP-PA14 <i>exoU</i> correlation (%) |
|----------------|--|--------------|---|
| 123 | AGCCCCCGCCTGAATCAGGGAGCCTGTCGAAACCCCGTCGCAGGCAGCGCATAAGTCCGCCAGCTTGCGTCAGCCAACCTTCA GGGCAAGGTCTCGGGGTGCCCCTAAAGAGCACGCCGGAATACTTTCCGGGAAGTTGCCGAAAGCGTTAGTGACGTGCGTTTC AGCAGTCCCCAAGGGCAAGGGGAGTCCCGTACTCTGACTGACTCGGCAGGGCCGCGGCAGATCACTCTGCGCCAGTTTGAGAAC GGAGTCACCTAGCTACAGCTCAGTCAGCCACCATTGACCAGTCTGGTCCTAAGCAGCTGTGGTGCCAAAG | 322 | 97 |
| 123 | TCGCGGTCTAATCGTGCAGCGGTTCAAGTGCATCCACATCGTCAACGCCATCACGTGCCTCAGCCCAGAGCACAATTCTACCGGC ATCGTCCGCCGCAAGTTTACCGGTGCCAAGGATGAGAATGGAGAGTTCACTTGTAACAATTGCAAGATCACACCCAGCGGTAA CCCTGAAGGCACAAGTCCCAACTCGACGACGAAAAGCCAACCGCCCTCGAGGCGCTCCATAACCAGTTCCGGTCCCTCTGCCGG TTGCAGCCGCAACGTGGCATCGTTGCTCGAGGGAAGTCTCAAGCCCCACTGTGCCAGCCATGTATCAATCATGTGAACTCCTTAT TCCGCCAAGCCTTAGCCATCTCAACGGTAGTCGAACTCAAGGGTTTGCCGAAACGCAGGAAGCCTCGTGCCGAGTAGTTGTCGA CGATATCGTTCAGCGCTTGATTGATTTCCGCCGACTGGTGGAATGCCGTAGCTGCTCCTCCGCCGACGTATTTGAGCTATGTTG GAATCCGGCTC | 519 | 98 |
| 124 | ACCGGCAGACTATCGGTGCAGCGGTTCAAGTGCATCCACATCGTCAACGCCATCACGTGCCTCAGCCCAGAGCACAAGTCTACCG GCATCGTCCGCCGCAAGTTTACCGGTGCCAAGGATGAGAATGGAGAGTTCACTTGTAACAATTGCAAGATCACACCCAGCGGT AACCTGAAGGCACAAGTCCCAACTCGACGACGAAAAGCCAACCGCCCTCGAGGCGCTCCATAACCAGTTCCGGTCCCTCTGCC GGTTGCAGCCGCAACGTGGCATCGTTGCTCGAGGGAAGTCTCAAGCCCCACTGTGCCAGCCATGTATCAATCATGTGAACTCCTT ATTCCGCCAAGCCTTAGCCATCTCAACGGTAGTCGAACTCAAGGGTTTGCCAAAACGCAGGAAGCCTCGTGCCGAGTAGTTGTCTG ACGATTTCCTCAGCGCG | 440 | 98 |
| PA103 | CTGCCCCCTCGTGTATCAGGAGCCTGTCGGAACCCCGTCGCAGGCAGCGCATAAGTCCGCCAGCTTGCGTCAGGAATCTTCAGGG CAAGGTCTCGGGGTGCCCCTAAAGAGTACGACTTGAATACTTTCCGGGATGTTGCCCCCAAAGCATTAGTGACCAAGTTTCATTT GTCTTCTTGCTCCAGGGTAGTCCATGACTCTG | 202 | 91 |
| PA103 | TGTAGGGCCTAATCGTGCAAGCAGGGTAGTGCATCCACATCGGTACGCCATCACGTGCCTCAGGCTTGAGAAAAAAAAGGT TGCATCCGCCGCCGCAAGTTTCATCAGTGAAACTTAGTAAAATTACCTGTTAATAAACCGT | 145 | 94 |

Table 8.1: Partial sequences resulting from DNA sequencing of *exoU* PCR products of PA103 and two environmental isolates and the percentage correlation of these sequences to the *exoU* locus of the *P. aeruginosa* UCBPP-PA14 the *Pseudomonas aeruginosa* PA14 whole genome recorded in the NCBI database (<http://www.ncbi.nlm.nih.gov>).

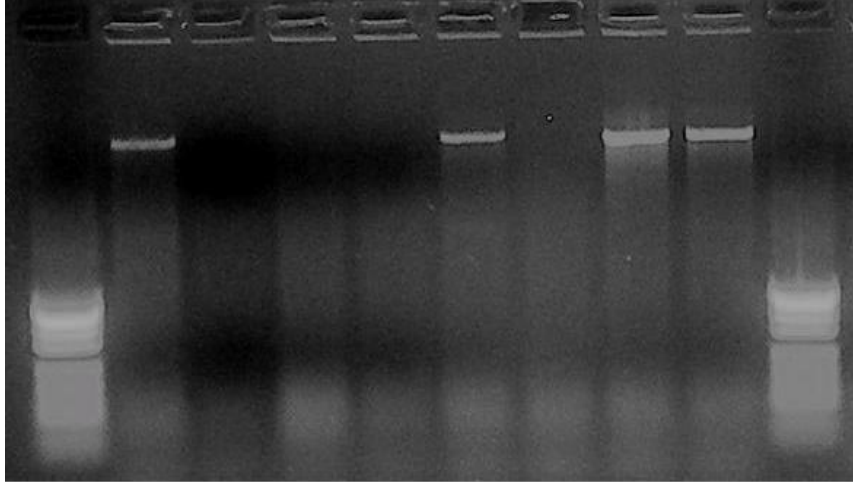


Figure 8.1: *exoU* PCR gel (L-R): *puc19* MW marker (Biorad), PA1O3, ddH₂O Negative control, PAO1, 23, 68, 108, 123, 124, *puc19* MW marker (Biorad)

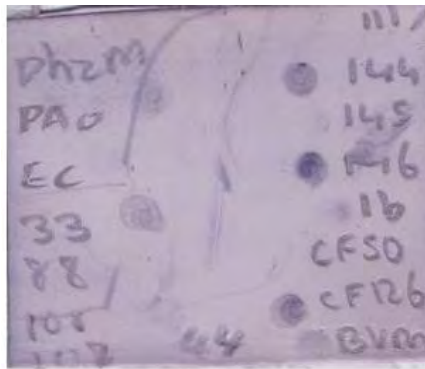


Figure 8.2: DNA-DNA hybridisation (44°C) of *phzM* (Anticlockwise from top left): PAO1, E. coli ATCC 10418, 33, 88, 108, 111, 144, 145, 146, U1b, U50, U126, AES1.

| Isolate | Patient/Site | Place of | Site of Isolation | Mucoidity | apr | lasB | phzI | phzII | phzM | phzH | phzS | exoS | exoY | exoT | exoU |
|---------------------|--------------|-------------|-------------------|-----------|-----|------|------|-------|------|------|------|------|------|------|------|
| 120 | DJ | ICU | SINK | non | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS |
| 121 | DK | NSU | SINK | non | POS | POS | POS | POS | POS | NEG | POS | POS | POS | POS | NEG |
| 122 | DL | NSU | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 123 | DM | NSU | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS |
| 124 | DN | NSU | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS |
| 127 | DO | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | NEG | POS | NEG |
| 128 | DP | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 130 | DQ | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 134 | DR | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 135 | DS | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS |
| 136 | DT | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 141 | DU | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 142 | DV | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 144 | DW | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 145 | DX | Other Ward | Shower | non | POS | POS | POS | POS | NEG | POS | POS | POS | POS | POS | NEG |
| 146 | DX | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 147 | DY | Community | Pool | non | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS |
| 149 | DZ | Community | Pool | non | POS | POS | POS | POS | POS | NEG | POS | POS | POS | POS | NEG |
| 150 | DZ | Community | Spa | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 151 | EA | Community | Pool | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 153 | EB | Community | Spa | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 154 | EC | Community | Effluent inlet | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 156 | ED | Community | Effluent spillage | non | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS |
| 157 | EE | Community | Effluent spillage | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 158 | EE | Community | Groundwater | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| 160 | EF | Community | Stream | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U1a | CH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U1b | CH | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U3a | EG | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U3b | EG | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U5a | EH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U5b | EH | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | NEG | POS | NEG |
| R14a | EI | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R14b | EI | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U16a | EJ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U16b | EJ | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U17a | EK | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U17b | EK | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U19a | EL | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U19b | EL | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U20 | EM | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U21 | EN | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U26 | EO | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R29b | EK | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U32a | AJ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U32b | AJ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U33a | AS | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U33b | AS | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R43 | EP | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R44a | EQ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R44b | EQ | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U47a | ER | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U47b | ER | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U50 | ES | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | NEG | NEG | POS | POS | NEG | NEG | NEG |
| U51 | ET | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R58 | ER | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U61a | EU | Out-patient | CF Respiratory | muc | POS | POS | NEG | POS | POS | POS | NEG | POS | POS | NEG | NEG |
| U61b | EU | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U66a | ET | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U66b | ET | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U73 | EW | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U80a | EX | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U80b | EX | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U81a | EY | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U81b | EY | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R86a | EZ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R86b | EZ | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U108a | FA | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U108b | FA | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U108c | FA | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U110 | FB | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U111a | AH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U111b | AH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R112 | CH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| R113a | AG | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | NEG | POS | POS | POS | POS | NEG |
| R113b | AG | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U115 | FC | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | NEG |
| U126 | FD | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U156 | FE | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS |
| U162 | FF | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U171 | FG | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U175 | FH | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U184 | FI | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U194a | FJ | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U194b | FJ | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U195a | FK | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| U195b | FK | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | NEG |
| U240 | FL | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | NEG | POS | POS | POS | POS | NEG |
| RWE2 | FM | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| AES1 | | | Control Strain | | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| AES2 | | | Control Strain | | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| P. aeruginosa PA01 | | | Control Strain | | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG |
| P. aeruginosa PA103 | | | Control Strain | | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS |

Table 8.2: Results of virulence gene PCRs.

AES1 isolate numbers are highlighted in purple, AES2 isolate numbers in pink, AES3 isolate numbers in orange and Tasmanian CF cluster isolate numbers in blue. muc: mucoid on first isolation; non: non-mucoid on first isolation.

| | All Clinical | Environment | All Isolates |
|--------------------------|--------------|-------------|--------------|
| | Isolates | Isolates | |
| Virulence Factor Gene | Present (n) | Present (n) | Present (n) |
| <i>apr</i> | 149 | 35 | 184 |
| <i>lasB</i> | 149 | 35 | 184 |
| <i>phzI</i> | 148 | 35 | 183 |
| <i>phzII</i> | 149 | 35 | 184 |
| <i>phzM</i> | 148 | 32 | 180 |
| <i>phzH</i> | 142 | 33 | 175 |
| <i>phzS</i> | 142 | 35 | 177 |
| <i>exoS</i> | 142 | 29 | 171 |
| <i>exoY</i> | 148 | 34 | 182 |
| <i>exoT</i> | 143 | 35 | 178 |
| <i>exoU</i> | 13 | 10 | 23 |
| Total (n) | 149 | 35 | 184 |

Table 8.3: Prevalence of virulence genes according to clinical or environmental source.

| | CF Isolates | AES3 | Respiratory | Skin/Soft tissue | Urinary | Ear Isolates | All Clinical |
|--------------------------|-------------|-------------|-------------|---------------------|-------------|--------------|--------------|
| | Isolates | Isolates | Isolates | Isolates | Isolates | | Isolates |
| Virulence Factor Gene | Present (n) | Present (n) | Present (n) | Present (n) | Present (n) | Present (n) | Present (n) |
| <i>apr</i> | 69 | 12 | 25 | 26 | 24 | 5 | 149 |
| <i>lasB</i> | 69 | 12 | 25 | 26 | 24 | 5 | 149 |
| <i>phzI</i> | 68 | 12 | 25 | 26 | 24 | 5 | 148 |
| <i>phzII</i> | 69 | 12 | 25 | 26 | 24 | 5 | 149 |
| <i>phzM</i> | 68 | 11 | 25 | 26 | 24 | 5 | 148 |
| <i>phzH</i> | 65 | 11 | 24 | 25 | 23 | 5 | 142 |
| <i>phzS</i> | 67 | 12 | 23 | 26 | 24 | 5 | 145 |
| <i>exoS</i> | 68 | 12 | 22 | 26 | 21 | 5 | 142 |
| <i>exoY</i> | 68 | 11 | 25 | 26 | 24 | 5 | 148 |
| <i>exoT</i> | 64 | 10 | 24 | 26 | 24 | 5 | 143 |
| <i>exoU</i> | 1 | 0 | 4 | 3 | 5 | 0 | 13 |
| Total (n) | 69 | 12 | 25 | 26 | 24 | 5 | 149 |

Table 8.4: Prevalence of virulence genes in clinical isolates according to site of infection.

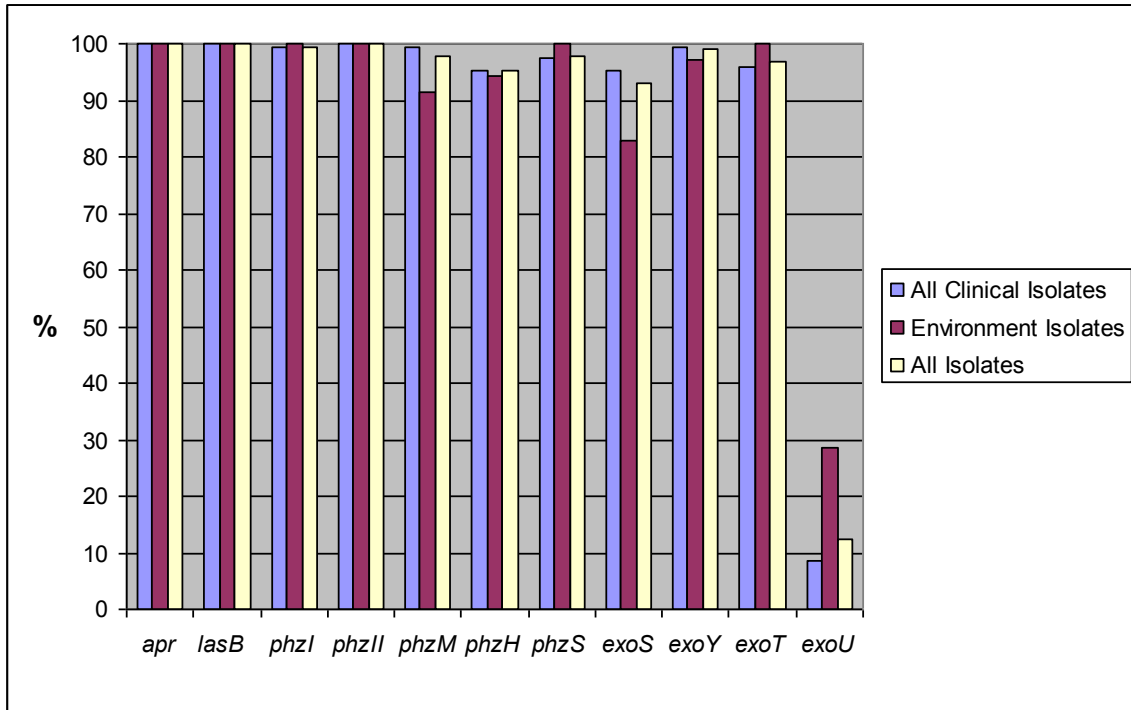


Figure 8.3: Percentage prevalence of virulence genes according to clinical or environmental source in clinical isolates

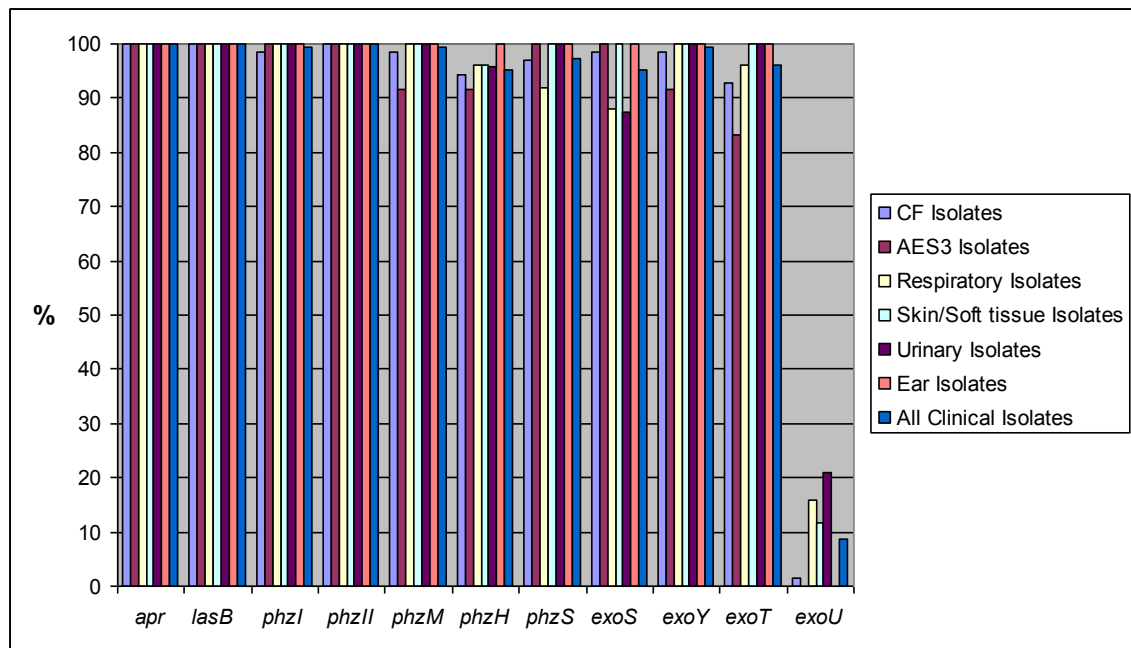


Figure 8.4: Percentage prevalence of virulence genes in clinical isolates according to site of infection.

| | ICU Clinical Isolates | NSU Clinical Isolates | Other Ward Clinical Isolates | Outpatient Isolates | Community Clinical Isolates |
|----------------|--------------------------|--------------------------|------------------------------------|------------------------|-----------------------------------|
| Virulence Gene | Present (n) | Present (n) | Present (n) | Present (n) | Present (n) |
| <i>apr</i> | 4 | 5 | 52 | 80 | 8 |
| <i>lasB</i> | 4 | 5 | 52 | 80 | 8 |
| <i>phzI</i> | 4 | 5 | 52 | 79 | 8 |
| <i>phzII</i> | 4 | 5 | 52 | 80 | 8 |
| <i>phzM</i> | 4 | 5 | 52 | 79 | 8 |
| <i>phzH</i> | 3 | 5 | 50 | 76 | 8 |
| <i>phzS</i> | 3 | 5 | 52 | 77 | 8 |
| <i>exoS</i> | 4 | 5 | 48 | 78 | 7 |
| <i>exoY</i> | 4 | 5 | 52 | 79 | 8 |
| <i>exoT</i> | 3 | 5 | 52 | 75 | 8 |
| <i>exoU</i> | 0 | 0 | 5 | 2 | 2 |
| Total (n) | 4 | 5 | 52 | 80 | 8 |

Table 8.5: Prevalence of virulence genes in clinical isolates according to source of isolation.

| | Community Environment Isolates | ICU Environment Isolates | NSU Environment Isolates | Hospital Environment Isolates | All Environment Isolates |
|--------------------------|--------------------------------------|--------------------------------|--------------------------------|-------------------------------------|--------------------------------|
| Virulence Factor Gene | Present (n) | Present (n) | Present (n) | Present (n) | Present (n) |
| <i>apr</i> | 10 | 8 | 4 | 25 | 35 |
| <i>lasB</i> | 10 | 8 | 4 | 25 | 35 |
| <i>phzI</i> | 10 | 8 | 4 | 25 | 35 |
| <i>phzII</i> | 10 | 8 | 4 | 25 | 35 |
| <i>phzM</i> | 10 | 7 | 4 | 22 | 32 |
| <i>phzH</i> | 9 | 8 | 3 | 24 | 33 |
| <i>phzS</i> | 10 | 8 | 4 | 25 | 35 |
| <i>exoS</i> | 10 | 5 | 4 | 19 | 29 |
| <i>exoY</i> | 10 | 8 | 4 | 24 | 34 |
| <i>exoT</i> | 10 | 8 | 4 | 25 | 35 |
| <i>exoU</i> | 2 | 5 | 2 | 8 | 10 |
| Total (n) | 10 | 8 | 4 | 25 | 35 |

Table 8.6: Prevalence of virulence genes in environmental isolates according to isolation source.

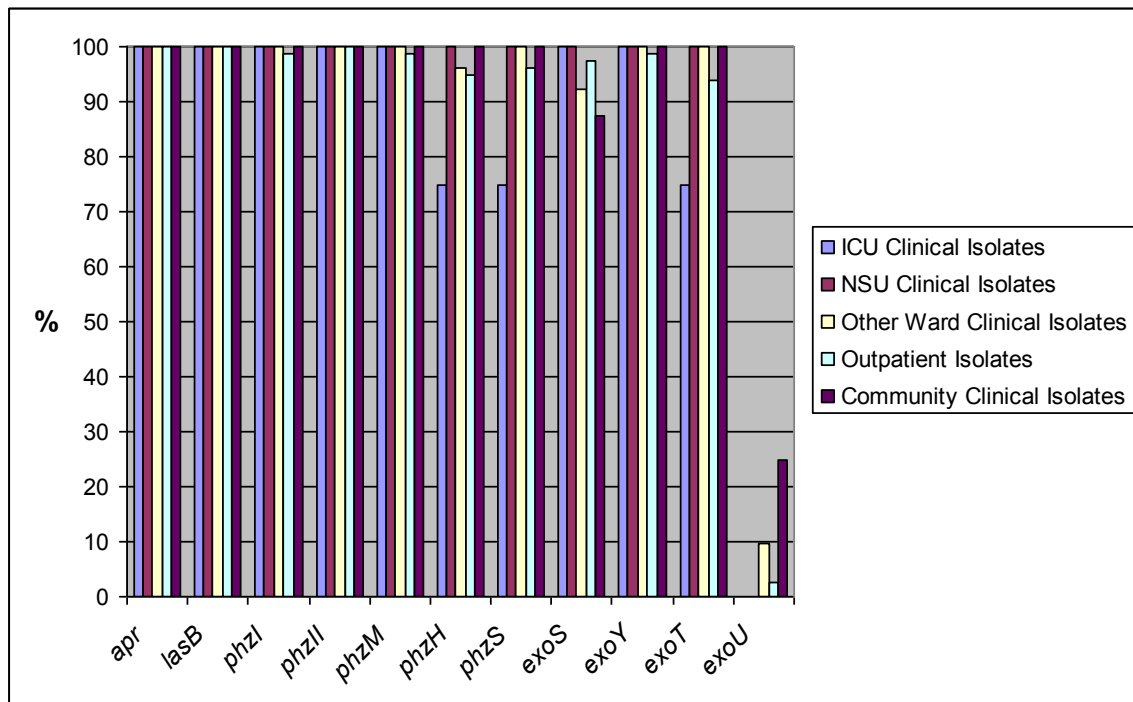


Figure 8.5: Prevalence of virulence genes in clinical isolates according to source of isolation.

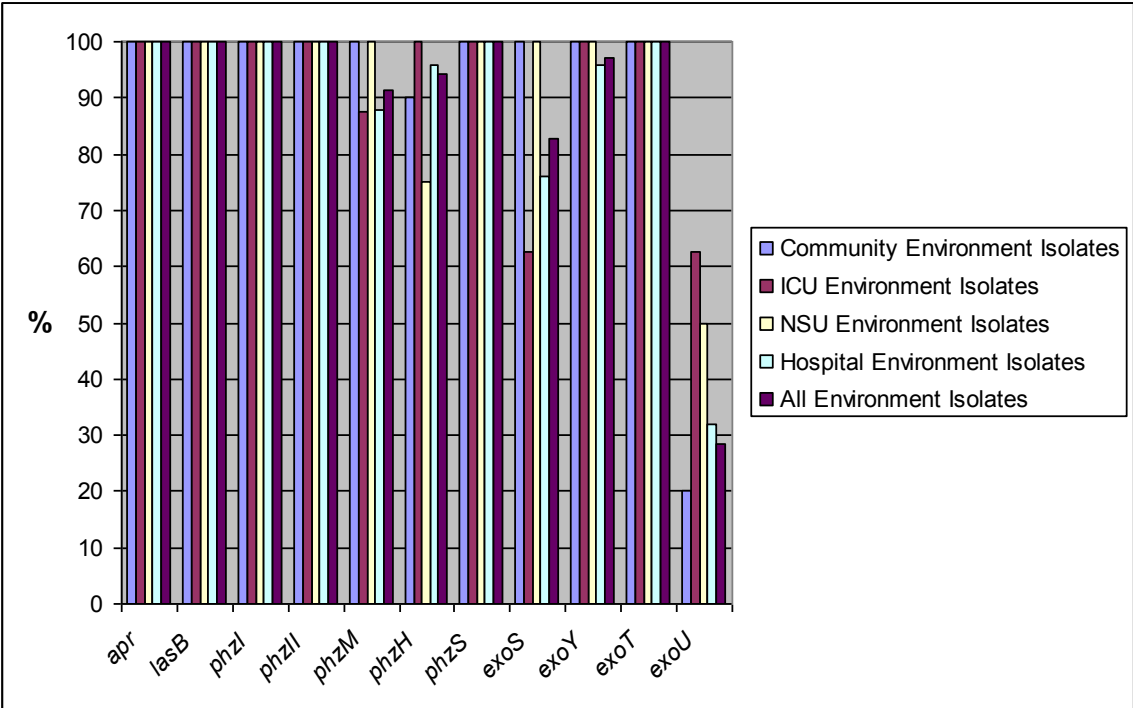


Figure 8.6: Percentage prevalence of virulence genes in environmental isolates according to source of isolation.

| | <i>exoS</i> ⁺ | <i>exoS</i> ⁻ | <i>exoS</i> ⁺ | <i>exoS</i> ⁻ <i>exoU</i> ⁻ | χ ² test |
|-----------|--------------------------|--------------------------|--------------------------|---|---------------------|
| | <i>exoU</i> ⁺ | <i>exoU</i> ⁺ | <i>exoU</i> ⁻ | | p value |
| Total (n) | 5 | 7 | 87 | 1 | <0.001 |

Table 8.7: Prevalence and comparison of *exoU* and *exoS* genotypes in all isolates.

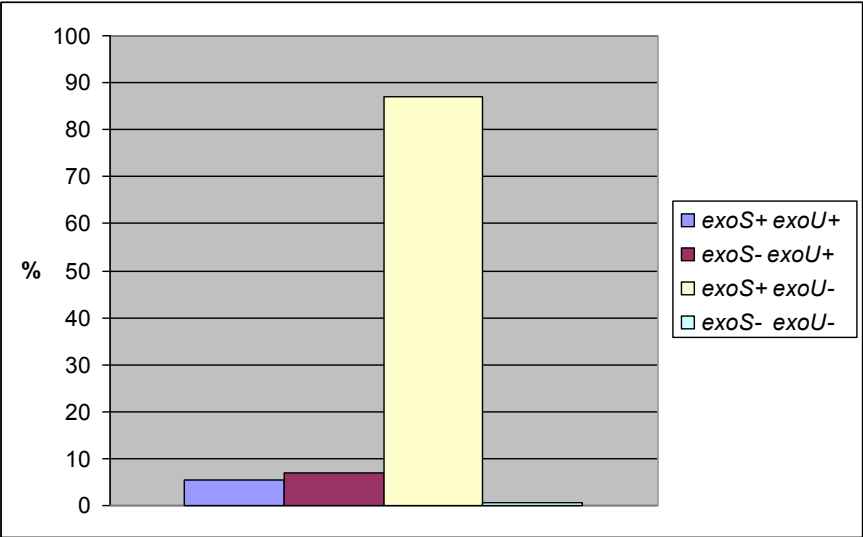


Figure 8.7: Percentage prevalence of *exoU* and *exoS* genotypes in all isolates.

| <i>exoU</i> Genotype | Susceptibility to Ciprofloxacin by CLSI Method | | | χ^2 test (p) |
|-------------------------|--|--------------|-----------|-------------------|
| | Sensitive | Intermediate | Resistant | |
| <i>exoU</i> + | 19 | 0 | 4 | 0.16564603 |
| <i>exoU</i> - | 124 | 21 | 16 | 0.77348952 |
| total (n=184) | 143 | 21 | 20 | |

Table 8.8: Prevalence of *exoU* in relation to ciprofloxacin sensitivity by the CLSI method

8.4 Discussion

8.4.1 Virulence factor genes are highly conserved

Virulence factor genes tested were highly conserved in the *P. aeruginosa* species genome. In cases where strains lacking specific virulence genes were identified, no statistically significant propensity towards a type of disease or site of origin was noted. A significant association between possession of *exoU* and loss of *exoS* was observed. These negative results were found to be due to gene deletions rather than mutation of the gene at the PCR primer site, as DNA-DNA hybridisation studies did not identify any discrepancy with the PCR results. These findings suggest that where such events occurred they do not predispose that organism to causing any specific type of disease or to colonising any specific environmental niche.

All isolates investigated in this study possessed both *apr* and *lasB*. These findings were in concordance with a comprehensive study carried out on 145 isolates of *P. aeruginosa* finding these genes to be universally present (Lomholt, Poulson *et al.* 2001), but in significant variation with another study involving a much smaller sample of eleven CF and five environmental *P. aeruginosa* isolates (Finnan, Morrissey *et al.* 2004). This study also found two environmental isolates, both from hospital sinks in Belgium with significant loss of the phenazine group genes (Finnan, Morrissey *et al.* 2004), no such isolates were identified in our environmental strains

The greatest heterogeneity was observed in the T3SS effector exo-enzyme genes. Regarding the chromosomal T3SS effector genes (*exoS*, *exoT*, *exoY*), the findings of this study were in concordance with other studies, in which a high prevalence of *exoT* and *exoY* was identified (Feltman, Schulert *et al.* 2001; Lomholt, Poulson *et al.* 2001; Finnan, Morrissey *et al.* 2004). Once again, discrepancies existed

between this study and one other study, in which *exoT* was found to be of very low prevalence (Finnan, Morrissey *et al.* 2004).

8.4.2 *exoU* and *exoS* are not mutually exclusive

Ten isolates in this study were found to harbour both *exoS* and *exoU*. These isolates included four isolates from the same patient and with identical genotype. Therefore, previous assertions that these genes are mutually exclusive are disproved by this study, as they have been by a number of previous studies. Despite this, a significant ($p < 0.001$) trend towards mutual exclusivity in these two genes was observed, with isolates possessing one gene far less likely to possess the other. This phenomenon is surprising, as no easily identifiable linkages between these genes are observable in the *P. aeruginosa* genome, as one gene (*exoS*) is chromosomal, whilst the other is acquired by horizontal transmission (*exoU*), also, these genes are found at points distant from each other on the *P. aeruginosa* genome.

8.4.3 Prevalence of *exoU*

The only known horizontally acquired virulence gene in *P. aeruginosa* is *exoU*. Given this, it is unsurprising that this particular gene shows many significantly different traits with regard to prevalence in the *P. aeruginosa* genome when compared to the chromosomal virulence genes. It has been shown to have a significantly lower overall prevalence in *P. aeruginosa* isolates when compared to other T3SS effector genes in a number of studies (Feltman, Schulert *et al.* 2001; Lomholt, Poulson *et al.* 2001; Wong-Beringer, Wiener-Kronish *et al.* 2007), a finding supported by this investigation. Once again, an exception to this was the findings of Finann, *et al.*, in which *exoU* was found to have a prevalence second only to *exoS* in the T3SS genotype of the isolates tested (Finnan, Morrissey *et al.* 2004).

Two possible explanations for the discrepancy in the results of this and other studies with those of Finnan, *et al*, exist. Firstly, as demonstrated during the course of this study, the *exoU* PCR forward primer employed by Finnan, *et al* was specific to a sequence outside of the target gene. It is possible that the particular strains involved in Finnan's studies had an unusually high prevalence of the *exoU* gene. Also possible is that the product observed was an amplification of some other area of the *P. aeruginosa* genome. Had these isolates been recovered from a source within a limited geographical area over a limited period of time, in which many isolates could have acquired *exoU*, the former explanation would seem feasible. Given that the eleven CF isolates employed in Finnan's study were isolated in Ireland between 1993 and 1994, and that the environmental isolates were isolated in Brussels in 1997, the unusually elevated prevalence of *exoU*, and the inordinately decreased prevalence of *exoT* in Finnan's strains remains puzzling.

A significantly ($p=0.005$) decreased prevalence of *exoU* in CF strains was observed in this study. This finding is supported by one previous T3SS gene study (Feltman, Schulert *et al.* 2001). A different study involving 137 *P. aeruginosa* isolates, 81 of which were recovered from the CF lung found a significant increase the prevalence of *exoS* in CF isolates (Lanotte, Watt *et al.* 2004). Unfortunately, no data is available from that study regarding the relative prevalence of *exoU* in these isolates, but given the demonstrated propensity for the *exoS*^{+*exoU*⁻ phenotype in *P. aeruginosa*, it is not unreasonable to elaborate that a conversely decreased prevalence of *exoU* in the CF strains studied may have occurred.}

Converse to the CF respiratory isolates, environmental samples recovered from the ICU and NSU were found to have an increased prevalence of *exoU*. This increased prevalence may be attributed to the increased selective pressures that will apply in the environment of these highly specialised wards. Both

the ICU and NSU environments are subject to more stringent disinfection protocols and higher antimicrobial usage than other environments within the hospital; which may facilitate and promote the acquisition of *exoU* by horizontal transfer between environmental strains of *P. aeruginosa*. It may be speculated that acquisition of other resistance mechanisms which may assist survival in the ICU environment, such as genes coding for disinfectant resistance, might also positively modify the *exoU* acquisition rate. The probability of increased *exoU* prevalence in the hospital environment being due to the selective pressure of disinfectant and antibiotic use is increased when the previously determined relationship between multi-drug resistance and the presence of *exoU* in *P. aeruginosa* is considered (Zaborina, Kohler *et al.* 2006).

8.5 Conclusion

Associations found by previous studies between disease type and T3SS virulence gene prevalence were not supported by this investigation. However, a concerning increased prevalence of *exoU* was noted in environmental isolates from intensive therapy units. The relative heterogeneity of the virulence genes studied in this work is concordant with the conserved nature of the *P. aeruginosa* genome in both disease and the environment. That *exoU* was the source of all significant variability within virulence genome correlates with its mobile nature and capacity to be acquired under the selective pressure of antibiotic use.

CHAPTER NINE

Whole Cell Virulence in a Novel Modification of the

***Dictyostelium discoideum* Eukaryotic**

Cell Model

9.1 Introduction

The previous chapter of this thesis demonstrated the relatively conserved nature of virulence genes in a large cohort of *P. aeruginosa* strains. Only a very small number of genotypic trends towards a particular environmental niche or disease process were identified. However, presence of specific virulence genes in an organism by no means relates to the degree of expression of the given genes in a given environment. As discussed in the first chapter, virulence in *P. aeruginosa* is a multi-factorial and dynamic process, involving the interaction of many cellular mechanisms of both the bacteria itself and its host. Indeed, from a clinical disease perspective, the expression of a given virulence is far more important than its presence or absence.

Expression of virulence may be analysed on a factor by factor basis *in vitro*. Often this is performed by the quantitative or qualitative measurement of a small number of virulence factors of the test bacteria, grown in isolation from a host. Whilst the information gained from such studies is relevant and useful in determining the role and action of specific virulence factors, it does not illuminate the role of these factors in a given strain or set of strains as a whole when interacting with the eukaryotic host, as occurs in natural infection of humans and other eukaryotes.

In order to find relevant information regarding the virulence and interactions of a given bacterial strain in a host organism, it is essential to introduce the test strain to such a host. Such testing has traditionally been performed in mammals, such as mice, but ethical and logistical considerations will often limit the scope of these studies. In response to this, a number of lower eukaryotes have been used to analyse the whole cell virulence of bacteria in a eukaryotic host. Such eukaryotic models have included *Arabidopsis thaliana* (thale cresse, or mouse ear cresse), *Drosophila melanogaster* (fruit fly),

Caenorhabditis elegans (soil nematode), *Acanthamoeba polyphaga* (free living soil amoeba) and *Dictyostelium discoideum* (Pukatzki, Kessin *et al.* 2002).

D. discoideum is a soil organism which is outstanding amongst the lower eukaryotes due to its capacity to convert between a unicellular amoeboid form and a multi-cellular mould form. In the single celled free-living amoeboid form, it exists by scavenging nutrients from environmental particles, including bacteria and yeasts, via phagocytosis (Gross 1994; Steinert and Heuner 2005). In axenic mutants, nutrient are acquired via pinocytosis from the immediate environment (Gross 1994; Parent and Devreotes 1996).

Even in the undifferentiated unicellular form, a great deal of chemical signalling has been shown to occur between *Dictyostelium* cells. With the onset of starvation following utilisation of all available nutrient sources in the immediate environment, these amoeboid forms will release “pulses” of adenosine 3’-5’ cyclic monophosphate (cyclic- AMP) at six minute intervals (Parent and Devreotes 1996). In response to this chemotactic signal, amoebae stream towards this source, where mounds of approximately 100, 000 cells encased in an acellular extracellular matrix will be formed within ten hours (Gross 1994). Over the following 24 hours, the mounds will differentiate into a cellulose stalk topped by spores encased in extracellular fluid. First, a barely discernable tip develops in the aggregated mound, which will then develop into a pre-stalk, or “finger” of differentiated cells. This “finger” is capable of acting as a motile “slug” form to seek out the most appropriate area for further development (including light sources), or may further develop *in situ* if the appropriate conditions are met. The final stage of cell development, culmination, occurs when the tip of the “finger” or “slug”

form differentiates into a spore, which is raised from the surface of the agar on a cellulose stalk to form a fruiting body (fig 9.1) (Gross 1994; Parent and Devreotes 1996; Steinert and Heuner 2005).

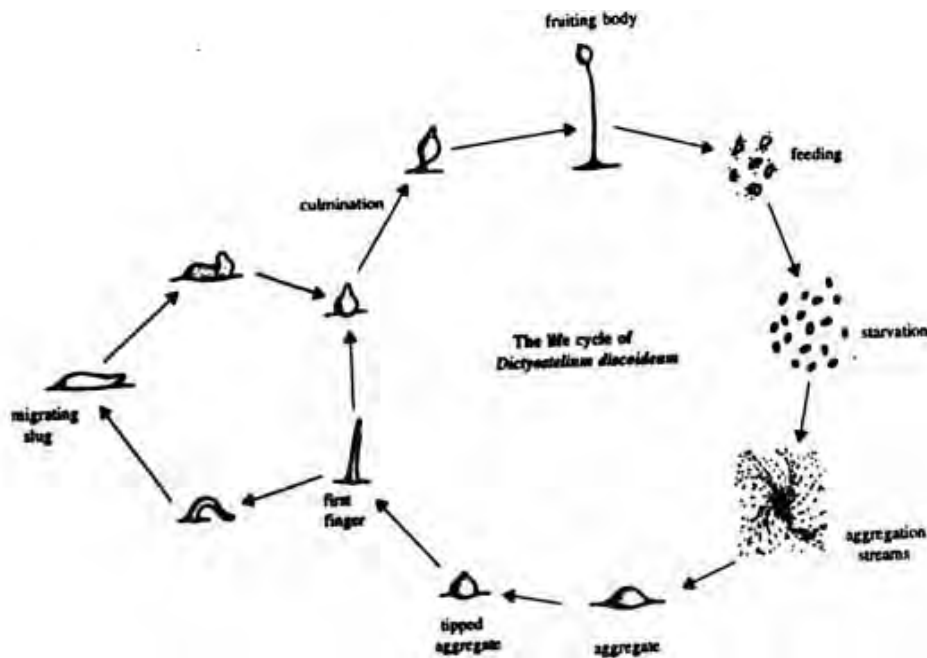


Figure 9.1: Developmental cycle of *Dictyostelium discoideum*, (Gross 1994).

The fruiting body itself is composed of 80% spores and 20% dead stalk cells (Steinert and Heuner 2005).

Chemical signalling plays an intrinsic role in the developmental cycle of *D. discoideum*, initiating and controlling each phase of development in the organism. The primary signalling chemical involved in initiating this process is cyclic AMP; controlling both chemotaxis of the unicellular forms and development into the multicellular form. Other signalling pathways, such as those involving guanylyl cyclase, adenylyl cyclase and triphosphate are initiated or shut down by the cyclic-AMP stimulus (Gross 1994; Parent and Devreotes 1996). Receptors for cyclic AMP also accept cyclic GMP and phospholipase C, and appear to have a role in controlling calcium ion transport into the cell (Gross 1994). Extracellular pH has been shown to play a significant role in the development of the fruiting body. A considerable amount of ammonia (NH_3), a weak base, is produced through protein catabolism

in the early stages of *D. discoideum* development. This lowers the extracellular pH which catalyses the development from aggregates into the slug form. Further to this, the ammonia must be reduced to the acidic form, ammonium (NH_4^+) for further development into the fruiting body. Increasing environmental ammonia concentration has been shown to significantly slow culmination in “slugger” mutants of *D. discoideum*. Conversely, incubation in a CO_2 rich environment stimulates development to the fruiting body stage due to the increased atmospheric acidity (Gee, Riussell *et al.* 1994).

Interference with any of the chemical signalling pathways in *D. discoideum* will alter or halt development of the organism. Previous studies of the interactions between *D. discoideum* and *P. aeruginosa* have focused on the capacity of the amoeboid form of the organisms to form plaques on a *P. aeruginosa* lawn. None have examined the prolonged effects of interaction with *P. aeruginosa* in the development of *D. discoideum*. Some strains of *P. aeruginosa* are capable of producing large amounts of ammonia from urea via the enzymatic action of urease, and whether sufficient ammonia may be synthesised to have an inhibitory effect on development in *D. discoideum* is currently unknown. The *P. aeruginosa* T3SS effector exoenzyme ExoT is an adenylate cyclase; any role it may play in altering *D. discoideum* development through the inhibition of cyclic AMP signalling has not been investigated to date.

The nature of interactions between *D. discoideum* amoeboid forms and bacterial cells is not unlike that of phagocytic cells of the human immune system (Pukatzki, Kessin *et al.* 2002). Indeed, it is postulated that a number of bacteria causing disease in humans have adapted many virulence determinants in order to escape predation by free-living soil amoebae (Steinert and Heuner 2005). These hypotheses, and the fact that *D. discoideum* may act as a host eukaryotic cell for multiple

bacteria causing human disease led to the development of a *D. discoideum* whole cell bacterial virulence assay.

Little work has been carried out on the virulence of clinical and environmental *P. aeruginosa* in amoeboid models. The one study performed thus far (in *A. polyphaga*) utilised a very limited range of clinical isolates. It is proposed that observations of the interactions of *D. discoideum* and *P. aeruginosa* from a wide range of sources and types of infection may lead to new findings in relation to the expression, distribution and action of virulence in *P. aeruginosa*.

9.2 Methods

Strains of *P. aeruginosa* were selected to provide a broad range of antimicrobial resistance properties, virulence genotype and clinical or environmental sources (table 9.1). An antimicrobial resistance score of between one and eleven was assigned to each isolate, with a score of one representing resistance to only one antimicrobial agent tested by the CLSI method, and a score of eleven representing resistance to all antimicrobials tested. Similarly, a virulence gene score between one and eleven was assigned, with each virulence gene present of the eleven studied providing a score of one, up to a maximum score of eleven. Strains were cultured from MMM onto TSAY for 48 hours. Urease production was measured by a simple qualitative method using urea agar slopes (method 3.23).

To avoid concerns regarding the influence of host *Klebsiella* virulence affecting results in *D. discoideum* models, and difficulties in producing a reproducible inoculum using amoebae in solution, a novel modification of the *D. discoideum* virulence assay was developed (methods 3.24). In this method, commercial filter paper discs impregnated with spores of *D. discoideum* were applied to a

standard inoculum of *P. aeruginosa* in lawn on an SM agar plate. Following several days incubation, the occurrence of spore germination and plaque formation allowed measurement of virulence properties. Three separate methods were investigated to purify *D. discoideum* spores cultured on *K. aerogenes* and impregnated into paper discs (ethanol shock treatment, heat shock treatment and desiccation; method 3.24.2).

The strains were then subjected to the seven day *Dictyostelium* disc virulence test using *D. discoideum* strains AX2 and X22 (method 3.24.3) on SM agar. Virulence towards *Dictyostelium* was also tested following the incorporation of antimicrobials the SM agar. All clinical and one hospital environmental isolate were tested for the effects of the presence of sub-MIC concentrations of azithromycin (4 mg/L) and the effects of ceftazidime and tobramycin at MIC₉₀ levels (6.25 mg/L and 25 mg/L, respectively) (Tessin, Thiringer *et al.* 1988). Where plaque formation occurred in the virulence assay, the annular radius of the plaque zone was measured and recorded to the nearest 0.5 mm. The degree of development of the *D. discoideum* within the plaques was recorded using a simple scoring method (figures 9.2-9.5).

9.2.1 Statistical analysis

All *Dictyostelium* virulence assays were performed in triplicate, with the mean annular radius and development score being recorded, as well as the standard deviation of the assay for each isolate tested. To test the reproducibility of results overall, the mean standard deviation of the assay overall was calculated. *Dictyostelium* zone sizes and development were compared according to *D. discoideum* strain, isolate source or site of isolation, type of disease isolated from, The relationship between results found in the AX2 and X22 *Dictyostelium* virulence tests, and these tests with virulence and

antimicrobial resistance score were compared by a Pearson's co-efficient of correlation. The strength of these correlations was tested by using the square of the Pearson's co-efficient of correlation and significance of the relationships by the student's t test.

Comparison of *D. discoideum* virulence assay results with Urease production, antimicrobial resistance score and virulence gene score were carried out using the Pearson's co-efficient of correlation and significance of the relationships by the student's t test. As the latter tests were qualitative, in order to facilitate these comparisons, quantitative values of plaque formation and development of *D. discoideum* in the virulence assay were altered to qualitative values prior to analysis.

9.3 Results

9.3.1 Removal of *Klebsiella aerogenes* contamination

Three methods were used to remove *K. aerogenes* contamination from the *D. discoideum* discs, of which only desiccation effectively removed the bacteria without also inactivating the *Dictyostelium* spores (table 9.1). Following this finding, all test discs were prepared by removal of *K. aerogenes* through desiccation.

9.3.2 *Dictyostelium discoideum* virulence testing results

In all assays performed, *D. discoideum* AX2 and X22 demonstrated reproducible plaque formation and development on *Klebsiella spp.* virulence negative control strains and reproducible inhibition of plaque formation and development by the *P. aeruginosa* PAO1 virulence positive control strain. A number of test *P. aeruginosa* strains showed a capacity to support growth of both *D. discoideum* AX2 and X22. Comparison of the results with regard to both plaque formation and development (tables 9.12 and 9.13)

showed a strong and significant correlation between *D. discoideum* strains in the results obtained from *P. aeruginosa* co-culture. Discrepant results with regard to the capacity of strains to support the growth of *D. discoideum* were only identified in two isolates (isolates U50 and 147). Whilst the capacity of particular strains to support the growth of *D. discoideum* was highly reproducible, the size and degree of development within the plaques formed varied greatly between runs, with the resultant standard deviations being sizeable. Only two isolates which supported the growth of X22 did not support the growth of AX2, these being isolates U50 and 147 (tables 9.3, 9.4 and figures 9.8, 9.9). Overall, there was a strong correlation between zone size and development scores between both strains of *D. discoideum* employed in testing (table 9.13).

9.3.3 Growth and development of *Dictyostelium discoideum*

A striking result of these two assays was that the capacity to support the growth of *D. discoideum* AX2 was restricted to CF isolates, and only one non-CF isolate (a hospital environmental strain, isolate 147) supported the growth of *D. discoideum* X22. These findings were highly significant ($p=0.016$ for growth of AX2 and $p=0.036$ for growth of X22; table 9.14). No difference was noted in the capacity of CF clonal complex and unique CF strains to support *D. discoideum* growth, and 31% of CF strains tested (AES1, U32a, U3a, U61a, U156 and 82) remained unsupportive of growth (tables 9.3, 9.4 and figures 9.8, 9.9). All six non-CF clinical isolates tested did not support the growth of either strain of *D. discoideum*.

A number of strains supported the growth but not the development of *D. discoideum*. Isolate 115 supported the development of X22, but not AX2, whilst isolate the reverse was observed with regard to isolate 19b. Both isolates had average plaque annular radii below 5 mm. Interestingly, isolate 43

supported luxuriant growth of both AX2 and X22, but did not support their development (tables 9.3, 9.4 and figures 9.8, 9.9).

9.3.4 Urease, antimicrobial resistance and virulence gene scores

Eighteen out of thirty two *P. aeruginosa* isolates (56%) involved in this study produced detectable levels of the urease enzyme on urea agar slopes. The only urease negative non-CF strain was isolate 147. Whilst a number of CF strains were able to elaborate significant amounts of the urease enzyme, 13 out of 19 CF isolates (68%) were urease negative. Discrepant urease testing results were obtained in duplicate tests of isolates U33b and R44a, consequently, a third run was employed and an average result of the three runs was used for statistical analyses. A weak negative correlation existed between urease production and capacity to support the growth and development of *D. discoideum*. A similar negative correlation between urease production and virulence gene score was also noted (table 9.13). No significant correlations between virulence score or antimicrobial resistance score and the growth or development of *D. discoideum* were noted (table 9.13). Despite this, it was observed that all isolates with an antimicrobial resistance score >6 supported the growth of *D. discoideum*.

9.3.5 Effect of antibiotics on virulence

Assays for all clinical isolates and one hospital environment isolate were repeated on SM agar infused with 4 mg/L azithromycin (tables 9.5 and 9.6, figures 9.12-9.15). This concentration of azithromycin inhibited growth of the control strain of *K. aerogenes*, and thus only the *K. pneumoniae* control strain was employed. Similarly, clinical isolates capable of growth on SM agar infused with 6.25 mg/L of ceftazidime (tables 9.7 and 9.8, figures 9.16-9.19) and 25 mg/L of tobramycin (tables 9.9 and 9.10 and figures 9.20-9.23) were tested. The control strain of *K. pneumoniae* was incapable of growth at the

concentration of tobramycin employed, and thus no negative virulence control was employed in the tobramycin assay. Whilst the lack of a negative control negates the validity of the tobramycin assay, the results for test strains did correlate with those found in the other, controlled assays, and have been included and discussed in this thesis. No significant variation in results was observed for isolates grown at sub-MIC concentrations of azithromycin, or excess concentrations of ceftazidime or tobramycin when compared to assays carried out without antibiotics (Tables 9.5-9.10, Figures 9.12-9.19).

| Method | Bacterial growth in trypticase soy broth 24 hrs | <i>D. discoideum</i> growth on SM agar with <i>K. aerogenes</i> 7 days |
|--------------------|---|--|
| 100% EtOH 5 min | 0 | 0 |
| 70°C 5 min | + | 0 |
| Desiccation 24 hrs | 0 | + |

Table 9.1: *D. discoideum* and *K. aerogenes* growth following three methods to eliminate *K. aerogenes* contamination from *Dictyostelium* virulence test paper discs.

| Isolate | Identifier | Age | Place of Isolation | Site of Isolation | Mucoidy | apr | lasB | phzI | phzII | phzM | phzH | phzS | exoS | exoY | exoT | exoU | Vir Score | Car | Tic | Tim | Caz | Fep | Atm | Ipim | Ak | Cn | Tob | Cip | Ab Score | |
|---------|------------|-----|--------------------|-------------------|---------|-----|------|------|-------|------|------|------|------|------|------|------|-----------|-----|-----|-----|-----|-----|-----|------|----|----|-----|-----|----------|----|
| PAO1 | | | | | | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 23 | 25 | 26 | 30 | 31 | 27 | 26 | 25 | 20 | 25 | 36 | 0 | |
| AES1 | | | | | | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 30 | 30 | 24 | 30 | 21 | 30 | 12 | 0 | 0 | 12 | 25 | 4 | |
| U17b | EK | 22 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 0 | 8 | 0 | 10 | 13 | 0 | 13 | 20 | 19 | 10 |
| U19b | EL | 19 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 13 | 19 | 11 | |
| U32b | AJ | 26 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 8 | 0 | 0 | 0 | 32 | 20 | 20 | 24 | 22 | 6 | | |
| U33b | AS | 26 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 13 | 11 | |
| R44a | EQ | 22 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 0 | 13 | 0 | 9 | 11 | 9 | 17 | 17 | 10 | |
| R44b | EQ | 22 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 11 | 0 | 0 | 0 | 0 | 0 | 9 | 0 | 11 | 0 | 16 | 18 | 10 | |
| U50 | ES | 33 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | NEG | NEG | POS | POS | NEG | NEG | NEG | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 12 | 12 | 20 | 20 | 10 | |
| U115 | FC | 25 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | NEG | 9 | 0 | 0 | 12 | 25 | 12 | 30 | 11 | 15 | 12 | 19 | 16 | 8 | |
| U32a | AJ | 23 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 9 | 0 | 0 | 15 | 9 | 20 | 0 | 9 | 10 | 19 | 20 | 10 | |
| U3a | EG | 19 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 26 | 25 | 24 | 25 | 21 | 26 | 30 | 15 | 13 | 19 | 30 | 2 | |
| R29b | EK | 22 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 9 | 0 | 0 | 0 | 0 | 8 | 0 | 9 | 11 | 8 | 20 | 15 | 10 | |
| R43 | EP | 41 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 28 | 26 | 30 | 30 | 22 | 30 | 30 | 17 | 13 | 22 | 28 | 1 | |
| U16a | EJ | 23 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 9 | 0 | 0 | 0 | 15 | 16 | 0 | 0 | 18 | 0 | 0 | 18 | 10 | |
| U61a | EU | 16 | Out-patient | CF Respiratory | muc | POS | POS | NEG | POS | POS | POS | NEG | POS | POS | NEG | NEG | 7 | 20 | 22 | 24 | 30 | 25 | 29 | 25 | 22 | 17 | 20 | 25 | 0 | |
| U156 | FE | 16 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | NEG | POS | POS | POS | 10 | 44 | 37 | 44 | 37 | 26 | 44 | 34 | 18 | 19 | 22 | 32 | 0 | |
| U194a | FJ | 16 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 40 | 40 | 5 | 36 | 38 | 40 | 47 | 0 | 0 | 0 | 19 | 4 | |
| U194b | FJ | 16 | Out-patient | CF Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 48 | 36 | 46 | 44 | 32 | 40 | 44 | 0 | 0 | 10 | 18 | 4 | |
| 82 | CH | 26 | Out-patient | CF Respiratory | muc | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 9 | 25 | 30 | 26 | 28 | 26 | 26 | 30 | 20 | 16 | 20 | 30 | 0 | |
| 20 | AQ | 83 | Other Ward | Urine | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 11 | 11 | 8 | 13 | 21 | 10 | 28 | 24 | 22 | 26 | 33 | 5 | |
| 30 | AY | 43 | NSU | Respiratory | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 10 | 13 | 20 | 28 | 29 | 23 | 22 | 20 | 0 | 13 | 5 | |
| 46 | BK | 76 | Out-patient | Skin/soft tissue | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 22 | 29 | 28 | 31 | 32 | 30 | 27 | 26 | 22 | 27 | 32 | 0 | |
| 67 | BW | 64 | Other Ward | Skin/soft tissue | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 13 | 14 | 15 | 24 | 22 | 20 | 25 | 35 | 6 | |
| 74 | CC | na | Other Ward | Suction Unit | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 0 | 0 | 0 | 32 | 19 | 31 | 24 | 23 | 0 | 0 | 13 | 6 | |
| 77 | CC | 77 | NSU | Catheter Urine | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | 10 | 16 | 25 | 20 | 27 | 20 | 21 | 9 | 21 | 16 | 21 | 29 | 3 | |
| 99 | CU | 70 | Other Ward | Urine | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 7 | 21 | 34 | 25 | 27 | 31 | 26 | 33 | 24 | 20 | 25 | 27 | 0 | |
| 119 | DD | na | ICU | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 9 | 18 | 17 | 16 | 29 | 23 | 21 | 27 | 23 | 20 | 25 | 26 | 1 | |
| 123 | DM | na | NSU | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | 10 | 0 | 0 | 19 | 17 | 17 | 0 | 21 | 10 | 0 | 0 | 9 | 9 | |
| 127 | DO | na | Other Ward | SINK | non | POS | POS | POS | POS | POS | POS | POS | POS | NEG | NEG | NEG | 10 | 13 | 16 | 18 | 26 | 22 | 18 | 29 | 22 | 18 | 23 | 26 | 2 | |
| 147 | DY | na | Community | Pool | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | 10 | 20 | 24 | 22 | 29 | 24 | 26 | 33 | 21 | 16 | 22 | 31 | 0 | |
| 160 | EF | na | Community | Stream | non | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | 10 | 21 | 21 | 19 | 25 | 23 | 23 | 24 | 19 | 16 | 21 | 32 | 0 | |

Table 9.2: Source and site of isolation, patient details, virulence genotype and CLSI antimicrobial resistance patterns of isolates employed in this study. **Isolate colour codes:** White: Control strains; Orange: AES3 Isolates; Pink; 50% similarity to AES3 by RAPD PCR; Blue: Tasmanian CF cluster isolates; Green: Unique isolates. **CLSI antibiogram colour codes:** Green: sensitive; Amber: intermediate; Red: resistant



Figure 9.2: *D. discoideum* disc test development scale 0; complete inhibition of *Dictyostelium* growth.



Figure 9.3: *D. discoideum* disc test development scale 1; plaque formation and aggregation without development



Figure 9.4: *D. discoideum* disc test development scale 2; slug formation



Figure 9.5: *D. discoideum* disc test development scale 3; plaque formation with fruiting bodies

| | AX2 Annular Radius 1 | AX2 Annular Radius 2 | AX2 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (0-3) | (0-3) | (0-3) | (0-3) | (0-3) | (%) | |
| <i>K. aer</i> | 27.0 | 17.0 | 22.0 | 22.0 | 5.00 | 23 | 3.0 | 3.0 | 3.0 | 3.0 | 0.0 | 0 | |
| <i>K. pne</i> | 11.0 | 11.5 | 10.0 | 10.8 | 0.76 | 7 | 3.0 | 3.0 | 3.0 | 3.0 | 0.0 | 0 | |
| PA01 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| AES 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U17b* | 7.0 | 4.0 | 13.0 | 8.0 | 4.58 | 57 | 2.0 | 1.0 | 2.0 | 1.7 | 0.6 | 35 | |
| U19b* | 3.0 | 2.0 | 10.0 | 5.0 | 4.36 | 87 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 | 0 | |
| U32b* | 1.0 | 1.0 | 2.0 | 1.3 | 0.58 | 43 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U33b* | 6.5 | 4.0 | 13.0 | 7.8 | 4.65 | 59 | 2.0 | 2.0 | 2.0 | 2.0 | 0.0 | 0 | |
| R44a* | 0.5 | 0.5 | 1.0 | 0.7 | 0.29 | 43 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| R44b* | 8.0 | 4.0 | 1.0 | 4.3 | 3.51 | 81 | 2.0 | 1.0 | 0.0 | 1.0 | 1.0 | 100 | |
| U50* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U115* | 1.0 | 1.0 | 0.0 | 0.7 | 0.58 | 87 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U32a† | 0.0 | 0.0 | 2.0 | 0.7 | 1.15 | 173 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U3a‡ | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U29b‡ | 7.0 | 5.0 | 6.0 | 6.0 | 1.00 | 17 | 2.0 | 1.0 | 1.0 | 1.3 | 0.6 | 43 | |
| R43‡ | 6.0 | 6.0 | 16.0 | 9.3 | 5.77 | 62 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U16a | 5.0 | 4.0 | 1.0 | 3.3 | 2.08 | 62 | 2.0 | 0.0 | 0.0 | 0.7 | 1.2 | 173 | |
| U61a | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U156 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U194a | 5.0 | 4.0 | 6.0 | 5.0 | 1.00 | 20 | 3.0 | 2.0 | 3.0 | 2.7 | 0.6 | 22 | |
| U194b | 8.0 | 5.0 | 13.0 | 8.7 | 4.04 | 47 | 2.0 | 1.0 | 2.0 | 1.7 | 0.6 | 35 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 30 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 46 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 67 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 77 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 119 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 123 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 127 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 147 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 160 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 1.16 | 26 | Overall Mean SD | | | | | 0.13 | 12 |
| Mean SD For AX2 Supportive Isolates | | | | | 2.62 | 58 | Mean SD For AX2 Supportive Isolates | | | | | 0.30 | 27 |

Table 9.3: *Dictyostelium discoideum* AX2 virulence model results (SM agar, no antibiotics)

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

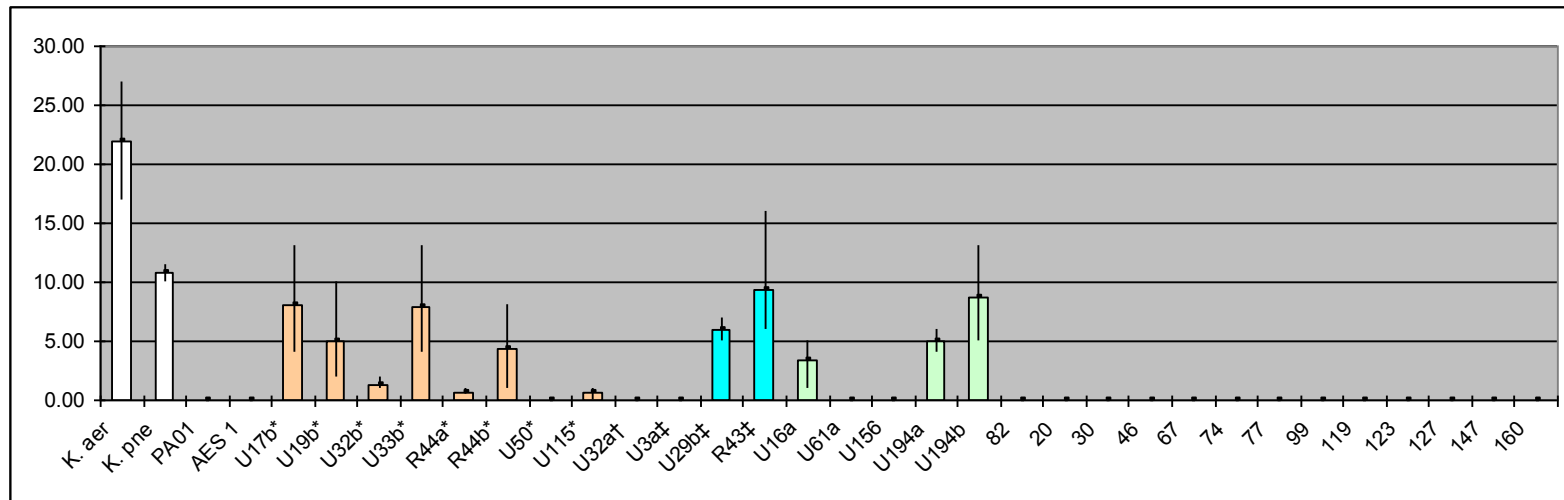


Figure 9.8: *Dictyostelium discoideum* AX2 virulence model annular radius of zones results (SM agar).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

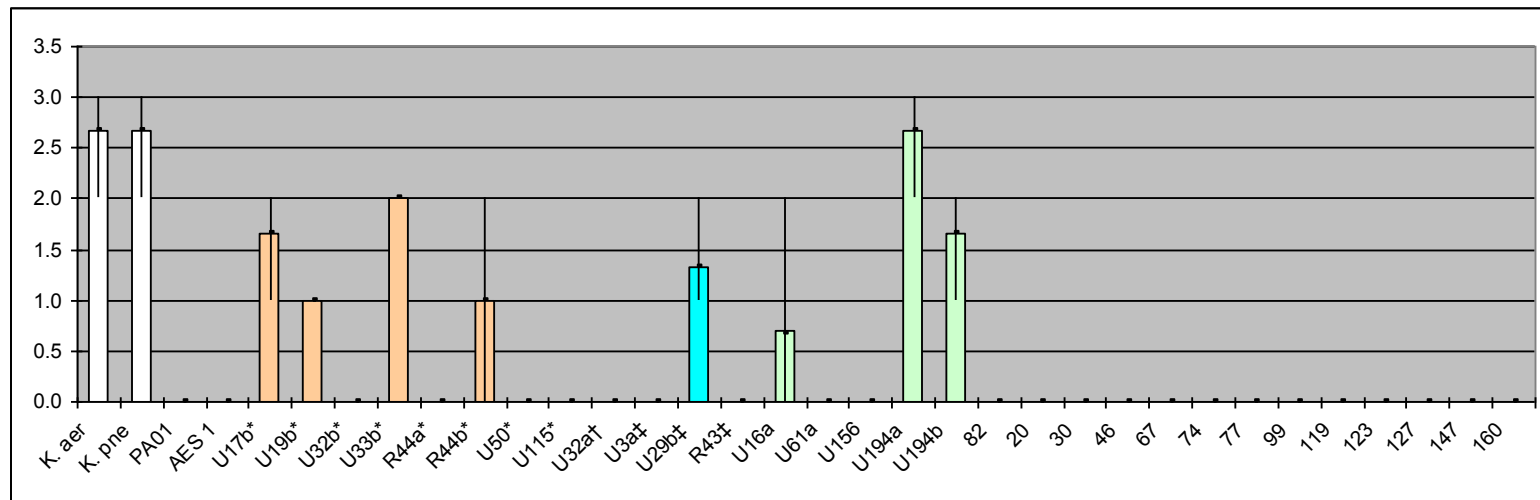


Figure 9.9: *Dictyostelium discoideum* AX2 virulence model development of plaques results (SM agar).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | X22 | X22 | X22 | Mean | Standard | Standard | Develop- | Develop- | Develop- | Develop- | Standard | Standard | |
|-------------------------------------|----------|----------|----------|---------|-----------|-----------|-------------------------------------|----------|----------|----------|-----------|-----------|----|
| | Annular | Annular | Annular | Annular | Deviation | Deviation | ment | ment | ment | ment | Deviation | Deviation | |
| Isolate | Radius 1 | Radius 2 | Radius 3 | Radius | (mm) | (%) | Scale 1 | Scale 2 | Scale 3 | Scale | (mm) | (%) | |
| | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (1-3) | (1-3) | (1-3) | Average | | | |
| | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (1-3) | (1-3) | (1-3) | (1-3) | (mm) | (%) | |
| K. aer | 10.0 | 3.0 | 12.0 | 8.3 | 4.73 | 57 | 3.0 | 2.0 | 3.0 | 2.7 | 0.51 | 19 | |
| K. pne | 13.0 | 4.0 | 13.0 | 10.0 | 5.20 | 52 | 3.0 | 2.0 | 3.0 | 2.7 | 0.51 | 19 | |
| PA01 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| AES 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U17b* | 7.0 | 4.0 | 5.0 | 5.3 | 1.53 | 29 | 2.0 | 1.0 | 2.0 | 1.7 | 0.51 | 31 | |
| U19b* | 4.0 | 4.0 | 2.0 | 3.3 | 1.15 | 35 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U32b* | 1.0 | 1.0 | 2.0 | 1.3 | 0.58 | 43 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U33b* | 7.0 | 4.0 | 3.0 | 4.7 | 2.08 | 45 | 2.0 | 2.0 | 2.0 | 2.0 | 0.00 | 0 | |
| R44a* | 0.5 | 0.5 | 1.0 | 0.7 | 0.29 | 43 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| R44b* | 4.0 | 6.0 | 12.0 | 7.3 | 4.16 | 57 | 2.0 | 1.0 | 2.0 | 1.7 | 0.51 | 31 | |
| U50* | 1.0 | 1.0 | 1.0 | 1.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U115* | 0.0 | 1.5 | 1.5 | 1.0 | 0.87 | 87 | 0.0 | 1.0 | 0.0 | 0.3 | 0.51 | 153 | |
| U32a† | 0.5 | 0.0 | 1.0 | 0.5 | 0.50 | 100 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U3a‡ | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U29b‡ | 7.0 | 5.0 | 13.0 | 8.3 | 4.16 | 50 | 2.0 | 1.0 | 2.0 | 1.7 | 0.51 | 31 | |
| R43‡ | 7.0 | 7.0 | 20.0 | 11.3 | 7.51 | 66 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U16a | 5.0 | 4.0 | 11.0 | 6.7 | 3.79 | 57 | 1.0 | 1.0 | 2.0 | 1.3 | 0.51 | 38 | |
| U61a | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U156 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| U194a | 6.0 | 4.0 | 8.0 | 6.0 | 2.00 | 33 | 3.0 | 1.0 | 3.0 | 2.3 | 1.02 | 44 | |
| U194b | 3.0 | 2.0 | 5.0 | 3.3 | 1.53 | 46 | 2.0 | 1.0 | 2.0 | 1.7 | 0.51 | 31 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 30 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 46 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 67 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 77 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 119 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 123 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 127 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| 147 | 6.0 | 5.0 | 4.0 | 5.0 | 1.00 | 20 | 2.0 | 2.0 | 2.0 | 2.0 | 0.00 | 0 | |
| 160 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | |
| Overall Mean SD | | | | | 1.21 | 24 | Overall Mean SD | | | | | 0.15 | 12 |
| Mean SD For X22 Supportive Isolates | | | | | 2.57 | 51 | Mean SD For X22 Supportive Isolates | | | | | 0.32 | 25 |

Table 9.4: *Dictyostelium discoideum* X22 virulence model results (SM agar, no antibiotics)

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

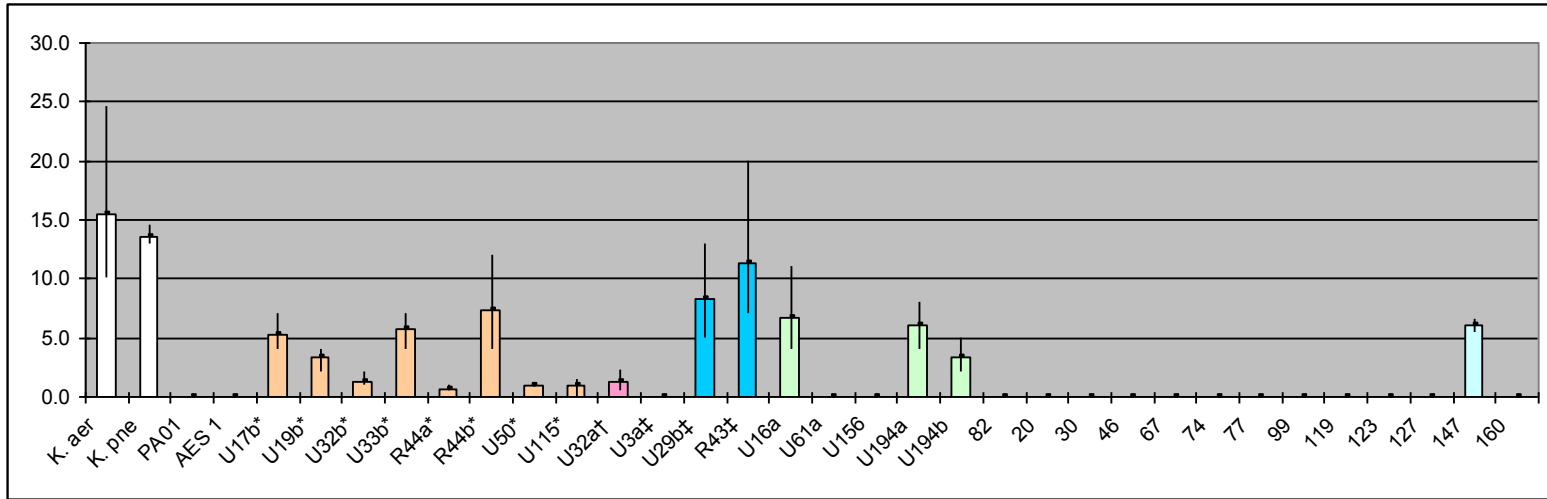


Figure 9.10: *Dictyostelium discoideum* X22 virulence model annular radius of plaques results (SM agar).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

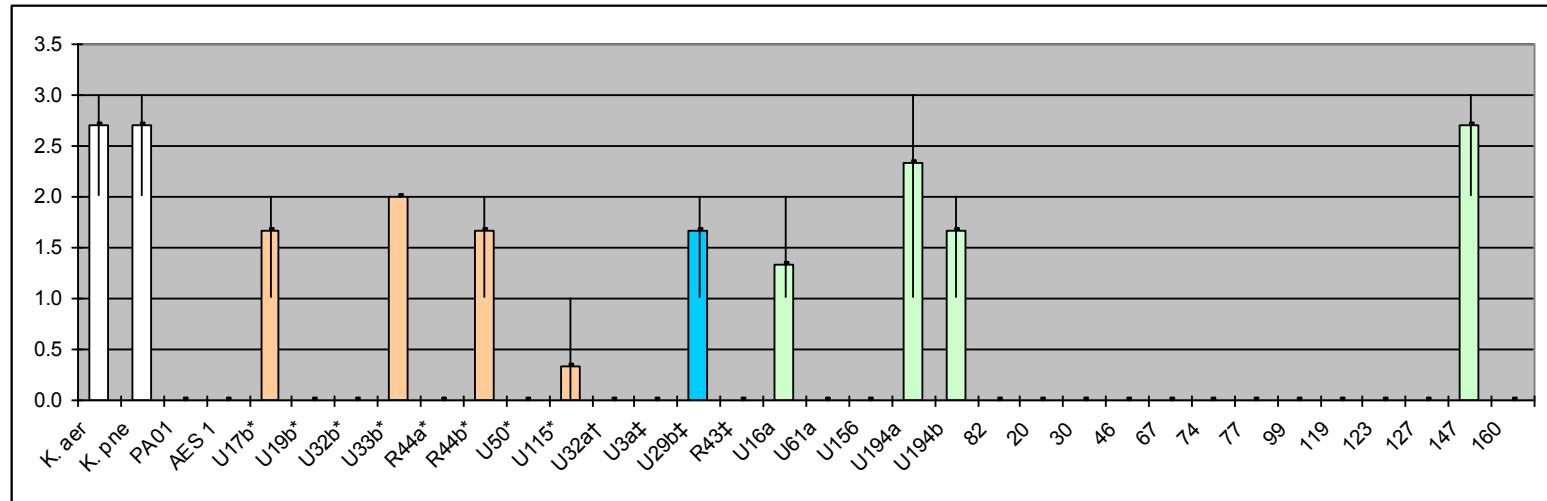


Figure 9.11: *Dictyostelium discoideum* X22 virulence model development within plaques results (SM agar).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | AX2 Annular Radius 1 | AX2 Annular Radius 2 | AX2 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (0-3) | (0-3) | (0-3) | (0-3) | (0-3) | (%) | |
| <i>K. pne</i> | 4.0 | 2.0 | 9.0 | 5.0 | 3.61 | 72 | 3.0 | 2.0 | 3.0 | 3.0 | 0.6 | 19 | |
| PA01 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| AES 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U17b* | 7.0 | 4.0 | 12.0 | 7.7 | 4.04 | 53 | 2.0 | 1.0 | 2.0 | 1.7 | 0.6 | 35 | |
| U19b* | 5.0 | 3.0 | 7.0 | 5.0 | 2.00 | 40 | 2.0 | 1.0 | 1.0 | 1.0 | 0.6 | 58 | |
| U32b* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U33b* | 7.0 | 5.0 | 13.0 | 8.3 | 4.16 | 50 | 2.0 | 2.0 | 2.0 | 2.0 | 0.0 | 0 | |
| R44a* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| R44b* | 6.0 | 4.0 | 0.0 | 3.3 | 3.06 | 92 | 2.0 | 1.0 | 0.0 | 1.0 | 1.0 | 100 | |
| U50* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U115* | 0.5 | 0.5 | 0.0 | 0.3 | 0.29 | 87 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U32a† | 0.0 | 0.0 | 2.0 | 0.7 | 1.15 | 173 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U3a‡ | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U29b‡ | 6.0 | 4.0 | 5.0 | 5.0 | 1.00 | 20 | 2.0 | 1.0 | 1.0 | 1.3 | 0.6 | 43 | |
| R43‡ | 7.0 | 7.0 | 6.0 | 6.7 | 0.58 | 9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U16a | 8.0 | 5.0 | 6.0 | 6.3 | 1.53 | 24 | 2.0 | 1.0 | 2.0 | 0.7 | 0.6 | 87 | |
| U61a | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U156 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U194a | 4.0 | 2.0 | 4.5 | 3.5 | 1.32 | 38 | 3.0 | 2.0 | 3.0 | 2.7 | 0.6 | 22 | |
| U194b | 10.0 | 7.0 | 11.0 | 9.3 | 2.08 | 22 | 2.0 | 3.0 | 2.0 | 1.7 | 0.6 | 35 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 30 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 46 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 67 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 77 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 0.89 | 24 | Overall Mean SD | | | | | 0.18 | 14 |
| Mean SD For AX2 Supportive Isolates | | | | | 2.07 | 42 | Mean SD For AX2 Supportive Isolates | | | | | 0.42 | 33 |

Table 9.5: *Dictyostelium discoideum* AX2 with azithromycin virulence model results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

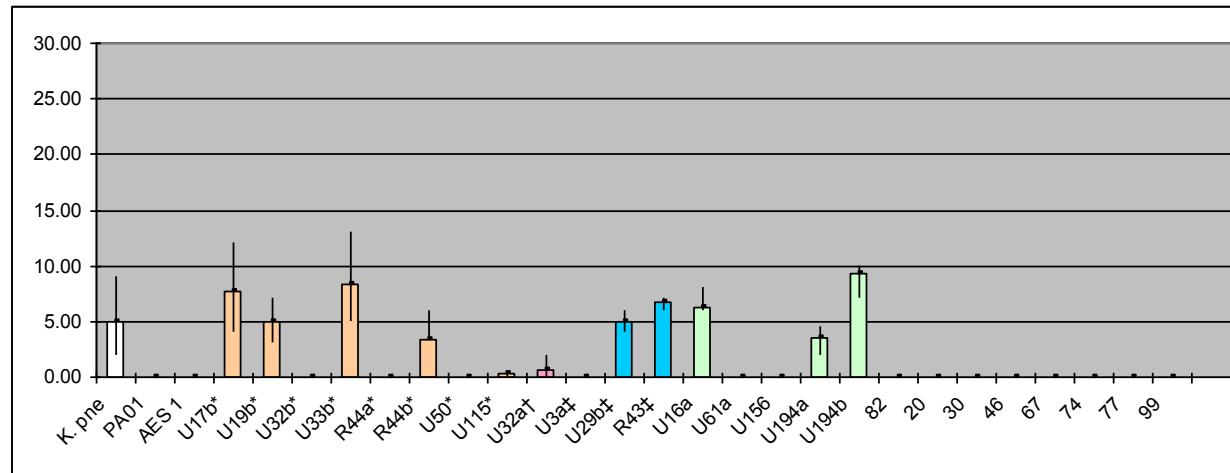


Figure 9.12: *Dictyostelium discoideum* AX2 virulence model with azithromycin annular radius of plaques results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

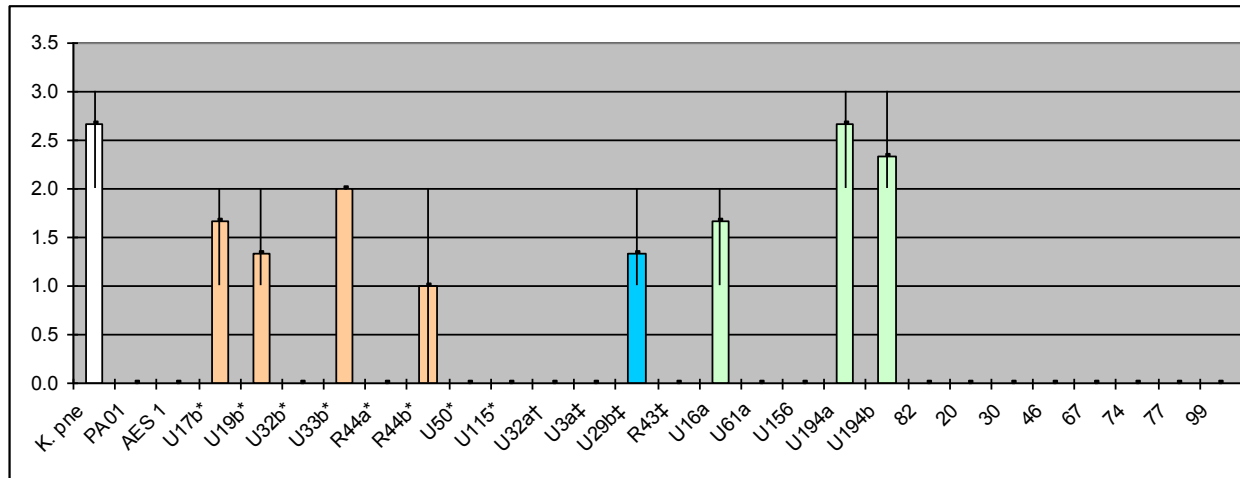


Figure 9.13: *Dictyostelium discoideum* AX2 virulence model with azithromycin development within plaques results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | X22 Annular Radius 1 | X22 Annular Radius 2 | X22 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (1-3) | (1-3) | (1-3) | (1-3) | (mm) | (%) | |
| <i>K. pne</i> | 4.0 | 1.0 | 4.0 | 3.0 | 1.73 | 58 | 3.0 | 2.0 | 3.0 | 3.0 | 0.6 | 19 | |
| PA01 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| AES 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U17b* | 6.0 | 4.0 | 5.0 | 5.0 | 1.00 | 20 | 2.0 | 1.0 | 1.0 | 1.7 | 0.6 | 35 | |
| U19b* | 4.0 | 3.0 | 1.0 | 2.7 | 1.53 | 57 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0 | |
| U32b* | 0.0 | 0.0 | 0.5 | 0.2 | 0.29 | 173 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U33b* | 8.0 | 4.0 | 1.0 | 4.3 | 3.51 | 81 | 2.0 | 2.0 | 2.0 | 2.0 | 0.0 | 0 | |
| R44a* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| R44b* | 6.0 | 4.0 | 5.0 | 5.0 | 1.00 | 20 | 2.0 | 1.0 | 2.0 | 1.0 | 0.6 | 58 | |
| U50* | 0.5 | 0.5 | 0.5 | 0.5 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U115* | 0.5 | 0.5 | 1.0 | 0.7 | 0.29 | 43 | 0.0 | 1.0 | 0.0 | 0.0 | 0.6 | 0 | |
| U32a† | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U3a‡ | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U29b‡ | 5.0 | 4.0 | 9.0 | 6.0 | 2.65 | 44 | 2.0 | 0.0 | 1.0 | 1.3 | 1.0 | 75 | |
| R43‡ | 8.0 | 8.0 | 5.0 | 7.0 | 1.73 | 25 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U16a | 1.5 | 0.5 | 6.0 | 2.7 | 2.93 | 110 | 1.0 | 0.0 | 1.0 | 0.7 | 0.6 | 87 | |
| U61a | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U156 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U194a | 4.0 | 2.0 | 2.0 | 2.7 | 1.15 | 43 | 3.0 | 1.0 | 2.0 | 2.7 | 1.0 | 38 | |
| U194b | 1.0 | 1.0 | 7.0 | 3.0 | 3.46 | 115 | 1.0 | 1.0 | 3.0 | 1.7 | 1.2 | 69 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 30 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 46 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 67 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 77 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 0.76 | 28 | Overall Mean SD | | | | | 0.22 | 14 |
| Mean SD For X22 Supportive Isolates | | | | | 1.77 | 66 | Mean SD For AX2 Supportive Isolates | | | | | 0.50 | 32 |

Table 9.6: *Dictyostelium discoideum* X22 with azithromycin virulence model results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

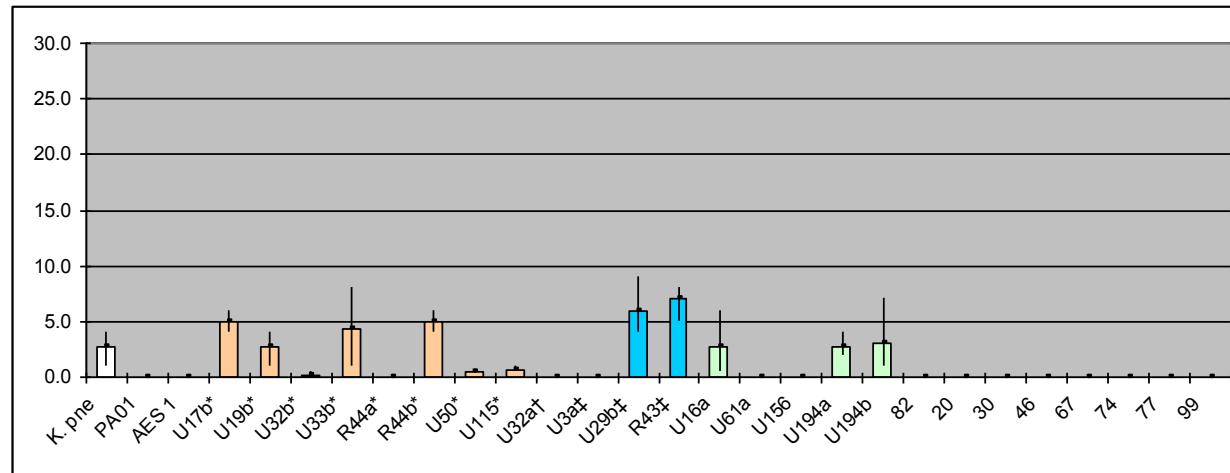


Figure 9.14: *Dictyostelium discoideum* X22 virulence model with azithromycin annular radius of plaques results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

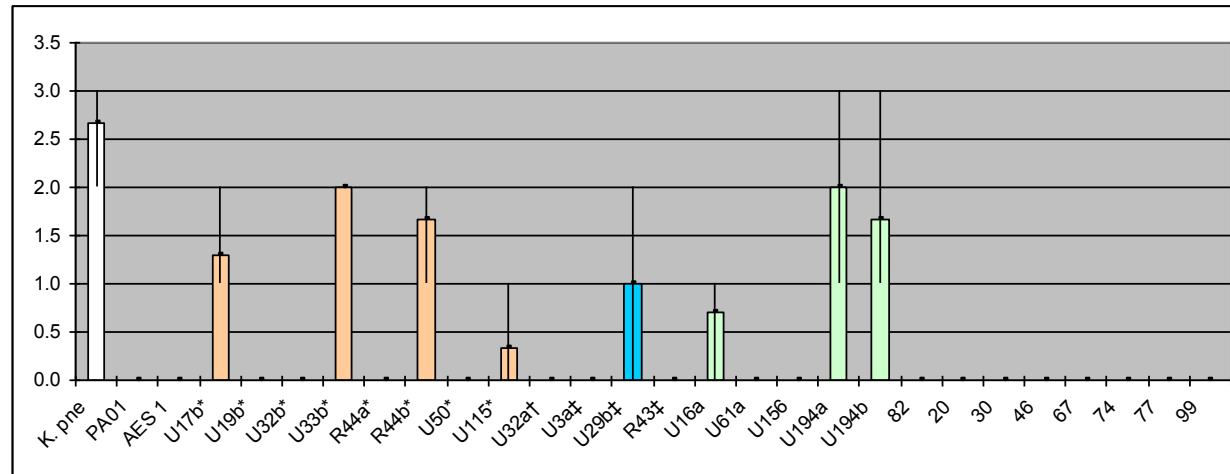


Figure 9.15: *Dictyostelium discoideum* X22 virulence model with azithromycin development within plaques results (SM agar plus 4 mg/L azithromycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | AX2 Annular Radius 1 | AX2 Annular Radius 2 | AX2 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (0-3) | (0-3) | (0-3) | (0-3) | (0-3) | (%) | |
| K. pne | 9.0 | 12.0 | 12.0 | 11.0 | 1.73 | 16 | 3.0 | 3.0 | 3.0 | 3.0 | 0.0 | 0 | |
| U17b* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 1.7 | 0.0 | 0 | |
| U19b* | 4.0 | 2.0 | 1.0 | 2.3 | 1.53 | 65 | 2.0 | 1.0 | 0.0 | 1.0 | 1.0 | 100 | |
| U32b* | 1.0 | 1.0 | 1.0 | 1.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U33b* | 8.0 | 5.0 | 13.0 | 8.7 | 4.04 | 47 | 2.0 | 2.0 | 2.0 | 2.0 | 0.0 | 0 | |
| R44a* | 1.0 | 0.5 | 1.0 | 0.8 | 0.29 | 35 | 1.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0 | |
| U50* | 1.0 | 1.0 | 0.0 | 0.7 | 0.58 | 87 | 1.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0 | |
| U115* | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U32a† | 1.0 | 1.0 | 1.0 | 1.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U194b | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 1.7 | 0.0 | 0 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| | | | | | | | | | | | | | |
| Overall Mean SD | | | | | 0.74 | 23 | Overall Mean SD | | | | | 0.20 | 9 |
| Mean SD For AX2 Supportive Isolates | | | | | 1.63 | 50 | Mean SD For AX2 Supportive Isolates | | | | | 0.43 | 20 |

Table 9.7: *Dictyostelium discoideum* AX2 with ceftazidime virulence model results (SM agar plus 6.25 mg/L ceftazidime).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | X22 Annular Radius 1 | X22 Annular Radius 2 | X22 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (1-3) | (1-3) | (1-3) | (1-3) | (mm) | (%) | |
| K. pne | 9.0 | 5.0 | 20.0 | 11.3 | 7.77 | 69 | 3.0 | 2.0 | 3.0 | 3.0 | 0.6 | 19 | |
| U17b* | 3.0 | 3.0 | 3.0 | 3.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 1.7 | 0.0 | 0 | |
| U19b* | 6.0 | 1.0 | 4.0 | 3.7 | 2.52 | 69 | 1.0 | 0.0 | 1.0 | 1.0 | 0.6 | 58 | |
| U32b* | 1.0 | 1.0 | 2.0 | 1.3 | 0.58 | 43 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U33b* | 9.0 | 6.0 | 4.0 | 6.3 | 2.52 | 40 | 2.0 | 1.0 | 2.0 | 2.0 | 0.6 | 29 | |
| R44a* | 1.0 | 0.5 | 4.5 | 2.0 | 2.18 | 109 | 1.0 | 0.0 | 1.0 | 0.0 | 0.6 | 0 | |
| U50* | 2.0 | 2.0 | 3.0 | 2.3 | 0.58 | 25 | 1.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0 | |
| U115* | 0.0 | 0.0 | 1.0 | 0.3 | 0.58 | 173 | 0.0 | 1.0 | 0.0 | 0.0 | 0.6 | 0 | |
| U32a† | 1.0 | 1.0 | 4.0 | 2.0 | 1.73 | 87 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| U194b | 4.0 | 0.5 | 7.0 | 3.8 | 3.25 | 85 | 2.0 | 1.0 | 3.0 | 1.7 | 1.0 | 60 | |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 1.97 | 64 | Overall Mean SD | | | | | 0.35 | 15 |
| Mean SD For AX2 Supportive Isolates | | | | | 3.11 | 62 | Mean SD For AX2 Supportive Isolates | | | | | 0.46 | 21 |

Table 9.8: *Dictyostelium discoideum* X22 with ceftazidime virulence model results (SM agar plus 6.25 mg/L ceftazidime).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

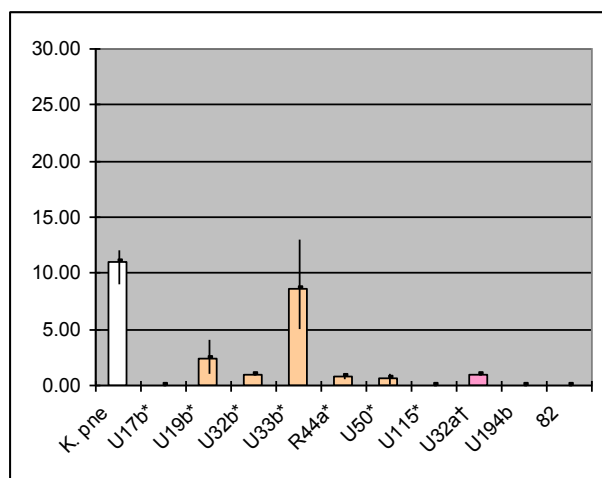


Figure 9.16: *Dictyostelium discoideum* AX2 virulence model with ceftazidime annular radius of plaques results (SM agar plus 6.25 mg/L ceftazidime).

*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

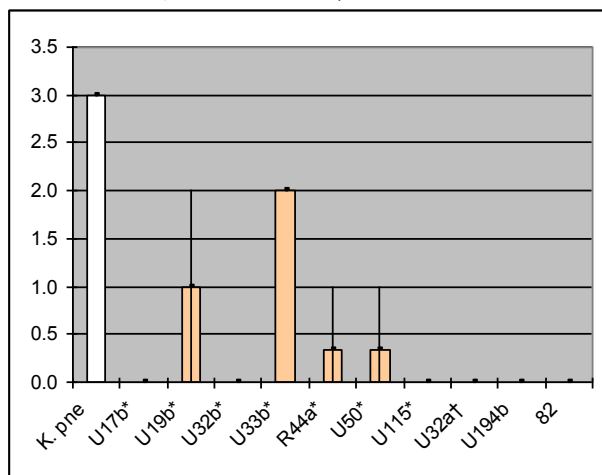


Figure 9.17: *Dictyostelium discoideum* AX2 virulence model with ceftazidime development within plaques results (SM agar plus 6.25 mg/L ceftazidime).

*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

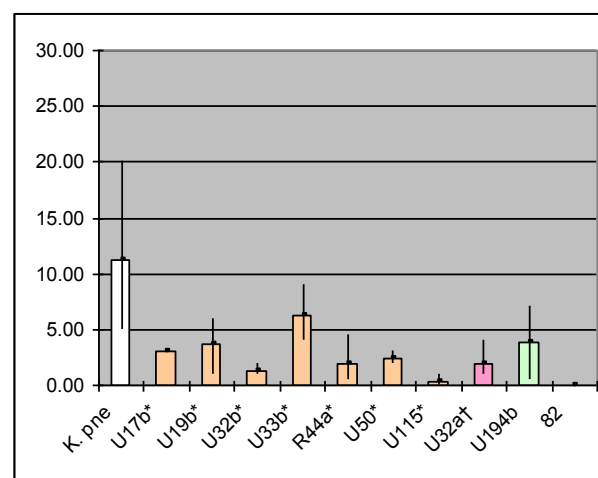


Figure 9.18: *Dictyostelium discoideum* X22 virulence model with ceftazidime annular radius of plaques results. (SM agar plus 6.25 mg/L ceftazidime).

*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

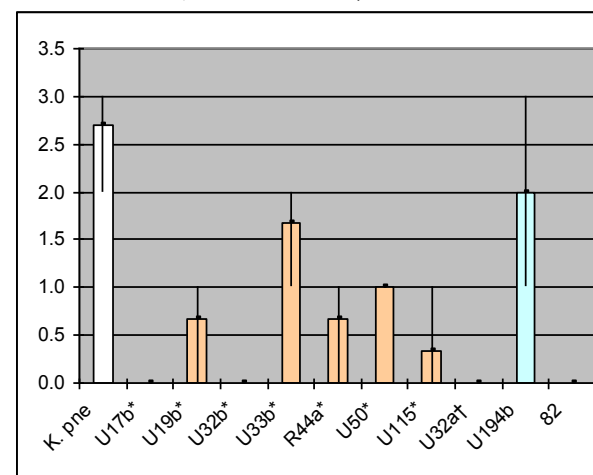


Figure 9.19: *Dictyostelium discoideum* X22 virulence model with ceftazidime development within plaques results. (SM agar plus 6.25 mg/L ceftazidime).

*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

| | AX2 Annular Radius 1 | AX2 Annular Radius 2 | AX2 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average (0-3) | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|---|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (0-3) | (0-3) | (0-3) | (0-3) | (0-3) | (%) | |
| U33b* | 6.0 | 4.0 | 11.0 | 7.0 | 3.61 | 52 | 2.0 | 2.0 | 1.0 | 2.0 | 0.6 | 29 | |
| U16a | 3.0 | 2.0 | 6.0 | 3.7 | 2.08 | 57 | 2.0 | 1.0 | 0.0 | 0.7 | 1.0 | 150 | |
| U194a | 3.0 | 1.0 | 7.0 | 3.7 | 3.06 | 83 | 2.0 | 2.0 | 0.0 | 2.7 | 1.2 | 43 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 1.75 | 38 | Overall Mean SD | | | | | 0.55 | 44 |
| Mean SD For AX2 Supportive Isolates | | | | | 2.91 | 64 | Mean SD For AX2 Supportive Isolates | | | | | 0.91 | 74 |

Table 9.9: *Dictyostelium discoideum* AX2 with tobramycin virulence model results (SM agar plus 25 mg/L tobramycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| | X22 Annular Radius 1 | X22 Annular Radius 2 | X22 Annular Radius 3 | Mean Annular Radius | Standard Deviation | Standard Deviation | Develop- ment Scale 1 | Develop- ment Scale 2 | Develop- ment Scale 3 | Develop- ment Scale Average (1-3) | Standard Deviation | Standard Deviation | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|-----------------------|-----------------------|-------------------------------------|-----------------------------|-----------------------------|---|-----------------------|-----------------------|----|
| Isolate | (mm) | (mm) | (mm) | (mm) | (mm) | (%) | (1-3) | (1-3) | (1-3) | | (mm) | (%) | |
| U33b* | 8.5 | 6.0 | 5.0 | 6.5 | 1.80 | 28 | 1.0 | 1.0 | 1.0 | 2.0 | 0.0 | 0 | |
| U16a | 5.0 | 4.0 | 2.0 | 3.7 | 1.53 | 42 | 1.0 | 0.0 | 1.0 | 0.7 | 0.6 | 87 | |
| U194a | 3.0 | 1.0 | 2.0 | 2.0 | 1.00 | 50 | 2.0 | 2.0 | 2.0 | 2.7 | 0.0 | 0 | |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | |
| Overall Mean SD | | | | | 0.87 | 24 | Overall Mean SD | | | | | 0.12 | 17 |
| Mean SD For AX2 Supportive Isolates | | | | | 1.44 | 40 | Mean SD For AX2 Supportive Isolates | | | | | 0.19 | 29 |

Table 9.10: *Dictyostelium discoideum* X22 with tobramycin virulence model results (SM agar plus 25 mg/L tobramycin).

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

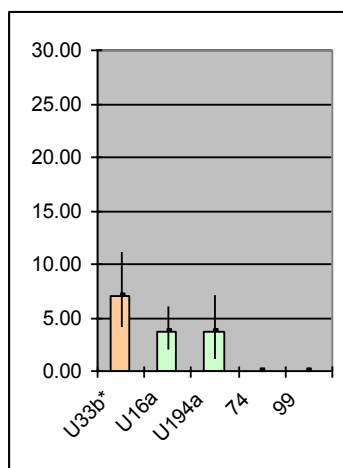


Figure 9.20: *Dictyostelium discoideum* AX2 virulence model with tobramycin annular radius of plaques results (SM agar plus 0.25 mg/L tobramycin).
*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

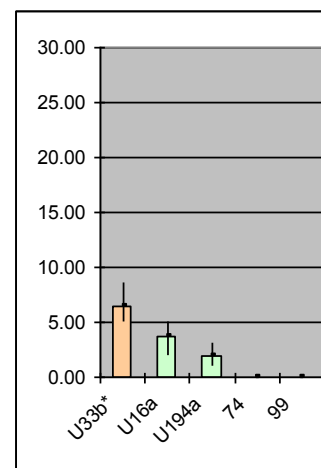


Figure 9.22: *Dictyostelium discoideum* X22 virulence model with tobramycin annular radius of plaques results (SM agar plus 25 mg/L tobramycin).
*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

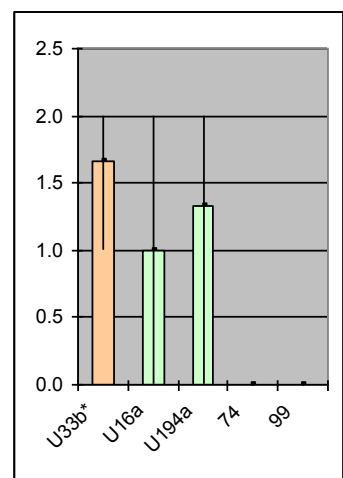


Figure 9.21: *Dictyostelium discoideum* AX2 virulence model with tobramycin development within plaques results (SM agar plus 25 mg/L tobramycin).
*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate

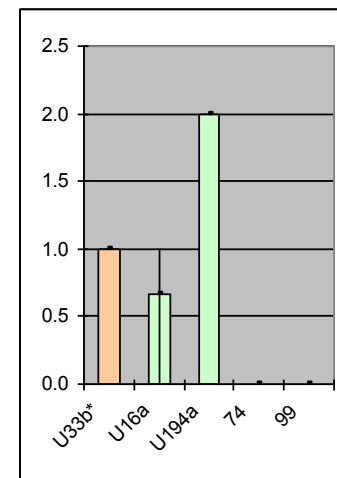


Figure 9.23: *Dictyostelium discoideum* X22 virulence model with tobramycin development within plaques results (SM agar plus 25 mg/L tobramycin).
*:AES3 isolate; † 50% RAPD PCR; ‡ Tasmanian CF cluster isolate



Figure 9.24: Urease reactions at 20°C without light over 7 days of selected isolates on Urea agar slopes. (L-R); *P. vulgaris* ATCC 3427, *E. coli* ATCC 10418, 44a, 33b, 67, 74.

| Isolate | <i>P. vulgaris</i> ATCC 8427 | <i>E. coli</i> ATCC 10418 | <i>K. aer</i> | <i>K. pne</i> | PA01 | AES 1 | U17b* | U19b* | U32b* | U33b* | R44a* | R44b* | U50* | U115* | U32a† | U3a‡ | U29b‡ | R43‡ |
|----------------------|---------------------------------|------------------------------|---------------|---------------|-------|-------|-------|-------|-------|-------|-------|-------|------|-------|-------|------|-------|------|
| Urease Result | POS | NEG | NEG | POS | POS | POS | NEG | NEG | NEG | NEGδ | NEGδ | NEG | NEG | NEG | NEG | POS | NEG | POS |
| Qualitative Score | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Isolate | U16a | U61a | U156 | U194a | U194b | 82 | 20 | 30 | 46 | 67 | 74 | 77 | 99 | 119 | 123 | 127 | 147 | 160 |
| Urease Result | NEG | POS | POS | NEG | NEG | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | POS | NEG | POS |
| Qualitative Score | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |

Table 9.11: Results of *P. aeruginosa* urease assays carried out at 20°C without light over 7 days.

POS: Strong colour change, NEG: No colour change

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate.

| Isolate | <i>D. discoideum</i> AX2 | | <i>D. discoideum</i> X22 | | Other Assays | | |
|---------------|--------------------------|---------------------------------|--------------------------|---------------------------------|--------------------|-----------------------------|------------------------------------|
| | Mean Annular Radius (mm) | Development Scale Average (0-3) | Mean Annular Radius (mm) | Development Scale Average (0-3) | Urease Score (0-2) | Virulence Gene Score (0-11) | Antibiotic Resistance Score (0-11) |
| <i>K. aer</i> | 22.0 | 3.0 | 8.3 | 2.7 | 0 | nd | nd |
| <i>K. pne</i> | 10.8 | 3.0 | 10.0 | 2.7 | 1 | nd | nd |
| PA01 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 0 |
| AES 1 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 4 |
| U17b* | 8.0 | 1.7 | 5.3 | 1.7 | 0 | 10 | 10 |
| U19b* | 5.0 | 1.0 | 3.3 | 0.0 | 0 | 10 | 11 |
| U32b* | 1.3 | 0.0 | 1.3 | 0.0 | 0 | 10 | 6 |
| U33b* | 7.8 | 2.0 | 4.7 | 2.0 | 0 | 10 | 11 |
| R44a* | 0.7 | 0.0 | 0.7 | 0.0 | 0 | 10 | 10 |
| R44b* | 4.3 | 1.0 | 7.3 | 1.7 | 0 | 11 | 10 |
| U50* | 0.0 | 0.0 | 1.0 | 0.0 | 0 | 6 | 10 |
| U115* | 0.7 | 0.0 | 1.0 | 0.3 | 0 | 9 | 8 |
| U32a† | 0.7 | 0.0 | 0.5 | 0.0 | 0 | 10 | 10 |
| U3a‡ | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 2 |
| U29b‡ | 6.0 | 1.3 | 8.3 | 1.7 | 0 | 9 | 10 |
| R43‡ | 9.3 | 0.0 | 11.3 | 0.0 | 1 | 10 | 1 |
| U16a | 3.3 | 0.7 | 6.7 | 1.3 | 0 | 9 | 10 |
| U61a | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 7 | 0 |
| U156 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 0 |
| U194a | 5.0 | 2.7 | 6.0 | 2.3 | 0 | 10 | 4 |
| U194b | 8.7 | 1.7 | 3.3 | 1.7 | 0 | 10 | 4 |
| 82 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 9 | 0 |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 5 |
| 30 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 5 |
| 46 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 0 |
| 67 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 6 |
| 74 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 6 |
| 77 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 3 |
| 99 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 7 | 0 |
| 119 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 9 | 1 |
| 123 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 9 |
| 127 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 2 |
| 147 | 0.0 | 0.0 | 5.0 | 2.0 | 0 | 10 | 0 |
| 160 | 0.0 | 0.0 | 0.0 | 0.0 | 1 | 10 | 0 |

Table 9.12: Summary of results in previous tables for comparison.

*: AES3 isolate; † 50% similarity to AES3 by RAPD PCR; ‡ Tasmanian CF cluster isolate

| Assays Compared | correlation co-efficient (r value) | correlation strength (r ² value) | t-test (p value) |
|---|--|---|---------------------|
| AX2 plaque annular radius and AX2 development score | 0.775 | 0.601 | 0.010 |
| X22 plaque annular radius and X22 development score | 0.690 | 0.476 | na |
| AX2 plaque annular radius and X22 plaque annular radius without antibiotics | 0.823 | 0.678 | 0.840 |
| AX2 development score and X22 development score without antibiotics | 0.837 | 0.701 | na |
| AX2 plaque annular radius with and without azithromycin | 0.957 | 0.916 | 0.843 |
| AX2 development score with and without azithromycin | 1.000 | 1.000 | 1.000 |
| X22 plaque annular radius with and without azithromycin | 0.957 | 0.916 | 0.304 |
| X22 development score with and without azithromycin | 0.932 | 0.868 | 0.908 |
| AX2 plaque annular radius with and without ceftazidime | 0.631 | 0.398 | 0.343 |
| AX2 development score with and without ceftazidime | 1.000 | 1.000 | 1.000 |
| X22 plaque annular radius with and without ceftazidime | 0.927 | 0.860 | 0.734 |
| X22 development score with and without ceftazidime | 0.826 | 0.682 | 0.734 |
| AX2 plaque annular radius with and without tobramycin | 0.984 | 0.969 | 0.859 |
| AX2 development score with and without tobramycin | 1.000 | 1.000 | 1.000 |
| X22 plaque annular radius with and without tobramycin | 0.691 | 0.477 | 0.602 |
| X22 development score with and without tobramycin | 0.955 | 0.912 | 0.930 |
| Urease and AX2 plaque annular radius | -0.520 | 0.270 | na |
| Urease and AX2 development score | -0.594 | 0.353 | na |
| Urease and X22 plaque annular radius | -0.530 | 0.281 | na |
| Urease and X22 development score | -0.662 | 0.438 | na |
| Virulence gene score and AX2 plaque annular radius | 0.211 | 0.045 | na |
| Virulence gene score and AX2 development score | 0.180 | 0.033 | na |
| Virulence gene score and X22 plaque annular radius | 0.160 | 0.026 | na |
| Virulence gene score and X22 development score | 0.184 | 0.034 | na |
| Antibiotic resistance score and AX2 plaque annular radius | 0.377 | 0.142 | na |
| Antibiotic resistance score and AX2 development score | 0.404 | 0.163 | na |
| Antibiotic resistance score and X22 plaque annular radius | 0.316 | 0.100 | na |
| Antibiotic resistance score and X22 development score | 0.308 | 0.095 | na |
| Urease and antibiotic resistance score | -0.008 | 0.000 | na |
| Urease and virulence gene score | -0.693 | 0.481 | na |

Table 9.13: Statistical correlation (r value), strength of correlation (r² value) and significance of correlation (two tailed t-test; samples of unequal variance) of all assays described in this chapter.

| Growth on <i>Dictyostelium</i> <i>discoideum</i> | CF Respiratory (n=19) | Non-CF Clinical (n=6) | Environmental (n=6) | χ ² test p value |
|--|--------------------------|--------------------------|------------------------|--------------------------------|
| AX2 | 13 | 0 | 0 | 0.016 |
| X22 | 14 | 0 | 1 | 0.036 |

Table 9.14: Significance of capacity of *P. aeruginosa* strains from different sites of isolation to support the growth of two strains of *D. discoideum*.

9.4 Discussion

9.4.1 The modified *Dictyostelium discoideum* virulence model

This is the first study to identify wild-type isolates of *P. aeruginosa* capable of supporting the growth of *D. discoideum*. Previously conducted studies into the virulence of a very small number of *P. aeruginosa* strains using *D. discoideum* DH1-10 and AX3 identified no such strains (Cosson, Zulianello *et al.* 2002; Pukatzki, Kessin *et al.* 2002; Alibaud, Kohler *et al.* 2008; Carilla-Latorre, Calvo-Garrido *et al.* 2008). However, *D. discoideum* modelling has been shown in some of these studies to be predictive of results obtained in virulence assays using higher eukaryotes, such as rat and mouse models of pneumonia (Cosson, Zulianello *et al.* 2002; Alibaud, Kohler *et al.* 2008) and *Drosophila* feeding assays (Alibaud, Kohler *et al.* 2008).

The abovementioned studies relied upon shorter incubation times and the delivery of amoeboid forms of *Dictyostelium* directly onto plates of either *P. aeruginosa* or a *Klebsiella/P. aeruginosa* co-culture. Whilst this allows shorter incubation times before plaque formation, these methods do require a droplet of amoeboid form in solution to be added to a lawn plate of bacteria. This method of inoculation does not allow significant control of the area over which the inoculating drop is spread, and thus the concentration of inoculating amoebae per mm² of agar surface inoculated. By using *Dictyostelium* spores impregnated into paper discs, an exact inoculum concentration was achieved, allowing better reproducibility of initial inoculum conditions. This method also eliminates any interference from the virulence *Klebsiella sp.* used in initial culturing of *D. discoideum* strains.

The primary detractor of the method presented in this study from previously described methods is the delayed time to plaque formation. Two factors extend this time. The first is that spore germination

must occur prior to amoeboid feeding. The second is that *P. aeruginosa* phagocytosis by *Dictyostelium* takes far longer than the consumption of *K. aerogenes* (Carilla-Latorre, Calvo-Garrido *et al.* 2008). *P. aeruginosa* has a far slower growth rate at 20°C than does *Klebsiella*. Thus, this modified assay takes seven days rather than the average of three to five days in previous studies to yield results. Another factor detracting from this novel method was the difficulty inherent in determining the percentage viability of spores used as an inoculum. Whilst fresh spores were used in all tests, the significant standard deviation seen in some tests may be due to a variation in the numbers of viable spores present.

9.4.2 Virulence between *Dictyostelium discoideum* strains is comparable

Two well defined laboratory mutants of *D. discoideum* were employed in this study. The virulence of *P. aeruginosa* in *D. discoideum* is reproducible between these two strain types. The axenic nature of AX2 predisposes this strain for use in virulence analyses, as maintenance and purity of axenic cultures is a simpler process than non axenic cultures. Which strain of *D. discoideum* will be used in a specific virulence assay will be guided by what measurements are to be made, and under what conditions they are made in. *D. discoideum* lends itself well to mutagenesis studies, and it may be possible to generate resistant or sensitive mutants to further investigate the mechanisms of virulence in this whole cell system.

9.4.3 Cystic Fibrosis isolates are less virulent in a *Dictyostelium discoideum* model

The most striking observation made in this study was the significantly decreased virulence of CF isolates of *P. aeruginosa* compared to those taken from other sources. The majority of CF isolates were capable of supporting the growth of both strains of *D. discoideum* tested, whilst only one non-CF

isolate (isolate 147) displayed this property, and then only in strain X22. Importantly, none of the clinical non-CF isolates tested displayed any capacity to support the growth of *D. discoideum*.

It has been shown by a number of previous investigators that bacterial T3SS virulence expression is irreversibly down regulated in CF infection (Dacheux, Toussaint *et al.* 2000; Roy-Burman, Savel *et al.* 2001; Jain, Ramirez *et al.* 2004; Filopon, Merieau *et al.* 2006). The capacity of CF strains to support the growth of *D. discoideum* corresponds with these findings. However, elements of the T2SS pathway controlled by *rhl* and *las* QS signalling have also been shown to induce death in *D. discoideum* (Cosson, Zulianello *et al.* 2002; Pukatzki, Kessin *et al.* 2002). The results of this work imply that global virulence, and not just the T3SS virulence pathway is down regulated in many CF isolates.

No significant variation in virulence was noted when virulence assays were repeated on SM agar infused with sub-MIC concentrations of azithromycin. Azithromycin has been shown to inhibit QS *in vivo* and *in vitro* (Nguyen, Louie *et al.* 2002; Gillis and Iglewski 2004), and the lack of disturbance in virulence by this method suggests that virulence in *D. discoideum* may be a QS independent activity. This suggests that T2SS may not be involved in the virulence of *P. aeruginosa* towards *D. discoideum*, as the T2SS secretory mechanism is controlled by a QS dependent pathway.

9.4.4 Virulence of AES3 in the *Dictyostelium discoideum* model

In chapter 7 of this thesis, examination of clinical data found that patients colonised with AES3 strains of *P. aeruginosa* in Tasmania showed poorer clinical outcomes than those colonised with unique isolates. However, in a *D. discoideum* model of eukaryotic infection, AES3 strains showed no difference in virulence when compared to Tasmanian CF cluster and unique CF isolates. This does

however not negate the significance of the results regarding increased patient morbidity in chapter 7. Whilst *D. discoideum* virulence assays have been shown to correlate with a mouse model of pneumonia, correlation studies of these models with clinical outcomes in human infection have not been performed. Also, in this assay, strains of *P. aeruginosa* are grown on solid agar at room temperature rather than human physiological temperature (*D. discoideum* will not survive at 37°C). Atmospheric partial pressures of gases will also differ from those in the CF lung. Therefore, this model does not necessarily reflect the virulence attributes of *P. aeruginosa* in the CF lung. Despite these shortcomings, analysis of *in vitro* virulence in *D. discoideum* remains an excellent guide as to the relative *in vivo* virulence of *P. aeruginosa* isolates, and in this it appears that there is little detectable disparity between AES3 strains and other CF isolates. As considered in chapter 7, it might be that increased severity of disease in CF patients is a predisposing factor for, rather than a consequence of, colonisation with AES3. Without further study of the virulence attributes of AES3 *in vivo*, the true role of such factors in disease presentation of colonised patients will not be entirely settled.

9.4.5 Environmental isolates may support the growth of *Dictyostelium discoideum*

Only one non-CF isolate in this study supported the growth of *D. discoideum*. This isolate was a community environmental strain recovered from a swimming pool (isolate 147). This loss of virulence expression was a particularly interesting observation, which may have further implications. There were no outstanding differences in virulence genotype or antimicrobial resistance of this isolate compared to the virulent environmental or clinical non-CF isolates tested. Importantly, this isolate only supported the growth of *D. discoideum* X22, whilst it did not support the axenic strain AX2. Isolate 147 was

urease negative, a property otherwise only seen in CF isolates, and it is possible that the elaboration of urease in *P. aeruginosa* may be a marker for virulence.

9.4.6 Urease production as a marker for virulence

A negative correlation between urease production and virulence was observed in this study, as was a lack of reproducibility of urease results for strains 44a and 33b. The latter degree of variation in urease results in consecutive runs of the same assay is analogous to the discrepancies noted in agar break point antimicrobial resistance testing in chapter 6 of this thesis. It is once again suggested that the hypermutable state of many CF isolates contributes this phenotypic switching. Such phenotypic switching may provide a further explanation for the significant standard deviation between *D. discoideum* virulence assay runs, particularly if it is applicable to expressed enzymes and proteins which are powerful virulence factors.

The use of environmental urea as a substrate for ammonia production by the urease enzyme provides an explanation for the lack of *D. discoideum* development observed on isolate R43, despite the large size of plaque produced. The ammonia product of urease will slow culmination of *Dictyostelium*, and in the case of R43 appears to have halted development altogether. Despite this, the strongly urease positive control strain *K. pneumoniae* allowed virtually complete growth and development of both AX2 and X22. This suggests that other factors are at play in the increased virulence of urease positive *P. aeruginosa* strains. The association of decreased virulence and urease negativity with CF isolates in particular points towards down-regulation of expression of urease as well as virulence factors. It is suggested that urease negativity is a marker of this down-regulation in *P. aeruginosa* strains.

9.4.7 Antimicrobial resistance and virulence in *Dictyostelium discoideum*

All isolates with an antimicrobial resistance score >6 supported the growth of *D. discoideum*. As MexEF-OprN multi drug resistance in *P. aeruginosa* has been shown to decrease virulence in a *D. discoideum* model, it is tempting to attribute the decreased virulence in MDR isolates to this mechanism. The relationship between the degree of antimicrobial resistance and *D. discoideum* plaque size was in no way linear, and no specific antibiogram was associated with decreased virulence. Secondly, only one isolate (U33b) showed the antibiogram pattern traditionally associated with MexEF-OprN resistance (table 1.1). Adding to the abovementioned factors, when exposed to high concentrations (MIC₉₀) of ceftazidime and tobramycin, designed to up-regulate any Mex-Opr related resistance mechanism, no alteration the virulence of these isolates was observed. Also, a number of antimicrobial sensitive strains also support *D. discoideum* growth. The mechanism by which the strains in this study were resistant to antimicrobial agents has not yet been investigated. Given current knowledge of decreased virulence in CF isolates, it is far more likely that the highly resistant isolates in this study showed decreased virulence in a *D. discoideum* assay because they were from the CF lung rather than because they carry a specific resistance mechanism.

9.4.8 Genotype and virulence in *Dictyostelium discoideum*

No significant relationship between the presence or absence of specific virulence genes or groups of virulence genes and capacity to support the growth of *D. discoideum* was identified in this study (table 9.13). It has been proven that expression of *exoU* is a major factor in *D. discoideum* virulence (Pukatzki, Kessin *et al.* 2002), and three isolates known to possess *exoU* were included in this study. Whilst two of these isolates (U156 and 77) did not support the growth of *D. discoideum*, the third isolate (147) did allow *Dictyostelium* growth. Thus the presence of *exoU* alone does not result in

killing of *D. discoideum*. Importantly, expression of *exoU* was not measured in this study, and it is entirely feasible that isolate 147 is not expressing *exoU*. Interestingly, isolate U156 is *exoU* positive, and was one of only two CF isolates which did not allow any growth of either AX2 or X22. Four *exoT*- and one *exoY*- strains were included in this study. Lack of these genes appeared to have no noticeable effect on *P. aeruginosa* virulence in *Dictyostelium*. Similar observations may be made regarding those isolates included which have lost one or more phenazine group genes.

Isolate U50 showed increased virulence in the X22 model than other CF isolates, and did not support the growth of AX2 at all. This strain of *P. aeruginosa* carried only the ExoS T3SS effector exoenzyme gene, conversely, U156 did not possess *exoS* and did not support the growth of either strain of *D. discoideum*. Beyond the statement that *exoS* is not essential for virulence in a *D. discoideum* model, further observations are difficult without analysis involving the use of knockout mutants and subsequent gene replacement in a test isolate.

The ineffectiveness of the presence of sub-MIC azithromycin to decrease virulence in the *D. discoideum* assay suggests that QS independent virulence factors (such as those belonging to the T3SS) are involved in virulence in the *D. discoideum* model. Without measurement of the level of expression and interactions of multiple known and potential unknown virulence factors of *P. aeruginosa* on *D. discoideum* growth, any conclusions regarding the specific role of individual virulence factors in this assay must remain, at least partially, speculative.

9.5 Conclusion

This work has presented a novel modification of the *D. discoideum* eukaryotic cell virulence assay for *P. aeruginosa* which excludes interference from virulence factors produced by *Klebsiella spp.* and allows an entirely reproducible initial inoculum concentration. It represents the first work comparing multiple isolates of *P. aeruginosa* from diverse clinical and environmental sources in a *D. discoideum* virulence assay. It also represents the first such assay to include observations regarding the effects of bacterial virulence on the morphological development of the eukaryotic host *D. discoideum* from spores through to fruiting bodies. This work has also definitively identified attenuated virulence in CF strains of *P. aeruginosa* when compared to those from all other clinical sources. No single virulence factor was identified as leading to virulence in *D. discoideum*, though loss of urease expression was identified as a potential marker for attenuated virulence in *P. aeruginosa* isolates.

CHAPTER TEN

Concluding Discussion

10.1 Introduction

The work presented in this thesis represents a comprehensive study of *P. aeruginosa* infections in a single geographical area. As such, it has provided a significant amount of data about the antimicrobial resistance, epidemiology, and virulence of *P. aeruginosa* infections. While the findings of this thesis may be extrapolated to *P. aeruginosa* strains worldwide, it should be noted that this study is representative of *P. aeruginosa* in Tasmania, Australia, and results of similar studies would vary in other geographic locales based upon local properties of endemic *P. aeruginosa* strains, such as rates of acquisition of antimicrobial resistance mechanisms, the presence or absence of CF clonal complexes of *P. aeruginosa* in that population, as well as rates of acquisition of horizontally transmitted virulence factor genes such as *exoU*. These factors will in turn be dependent upon the degree of regulation of antimicrobial use, the effectiveness of infection control procedures in local health care institutions, the prevalence of antimicrobial resistance genes in local isolates of *P. aeruginosa*, the morbidity, mortality and management of susceptible patients in local populations, and even environmental factors such as average annual rainfall and temperatures. This study provides a snap-shot of infections in a temperate, isolated island community with a large and well cared for CF population, effective and timely care for patients susceptible to *P. aeruginosa* infections, low rates of antimicrobial resistance in *P. aeruginosa* isolates, prudent protocols for the use of antimicrobials and a highly developed health care system.

10.2 The Need to Assess Virulence in Multiple Clinical Isolates From Diverse Sources

This study demonstrates the immense genotypic and phenotypic variability of *P. aeruginosa*, no doubt one of the reasons that it remains one of the most important clinical infectious agents. A number of the findings presented here suggest that the conclusions of some previous studies should be tempered due to the limited numbers and sources of *P. aeruginosa* strains studied. This reinforces the prudence of

testing hypotheses around infectivity and virulence of *P. aeruginosa* in a wide range of clinical and environmental isolates before broad statements, interpreted as being representative of the species as a whole, are made.

Assessment of selected *P. aeruginosa* isolates in the *D. discoideum* whole cell eukaryotic model demonstrated significantly decreased whole cell virulence in CF strains of *P. aeruginosa*. This is despite the negligible differences in virulence genes possessed between CF and non-CF strains tested. Therefore, virulence genotype alone does not predict the degree of whole cell virulence of a given isolate. Previous studies of bacterial virulence have generally focused on single virulence factors in isolation, and have not considered whole cell virulence and host/pathogen interactions. Some have relied upon the use of virulence gene knockout strains. While such testing is both necessary and useful to elucidate the role of individual virulence factors, it has a number of potential flaws. Primary amongst these is that when an individual secretion system or virulence factor is examined in isolation, its *in vivo* interactions with other virulence factors and secretion systems of *P. aeruginosa* are not considered. For instance; the difference in virulence between a wild-type strain and an *exoU* knockout strain may be considered as only attributable to the action of the product, ExoU. This does not consider how ExoU expression may affect the function of the overall regulation and control of *P. aeruginosa* global virulence. Thus, measurable differences in the virulence of an *exoU* knock-out strain, which are then attributed to the absence of *exoU*, may in fact be due to alterations in the expression of an entirely different virulence factor regulated by the ExoU expression. Furthermore, laboratory control strains, such as the much utilised PA01 (originally isolated from a burn wound) do not necessarily reflect the activity of clinical strains. Inferences about the virulence of *P. aeruginosa* in CF infection should not be made by observation of PA01. Such inferences do not take into account the remarkable differences

in phenotype displayed by chronic CF isolates compared to other clinical strains, or differences in the mechanisms and capacity to cause disease between such isolates. The conditions used in many *in vitro* experiments do not reflect in any way the conditions found *in vivo*, in which complex balances of ion gradients, cell density, osmolarity and gas partial pressures all interact to markedly affect the expression of *P. aeruginosa* virulence factors. Finally, virulence factor activity in many models is measured only once at a specific end time, with interpretation based upon viability of host cells at that specific moment in time. Rather, such complex host/bacteria interactions should be observed and measured regularly over an extended time period.

As discussed in the introduction to this thesis, virulence in *P. aeruginosa* is a dynamic system involving complex interactions between over thirty individual factors. The exact modes of action of many of these virulence factors remain obscure, and it is likely that further, as yet unidentified, virulence factors remain to be discovered. Therefore, examination of virulence as a global system rather than as a group of isolated factors is advantageous by virtue of its inclusive nature. The capacity to measure whole cell virulence is preferable to attempts to measure a complex system by examining the activity of a single, known virulence factor, or a handful of such factors. Similarly, by the use of multiple clinical and environmental isolates across a range of eukaryotic models, the complexities whole species *P. aeruginosa* virulence can be better elucidated than would be possible by the examination of one single laboratory control strain.

10.3 Evolution of Highly Virulent, Antimicrobial Resistant Isolates

Within the Hospital Environment

Rates of recovery of *P. aeruginosa* were far higher from the environment of intensive therapy wards than anywhere else in the hospital environment. Notable, rates of resistance and acquisition of *exoU* were also higher in this particular sub-set of isolates. It is most likely that acquisition of the resistant phenotype occurred *in situ* via horizontal transfer of genetic information from antimicrobial resistant enterobacteriaceae also present in the sinks of the intensive therapy wards. Ample opportunity exists for contact and exchange of genetic information between bacterial species in the hospital drains and sinks. The higher exposure to harsh disinfectants in such wards, combined with increased exposure to often highly resistant isolates of other Gram negative bacilli will inevitably lead to the evolution of more resistant strains of *P. aeruginosa*. Two non-CF isolates in this study conformed to the strict definition of multi-drug resistance applied; both of these having been isolated from the environment of the NSU. These isolates demonstrated both multi-drug resistance and the *exoU*⁺ genotype, and have been shown by another group to possess a horizontally acquired SHV ESBL gene (Pimay 2008, pers. com.). It is suggested that such selective pressures led to the horizontal acquisition of these genes in these isolates. Studies of virulence genotype in conjunction with antimicrobial resistance patterns have shown a link between resistance to multi-drug resistance and expression of the horizontally transferred *exoU* (Zaborina, Kohler *et al.* 2006). Therefore, acquisition of antimicrobial resistance in the hospital environment may also be linked to incorporation of new virulence factors into the genome of *P. aeruginosa* isolates within the hospital environment. This potential relationship of antimicrobial resistance and acquisition of virulence determinants in *P. aeruginosa* is of great concern, as it may lead to the development of a “super-bug” phenotype; a multi-drug resistant strain with significantly greater virulence when compared to other strains.

10.4 The Role of *P. aeruginosa* Virulence, Immunogenicity and Hypermutable in Cystic Fibrosis

Strains of AES1, AES2, AES3 and the Tasmanian CF cluster strain showed no detectable difference in the possession of specific virulence genes studied when compared to other CF isolates. Similarly, CF isolates showed few differences in their suite of virulence genes when compared to other *P. aeruginosa* strains. In contrast, significant differences in phenotypic virulence were apparent. Markedly decreased whole cell virulence was observed in CF isolates of *P. aeruginosa* when compared to virtually all other isolates. Such a distinct variation in phenotypic expression is only possible in a species with a genome as large and diverse as that of *P. aeruginosa*. This great capacity for phenotypic alteration must provide the organism a selective advantage for colonisation and persistence within environments. Further to this argument, it is proposed that the decreased global virulence of such strains may be predisposing to, rather than a consequence of, colonisation of the CF lung.

Bacterial virulence factors are potent ligands for the host immune system, and by down-regulating the expression of such factors, CF isolates of *P. aeruginosa* may be better able to evade the host immune response, and establish persistence in the CF lung. Such a hypothesis would explain the relatively low rate of new acquisitions of *P. aeruginosa* respiratory infections in CF patients, when it is considered that due to its prevalence in the environment, rates of exposure to this organism must be infinitely greater than rates of acquisition. The decreased phenotypic virulence of one environmental strain observed in this study provides further validity to such an argument, having demonstrated that low virulence isolates; which may preferentially infect the CF lung; do exist naturally in the community environment.

10.4.1 Decreased prevalence of *exoU* in cystic fibrosis isolates

CF isolates of *P. aeruginosa* frequently do not express their T3SS effector *exo*-enzymes after prolonged lung colonisation (Dacheux, Toussaint *et al.* 2000; Roy-Burman, Savel *et al.* 2001; Jain, Ramirez *et al.* 2004). This may be the means whereby *P. aeruginosa* avoids detection and clearance by the host immune system in CF, which suggests that expression of *ExoU* may have a deleterious effect with respect to long term persistence within the CF lung. Strains of *P. aeruginosa* elaborating *ExoU* may be both less capable of establishing infection, and less capable of persistence in the CF lung due to increased antigenicity, resulting in earlier detection and clearance by the host immune system.

10.4.2 Mutual exclusivity of *exoU* and *exoS*

Whilst *exoU* and *exoS* were not found to be mutually exclusive in this study, an there was an unexplained trend towards mutual exclusivity. It is possible that possession of both genes may in some way reduce the organism's fitness, encouraging the loss of one or the other from an individual organism's genome. It may also be possible that simultaneous production of both *exo*-enzyme products results in an increased or up-regulated host immune response. The fact that a higher relative percentage of the *exoS*⁺*exoU*⁺ isolates existed in some environmental strains provides tentative support for this argument; as the genotype of these isolates will not be selectively influenced by host immune response. Current understanding of the functional role of the *exo*-enzyme products of these genes and their interaction in both the virulence pathways and the global cellular mechanisms of *P. aeruginosa* is at present insufficient to identify an explanation for the this trend towards mutual exclusivity.

10.4.3 Decreased of virulence of cystic fibrosis isolates in the *D. discoideum* model

CF isolates exhibited reduced virulence when compared to the other clinical and environmental isolates studied in the *D. discoideum* model. The temporal loss of virulence in CF isolates of *P. aeruginosa* may correspond with other phenotypic changes seen in chronic CF infection, such as changes in LPS and mucoidy. As mentioned previously, CF isolates have been shown to irreversibly lose T3SS activity over time (Jain, Ramirez *et al.* 2004; Lee, Smith *et al.* 2005; Filopon, Merieau *et al.* 2006). As previously discussed, it is proposed that masking of cell surface antigens by alginate, and the loss of virulence factor expression not only allows *P. aeruginosa* to better colonise the CF lung, but will also facilitate chronicity of infection through avoidance of immune defence systems. Thus, a CF patient's lungs may be infected with a strain of low immunogenicity over a very prolonged period causing relatively mild and slowly progressing disease, whereas the lungs of a non-CF ICU patient, infected with a highly immunogenic and virulent strain will be rapidly damaged, progressing to invasion, sepsis, toxic shock and death. It is suggested that such interactions between host immune response and bacterial immunogenicity and ecology will assume primary importance in the investigation of the management of clinical disease caused by micro-organisms in future decades.

10.4.4 A potential role for hypermutability in cystic fibrosis clonal complex infections

CF clonal complexes of *P. aeruginosa* not only display apparent hyper-transmissibility, but also demonstrate an increased propensity towards antimicrobial resistance. Such increased antimicrobial resistance, when seen in *P. aeruginosa* isolates, is often associated with the existence of a hypermutable state (Morosini, Garcia-Castillo *et al.* 2005). The phenotypic switching observed in urease production in two AES3 isolates and the corresponding great variation in antimicrobial susceptibility patterns within the AES3 genotype suggest an increased degree of hypermutability in

these strains. CF isolates generally show a generally increased rate of hypermutability compared to non-CF *P. aeruginosa* isolates (Oliver, Levin *et al.* 2004), but this has not been explored in great depth. However, it is suggested that the key to a single genotype of *P. aeruginosa* emerging as a dominant hyper-infective CF clonal complex is this abnormally increased capacity for hypermutability over and above that normally associated with CF isolates. Hypermutable isolates would be better able to compete with other bacteria and supplant pre-existing genotypes of *P. aeruginosa* colonising the CF lung. This has been observed in LES infections, but the potential role of hypermutability was not considered. Abnormally elevated degrees of hypermutability certainly provide a feasible explanation for the apparently increased acquisition, multi-drug resistance and transmissibility associated with CF clonal complexes of *P. aeruginosa*.

10.5 Limitations of the Study

10.5.1 Population size, sample distribution, collection methods and Identification

While every attempt has been made to mitigate against conceivable shortfalls of analysis and technical methods throughout the course of this thesis, a number of limitations remain. The examination of the epidemiology and genotypic virulence of *P. aeruginosa* in Tasmania overall was performed on a moderately large sample size (184 isolates). However, only a small number (ten) of community environmental isolates were analysed. This was due to limited availability of community environmental isolates, but the lower numbers limits the power of the study with regard to this epidemiological group. More importantly, non-CF isolates were only collected in the Southern region of the island, and these were collected over a different time period to that in which CF isolates were collected. As a consequence of this, the two data sets should not, and were not interpreted for epidemiological surveillance in one dendrogram, as they represent two separate sample groups. It

remains possible that AES3 is present in low numbers in the community environment of Tasmania, in a manner analogous to clone C in Europe. It is particularly unfortunate that access could not be gained for environmental sampling at the site of the CF Summer camp where it is speculated that AES3 infection originated.

Collection of isolates from CF sputum in this thesis relied upon morphological criteria alone. As has been shown in this thesis isolates may share an identical genotype whilst being morphologically distinct, and *vice versa*. It is recommended that future studies employ random sampling of colonies on non-selective agar, with at least ten colonies from each sputum sample being genotyped. This will not only ensure that all *P. aeruginosa* genotypes within a patient's sputum sample are isolated, but may elucidate both major and subtle differences in genotype and antibiogram within a single patient's sample. Similar methods should be employed when sampling non-CF infections and environmental sites, although due to the lower prevalence of hypermutation and morphological variation in such isolates, it is considered that only five colonies per sample need be analysed.

Isolates of *P. aeruginosa* employed in this study were subjected to a battery of phenotypic tests prior to this identification being made. A single genotypic identification test was employed, this being a PCR targeting one element of the *P. aeruginosa* genome (Spilker, Coenye *et al.* 2004). Employment of a single target PCR for definitive identification has limitations in regard to the possibility of mutation at the primer site or along the length of the product that may result in no product or a product of incorrect size for an isolate truly belonging to the species *P. aeruginosa*. A more recently published identification protocol has addressed this source of error by employing a duplex PCR targeting two house keeping genes (*ecfX* and *gyrB*) unique to *P. aeruginosa* (Anuj, Whiley *et al.* 2009). This

protocol determine species identity in a stringent fashion by requiring any isolate not yielding a product of appropriate size for both targets to undergo partial 16S rRNA sequencing (Kidd, Ramsay *et al.* 2009) for definitive species identification.

10.5.2 Determination of Antibigram and Antimicrobial Susceptibility

As discussed above, variations in antibiogram within a single genotype of *P. aeruginosa* was identified frequently in this study, particularly amongst the AES3 clonal complex. In this study, a simple disc diffusion method was used for the determination of antibiogram. This method is easily performed, and allows a large number of antimicrobials to be simply tested against one isolate. The method is however, not as accurate as determination of MIC, and the gold standard for *P. aeruginosa* antimicrobial susceptibility testing remains determination of MIC by agar dilution testing (Saiman, Burns *et al.* 1999).

Further compounding the problems with antimicrobial susceptibility testing is the acknowledged difficulty in determining the antimicrobial susceptibility of such isolates due to phenotypic switching and hypermutable states common in CF isolates (Williams 1997; Foweraker and Laughton *et al.* 2005). One study found a mean number of four morphotypes per CF sputum. The same study performed antimicrobial susceptibility testing by the BSAC method and found a mean average of three differing antibiograms per morphotype (Foweraker and Laughton *et al.* 2005). These factors do not even consider the possibility of inducible resistance, such as derepression of the AmpC β -lactamase, which may not be detected by phenotypic methods. Current methods of *in vitro* phenotypic antimicrobial have many significant defects, but they do allow some significant and utilitarian indications to be made about the likely clinical outcome of treatment with a given antimicrobial agent.

10.5.3 Determination of Genotype

Typing data should be interpreted in the context of the source and clinical presentation of the isolates being typed, and knowledge of the applications and limitations of the typing methods used is imperative when interpreting epidemiological typing data (Arbeit, 1999). Interpretation methods rely on the assumptions that isolates involved in an outbreak will be of a common lineage, will yield identical or extremely similar macrorestriction patterns and that genetically unrelated isolates will yield markedly different results (Tenover, Arbeit *et al.* 1995). In practice, such assumptions are not always correct and are affected by a number of variables. PFGE produces extremely reliable results in all but those organisms with the most conserved genomes. This does not infer however that related strains will produce absolutely identical patterns, as events such as random mutations and genetic transfer may affect the final macrorestriction products of this method (Tenover, Arbeit *et al.* 1995). In order to address these variables, Tenover *et al.* (1995), described a method to interpret PFGE patterns that has since been widely employed by many research groups, a modification of which was employed in this thesis (method 3.15). In this method, isolates with only one band difference from the reference strain are considered to be genotypically indistinguishable, those with 2-3 bands different are considered closely related, whilst even those with up to six bands different are considered possibly related (Tenover, Arbeit *et al.* 1995). This method was subject to two limitations; that only one restriction endonuclease is used and that the strains being typed should represent an outbreak of less than one year in duration (Tenover, Arbeit *et al.* 1995).

In this study, RAPD PCR data was interpreted using dendrogram created through UPGMA with a Dice coefficients dendrogram. These methods are best applied to data such as nucleotide sequences, with multiple distinct characteristics (such as nucleotide position) and independent variables (such as

nucleotide substitutions) (Arbeit, 1999). As RAPD PCR amplification sites will vary between individual isolates, bands of same molecular size may not represent the same genetic locus, and areas of the same genetic locus may not yield bands of identical mobility. For these reasons, analysis by such statistical methods is only applicable in isolates that display minimal macrorestriction pattern differences (Arbeit, 1999). Therefore, whilst isolates found to be very similar or indistinguishable in the dendrograms presented in this thesis are reliably so, as percentage genotypic difference increases, a there is a proportional decrease in the accuracy of relatedness results obtained.

Whilst the RAPD PCR primer employed has been shown to produce highly reproducible and discriminatory results when applied to *P. aeruginosa* (Mahenthiralingam, Campbell *et al.* 1996), this method is generally considered to not be as reliable as PFGE or more recently described methods for genotyping of bacteria (Speert 2002). In order to avoid any error inherent in RAPD PCR, isolates showing >90% similarity by this method were subjected to *Spe1* PFGE, which remains the gold standard method for the genotyping of *P. aeruginosa* (Grundmann, Schneider *et al.*, 1995, Arbeit, 1999, Speijer, Savelkoul *et al.*, 1999). However, as discussed in the literature review of this thesis, even isolates showing identical macrorestriction band patterns on *Spe1* PFGE may not be sequence identical. Consequently, *Spe1* PFGE is now being challenged in accuracy by methods such as whole genome sequencing (Spencer, Kas *et al.* 2003) and microarray (Wiehlmann, Wagner, *et al.* 2007; Rakhimova, Wiehlmann *et al.* 2009) as the most discriminatory methods for determining isolate relatedness.

10.5.4 Adverse Clinical impact of AES3 on CF patients

In chapter five of this thesis, the presence of AES3 in a CF patient's lungs was found to correlate to adverse clinical outcomes such as number of admissions to hospital and number of bed days during

hospital stays. However, no differences between AES3 and genotypically unique *P. aeruginosa* isolates were observed in the *D. discoideum* virulence model. The number of patients carrying AES3 is small (nine), and as such the statistical power of any comparative analysis is lowered. As discussed previously in this thesis, such adverse clinical outcomes may provide a predisposing factor for AES3 infection, rather than being due to the infection. Increased rates of admission and longer hospital stays would also correlate to more exposure to *P. aeruginosa* strains in the hospital environment. Whilst no source of AES3 was found in the major tertiary referral hospital within the state of Tasmania, it remains a remote possibility that such a source did exist at one time and has since been replaced by new genotypes of *P. aeruginosa*. Also of concern in regard to this finding is that when comparing more objective measures of clinical outcome such as BMI, FEV1 and FVC, no significant difference between AES3 carriers and other CF patients were observable.

10.5.5 Determination of Virulence

As discussed in chapter eight of this thesis, whilst a great deal of information has been gained regarding virulence genotype in this study, it is expression rather than the presence of a virulence gene that is crucial in infection. Attempts to determine whole cell virulence expression were made in the *D. discoideum* virulence model, but this model itself suffers from some major limitations. The *D. discoideum* model employed yielded crude whole cell virulence results, but standard deviation of results were high, and interpretation of what this will mean in human infection difficult. Determination of which virulence factors are expressed at what levels during the course of *D. discoideum* experiments would be extremely useful to find what mechanisms are used to kill or inhibit *D. discoideum* growth and development. Even with such information, these experiments are carried out at room temperature on an agar substrate. Such conditions do not reflect the environment of the CF lung. It also remains

possible that *P. aeruginosa* may use a entirely different virulence pathway when interacting with *D. discoideum* to that which is employed in interactions with the CF lung environment. Whilst data generated from this model is indicative of lower virulence in CF *P. aeruginosa* isolates, many inherent variables occur between virulence *in vitro* and *in vivo*.

10.6 Further Investigations

A number of avenues for further study arise from the work presented in this thesis. A need to determine whether relationships exist between the length of time a strain has colonised a patient and the degree of virulence it expresses using the *D. discoideum* model is warranted. It has been suggested that the virulence of individual patient's strains should be followed longitudinally from time of acquisition, initial infection period and then over five to ten years. Changes in virulence or antimicrobial resistance observable at times of acute exacerbations, introduction of new treatment regimens or changes in the host physiology such as the onset of puberty or changes in clinical status would allow greater insights into the dynamic interactions between *P. aeruginosa* and the CF host.

Development of a multiplex PCR with targets for all identified Australian and European CF clonal complexes of *P. aeruginosa*, as well as the Tasmanian CF cluster strain would allow continuous monitoring of Tasmanian CF patients for the emergence of new clonal complexes. By the development and implementation of such testing, newly infected patients could be rapidly identified and segregated from the wider CF community to avoid further spread of these infections.

Analysis of the mechanisms of antimicrobial resistance in AES3, the degree of hypermutability in comparison to other CF isolates and non-CF isolates, the prevalence of integrons and other mobile

genetic elements and what genes are carried upon them are all needed to increase understanding of the mode by which CF clonal complexes of *P. aeruginosa* cause disease. Environmental sampling from the site of the Tasmanian CF summer camp which was potentially the initial source of AES3 might demonstrate its presence in that environment, despite the camps being held many years ago. Comparison of AES3 isolates from currently infected patients and those from the source environment employing methods such as DNA and RNA micro-array or whole genome analysis could well provide useful insights into the evolution of this particular clonal complex. Similarly, genome comparisons of all known CF clonal complexes with unique CF isolates might identify specific genetic elements associated with the hyper-infective state

10.6 Conclusion

The discovery of a new CF clonal complex (AES3) in a CF population without the previously recognised predisposing factors for emergence of such strains; ie: large CF centre care, has led to a revision of our understanding of CF *P. aeruginosa* clonal complex epidemiology. Studying the phenotypic and genotypic virulence of a large cohort of clinical and environmental strain of *P. aeruginosa* has clarified a number of presently held assumptions and observations regarding the virulence of *P. aeruginosa*. This work has demonstrated the presence of significantly decreased virulence in CF strains of *P. aeruginosa* tested in a eukaryotic whole cell virulence model, when compared to isolates from other sources. Overall, the studies presented in this thesis represent a thorough examination of *P. aeruginosa* strains associated with human infection in Tasmania, Australia.

APPENDICES

Appendix A

Antimicrobial Resistance Testing Methods Reference Values

| Antimicrobial | Disc Potency (µg) | Zone Diameter (Nearest Whole mm) | | |
|----------------------|-------------------|----------------------------------|-------|------|
| | | R | I | S |
| Ticarcillin | 75 | ≤ 14 | - | ≥ 15 |
| Carbenicillin | 100 | ≤ 13 | 14-16 | ≥ 17 |
| Timentin | 75/10 | ≤ 14 | - | ≥ 15 |
| Ceftazidime | 30 | ≤ 14 | 15-17 | ≥ 18 |
| Cefepime | 30 | ≤ 14 | 15-17 | ≥ 18 |
| Imipenem | 10 | ≤ 13 | 14-15 | ≥ 16 |
| Aztreonam | 30 | ≤ 15 | 16-21 | ≥ 22 |
| Ciprofloxacin | 5 | ≤ 15 | 16-20 | ≥ 21 |
| Amikacin | 30 | ≤ 14 | 15-16 | ≥ 16 |
| Gentamicin | 10 | ≤ 12 | 13-14 | ≥ 15 |
| Tobramycin | 10 | ≤ 12 | 13-14 | ≥ 15 |

Table A1: CLSI antimicrobial susceptibility disc test interpretative break point values (NB: timentin disc concentration of 75/10 refers to concentration of ticarcillin/clavulanic acid)

Appendix B

PCR Primers used in this study

| PCR Target | Direction | DNA Sequence (5' to 3') | Predicted Product Size (bp) | Annealing Temperature (°C) |
|-------------------------------|-----------|-------------------------|-----------------------------|----------------------------|
| <i>P. aeruginosa</i> specific | Forward | GGGGGATCTTCGGACCTCA | 956 | 58 |
| | Reverse | TCCTTAGAGTGCCACCCG | | |
| RAPD 272 | Random | AGCGGGCCAA | multiple, variable | 36 |
| | n/a | n/a | | |
| <i>apr</i> | Forward | TGTCCAGCAATTCTCTTGC | 1017 | 51 |
| | Reverse | CGTTTTCCACGGTGACC | | |
| <i>lasB</i> | Forward | ACAGGTAGAACGCACGGTTG | 1220 | 57 |
| | Reverse | GATCGACGTGTCCAACTCC | | |
| <i>phzI</i> | Forward | CATCAGCTTAGCAATCCC | 392 | 49 |
| | Reverse | CGGAGAACTTTCCCTC | | |
| <i>phzII</i> | Forward | CGCATTGACGATATGGAAC | 1036 | 51 |
| | Reverse | CGCATTGACGATATGGAAC | | |
| <i>phzH</i> | Forward | GGGTTGGGTGGATTACAC | 1752 | 51 |
| | Reverse | TGTCGTAGAGGATCTCCTG | | |
| <i>phzM</i> | Forward | ATGGAGAGCGGGATCGACAG | 875 | 53 |
| | Reverse | ATGCGGGTTTCCATCGGCAG | | |
| <i>phzS</i> | Forward | TCGCCATGACCGATACGCTC | 1752 | 63 |
| | Reverse | ACAACCTGAGCCAGCCTTCC | | |
| <i>exoS</i> | Forward | ATCCTCAGGCGTACATCC | 328 | 53 |
| | Reverse | ACGACGGCTATCTCTCCAC | | |
| <i>exoT</i> | Forward | CAATCATCTCAGCAGAACCC | 1159 | 53 |
| | Reverse | TGTCGTAGAGGATCTCCTG | | |
| <i>exoU</i> | Forward | ATGCATATCCAATCGTTGG | 2415 | 55 |
| | Reverse | CTAGCAATGGCACTAATCG | | |
| <i>exoY</i> | Forward | TATCGACGGTCATCGTCAGGT | 1035 | 61.8 |
| | Reverse | TTGATGCACTCGACCAGCAAG | | |

Table B1: Primer sequences, predicted product sizes and annealing temperatures for PCR reactions performed in this study (Finnan, Morrissey *et al.* 2004; Mahenthiralingam, Campbell *et al.* 1996; Spilker, Coenye *et al.* 2004)

Appendix C

Annealing Temperatures for DNA-DNA Hybridisation Assays

| Target Gene | Bacterial Strain Source of probe DNA | Annealing Temperature (°C) |
|-------------|--|----------------------------------|
| <i>phzI</i> | <i>P. aeruginosa</i> PAO1 | 35 |
| <i>phzH</i> | <i>P. aeruginosa</i> PAO1 | 35 |
| <i>phzM</i> | <i>P. aeruginosa</i> PAO1 | 44 |
| <i>phzS</i> | <i>P. aeruginosa</i> PAO1 | 35 |
| <i>exoS</i> | <i>P. aeruginosa</i> PAO1 | 44 |
| <i>exoT</i> | <i>P. aeruginosa</i> PAO1 | 35 |
| <i>exoU</i> | <i>P. aeruginosa</i> PA1O3 | 35 |
| <i>exoY</i> | <i>P. aeruginosa</i> PAO1 | 44 |

Table C1: Bacterial strain from which dig-labeled PCR product probes were produced and assay annealing temperatures for DNA-DNA hybridisation reactions performed in this study

Appendix D

Statistical Analysis of Virulence Factor Gene Distribution

| | CF Isolates | All Non-CF | χ^2 test | Clinical Non- | χ^2 test |
|-----------------------|-------------|-------------|---------------|---------------|---------------|
| | | Isolates | | CF Isolates | |
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 69 | 115 | 1.000 | 80 | 1.000 |
| <i>lasB</i> | 69 | 115 | 1.000 | 80 | 1.000 |
| <i>phzI</i> | 68 | 115 | 0.924 | 80 | 0.929 |
| <i>phzII</i> | 69 | 115 | 1.000 | 80 | 1.000 |
| <i>phzM</i> | 68 | 112 | 0.939 | 80 | 0.929 |
| <i>phzH</i> | 65 | 110 | 0.922 | 77 | 0.898 |
| <i>phzS</i> | 67 | 113 | 0.939 | 78 | 0.980 |
| <i>exoS</i> | 68 | 99 | 0.367 | 74 | 0.706 |
| <i>exoY</i> | 68 | 114 | 0.969 | 80 | 0.929 |
| <i>exoT</i> | 64 | 114 | 0.670 | 79 | 0.709 |
| <i>exoU</i> | 1 | 22 | 0.001 | 12 | 0.005 |
| Total (n) | 69 | 115 | | 80 | |

Table D1: Comparison of virulence gene prevalence in CF and non-CF isolates.

| | AES3 | Other | χ^2 test | Other CF | χ^2 test | All Other Clinical Isolates |
|-----------------------|-------------|-------------|---------------|-------------|---------------|-----------------------------|
| | Isolates | Isolates | | Isolates | | |
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value | Present (n) |
| <i>apr</i> | 12 | 172 | 0.817 | 57 | 1.000 | 137 |
| <i>lasB</i> | 12 | 172 | 0.817 | 57 | 1.000 | 137 |
| <i>phzI</i> | 12 | 171 | 0.821 | 56 | 0.956 | 136 |
| <i>phzII</i> | 12 | 172 | 0.817 | 57 | 1.000 | 137 |
| <i>phzM</i> | 11 | 169 | 0.720 | 57 | 0.792 | 137 |
| <i>phzH</i> | 11 | 164 | 0.776 | 54 | 0.921 | 131 |
| <i>phzS</i> | 12 | 168 | 0.825 | 55 | 0.911 | 133 |
| <i>exoS</i> | 12 | 159 | 0.766 | 56 | 0.956 | 130 |
| <i>exoY</i> | 11 | 171 | 0.697 | 57 | 0.792 | 137 |
| <i>exoT</i> | 10 | 168 | 0.556 | 54 | 0.709 | 133 |
| <i>exoU</i> | 0 | 23 | 0.197 | 1 | 0.646 | 13 |
| Total (n) | 12 | 172 | | 57 | | 137 |

Table D2: Comparison of virulence gene prevalence in AES3, other CF isolates and all other isolates.

| | non-CF Respiratory Isolates | All Non- Respiratory Isolates | χ^2 test | Clinical Non- Respiratory Isolates | χ^2 test |
|--------------------------|-----------------------------------|-------------------------------------|---------------|--|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 25 | 90 | 1.000 | 80 | 1.000 |
| <i>lasB</i> | 25 | 90 | 1.000 | 80 | 1.000 |
| <i>phzI</i> | 25 | 90 | 1.000 | 80 | 1.000 |
| <i>phzII</i> | 25 | 90 | 1.000 | 80 | 1.000 |
| <i>phzM</i> | 25 | 87 | 0.881 | 80 | 1.000 |
| <i>phzH</i> | 24 | 86 | 0.984 | 77 | 0.988 |
| <i>phzS</i> | 23 | 90 | 0.721 | 78 | 0.737 |
| <i>exoS</i> | 22 | 81 | 0.926 | 74 | 0.778 |
| <i>exoY</i> | 25 | 89 | 0.961 | 80 | 1.000 |
| <i>exoT</i> | 24 | 90 | 0.859 | 79 | 0.867 |
| <i>exoU</i> | 4 | 18 | 0.686 | 12 | 0.88 |
| Total (n) | 25 | 90 | | 80 | |

Table D3: Comparison of virulence gene prevalence in respiratory isolates, all other isolates and other clinical isolates (CF isolates excluded).

| | Skin/Soft tissue Isolates | Other Isolates | χ^2 test | Other Clinical Isolates | χ^2 test |
|--------------------------|---------------------------------|-------------------|---------------|-------------------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 26 | 158 | 1.000 | 123 | 1.000 |
| <i>lasB</i> | 26 | 158 | 1.000 | 123 | 1.000 |
| <i>phzI</i> | 26 | 157 | 0.976 | 122 | 0.970 |
| <i>phzII</i> | 26 | 158 | 1.000 | 123 | 1.000 |
| <i>phzM</i> | 26 | 154 | 0.904 | 122 | 0.970 |
| <i>phzH</i> | 25 | 150 | 0.953 | 117 | 0.961 |
| <i>phzS</i> | 26 | 154 | 0.904 | 119 | 0.879 |
| <i>exoS</i> | 26 | 145 | 0.687 | 116 | 0.787 |
| <i>exoY</i> | 26 | 156 | 0.952 | 122 | 0.970 |
| <i>exoT</i> | 26 | 152 | 0.855 | 117 | 0.818 |
| <i>exoU</i> | 3 | 20 | 0.881 | 10 | 0.593 |
| Total (n) | 26 | 158 | | 123 | |

Table D4: Comparison of virulence gene prevalence in skin and soft tissue isolates, all other isolates and other clinical isolates.

| | Urinary Isolates | All Non-Urinary | χ^2 test | Clinical Non-Urinary | χ^2 test |
|-----------------------|------------------|-----------------|---------------|----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 24 | 160 | 1.000 | 125 | 1.000 |
| <i>lasB</i> | 24 | 160 | 1.000 | 125 | 1.000 |
| <i>phzI</i> | 24 | 159 | 0.977 | 124 | 0.971 |
| <i>phzII</i> | 24 | 160 | 1.000 | 125 | 1.000 |
| <i>phzM</i> | 24 | 156 | 0.908 | 124 | 0.971 |
| <i>phzH</i> | 23 | 152 | 0.969 | 119 | 0.977 |
| <i>phzS</i> | 24 | 156 | 0.908 | 121 | 0.884 |
| <i>exoS</i> | 21 | 150 | 0.767 | 121 | 0.669 |
| <i>exoY</i> | 24 | 158 | 0.954 | 124 | 0.971 |
| <i>exoT</i> | 24 | 154 | 0.862 | 119 | 0.826 |
| <i>exoU</i> | 5 | 18 | 0.216 | 8 | 0.028 |
| <i>exoU*</i> | 4 | 18 | 0.474 | 8 | 0.105 |
| Total (n) | 24 | 160 | | 125 | |

Table D5: Comparison of virulence gene prevalence in urinary isolates, all other isolates and other clinical isolates. *: data for sample 96 excluded

| | Ear Isolates | Other Isolates | χ^2 test | Other Clinical Isolates | χ^2 test |
|-----------------------|--------------|----------------|---------------|-------------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 5 | 179 | 1.000 | 144 | 1.000 |
| <i>lasB</i> | 5 | 179 | 1.000 | 144 | 1.000 |
| <i>phzI</i> | 5 | 178 | 0.990 | 143 | 0.988 |
| <i>phzII</i> | 5 | 179 | 1.000 | 144 | 1.000 |
| <i>phzM</i> | 5 | 175 | 0.960 | 143 | 0.988 |
| <i>phzH</i> | 5 | 170 | 0.909 | 137 | 0.913 |
| <i>phzS</i> | 5 | 175 | 0.960 | 140 | 0.951 |
| <i>exoS</i> | 5 | 166 | 0.868 | 137 | 0.913 |
| <i>exoY</i> | 5 | 177 | 0.980 | 143 | 0.988 |
| <i>exoT</i> | 5 | 173 | 0.940 | 138 | 0.926 |
| <i>exoU</i> | 0 | 23 | 0.423 | 13 | 0.502 |
| Total (n) | 5 | 179 | | 144 | |

Table D6: Comparison of virulence gene prevalence in ear isolates, all other isolates and other clinical isolates.

| | All Environment Isolates | All Clinical Isolates | χ^2 test |
|--------------------------|--------------------------------|--------------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 35 | 149 | 1.000 |
| <i>lasB</i> | 35 | 149 | 1.000 |
| <i>phzI</i> | 35 | 148 | 0.971 |
| <i>phzII</i> | 35 | 149 | 1.000 |
| <i>phzM</i> | 32 | 148 | 0.671 |
| <i>phzH</i> | 33 | 142 | 0.956 |
| <i>phzS</i> | 35 | 145 | 0.885 |
| <i>exoS</i> | 29 | 142 | 0.492 |
| <i>exoY</i> | 34 | 148 | 0.907 |
| <i>exoT</i> | 35 | 143 | 0.827 |
| <i>exoU</i> | 10 | 13 | 0.003 |
| Total (n) | 35 | 149 | |

Table D7: Comparison of virulence gene prevalence in environmental isolates and all other isolates.

| | Community Environment Isolates | All Other Isolates | χ^2 test |
|--------------------------|--------------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 10 | 174 | 1.000 |
| <i>lasB</i> | 10 | 174 | 1.000 |
| <i>phzI</i> | 10 | 173 | 0.986 |
| <i>phzII</i> | 10 | 174 | 1.000 |
| <i>phzM</i> | 10 | 170 | 0.943 |
| <i>phzH</i> | 9 | 166 | 0.865 |
| <i>phzS</i> | 10 | 170 | 0.943 |
| <i>exoS</i> | 10 | 161 | 0.812 |
| <i>exoY</i> | 10 | 172 | 0.972 |
| <i>exoT</i> | 10 | 168 | 0.914 |
| <i>exoU</i> | 2 | 21 | 0.490 |
| Total (n) | 10 | 174 | |

Table D8: Comparison of virulence gene prevalence in community environmental isolates and all other isolates.

| | Hospital Environment Isolates | All Other Isolates | χ^2 test | Community Environment Isolates | χ^2 test |
|--------------------------|-------------------------------------|-----------------------|---------------|--------------------------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 25 | 159 | 1.000 | 10 | 1.000 |
| <i>lasB</i> | 25 | 159 | 1.000 | 10 | 1.000 |
| <i>phzI</i> | 25 | 158 | 0.977 | 10 | 1.000 |
| <i>phzII</i> | 25 | 159 | 1.000 | 10 | 1.000 |
| <i>phzM</i> | 22 | 158 | 0.593 | 10 | 0.737 |
| <i>phzH</i> | 24 | 151 | 0.961 | 9 | 0.869 |
| <i>phzS</i> | 25 | 155 | 0.906 | 10 | 1.000 |
| <i>exoS</i> | 19 | 152 | 0.345 | 10 | 0.481 |
| <i>exoY</i> | 24 | 158 | 0.875 | 10 | 0.914 |
| <i>exoT</i> | 25 | 153 | 0.858 | 10 | 1.000 |
| <i>exoU</i> | 8 | 15 | 0.003 | 2 | 0.549 |
| Total (n) | 25 | 159 | | 10 | |

Table D9: Comparison of virulence gene prevalence in hospital environmental isolates, all other isolates and community environmental isolates.

| | All ICU Isolates | All Other Isolates | χ^2 test |
|--------------------------|---------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 12 | 172 | 1.000 |
| <i>lasB</i> | 12 | 172 | 1.000 |
| <i>phzI</i> | 12 | 171 | 0.984 |
| <i>phzII</i> | 12 | 172 | 1.000 |
| <i>phzM</i> | 11 | 169 | 0.823 |
| <i>phzH</i> | 11 | 164 | 0.899 |
| <i>phzS</i> | 11 | 169 | 0.823 |
| <i>exoS</i> | 9 | 162 | 0.505 |
| <i>exoY</i> | 12 | 170 | 0.969 |
| <i>exoT</i> | 11 | 167 | 0.853 |
| <i>exoU</i> | 5 | 18 | 0.003 |
| Total (n) | 12 | 172 | |

Table D10: Comparison of virulence gene prevalence in ICU isolates and all other isolates.

| | ICU Clinical Isolates | All Other Isolates | χ^2 test |
|----------------|--------------------------|-----------------------|---------------|
| Virulence Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 4 | 180 | 0.822 |
| <i>lasA</i> | 4 | 174 | 0.824 |
| <i>lasB</i> | 4 | 180 | 0.822 |
| <i>phzI</i> | 4 | 179 | 0.823 |
| <i>phzII</i> | 4 | 180 | 0.822 |
| <i>phzM</i> | 4 | 176 | 0.825 |
| <i>phzH</i> | 3 | 172 | 0.617 |
| <i>phzS</i> | 3 | 177 | 0.585 |
| <i>exoS</i> | 4 | 167 | 0.807 |
| <i>exoY</i> | 4 | 178 | 0.824 |
| <i>exoT</i> | 3 | 175 | 0.598 |
| <i>exoU</i> | 0 | 23 | 0.465 |
| Total (n) | 4 | 180 | |

Table D11: Comparison of virulence gene prevalence in ICU clinical isolates and all other isolates.

| | ICU Environment Isolates | All Other Isolates | χ^2 test |
|--------------------------|--------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 8 | 176 | 1.000 |
| <i>lasB</i> | 8 | 176 | 1.000 |
| <i>phzI</i> | 8 | 175 | 0.987 |
| <i>phzII</i> | 8 | 176 | 1.000 |
| <i>phzM</i> | 7 | 173 | 0.763 |
| <i>phzH</i> | 8 | 167 | 0.885 |
| <i>phzS</i> | 8 | 172 | 0.949 |
| <i>exoS</i> | 5 | 166 | 0.361 |
| <i>exoY</i> | 8 | 174 | 0.975 |
| <i>exoT</i> | 8 | 170 | 0.924 |
| <i>exoU</i> | 5 | 18 | <0.001 |
| Total (n) | 8 | 176 | |

Table D12: Comparison of virulence gene prevalence in ICU environmental isolates and all other isolates.

| | All NSU Isolates | All Other Isolates | χ^2 test |
|--------------------------|---------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 9 | 175 | 1.000 |
| <i>lasB</i> | 9 | 175 | 1.000 |
| <i>phzI</i> | 9 | 174 | 0.986 |
| <i>phzII</i> | 9 | 175 | 1.000 |
| <i>phzM</i> | 9 | 171 | 0.945 |
| <i>phzH</i> | 8 | 167 | 0.846 |
| <i>phzS</i> | 9 | 171 | 0.945 |
| <i>exoS</i> | 9 | 162 | 0.820 |
| <i>exoY</i> | 9 | 173 | 0.973 |
| <i>exoT</i> | 9 | 169 | 0.918 |
| <i>exoU</i> | 2 | 21 | 0.333 |
| Total (n) | 9 | 175 | |

Table D13: Comparison of virulence gene prevalence in NSU and all other isolates.

| | NSU Clinical Isolates | All Other Isolates | χ^2 test |
|--------------------------|--------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 5 | 179 | 1.000 |
| <i>lasB</i> | 5 | 179 | 1.000 |
| <i>phzI</i> | 5 | 178 | 0.990 |
| <i>phzII</i> | 5 | 179 | 1.000 |
| <i>phzM</i> | 5 | 175 | 0.960 |
| <i>phzH</i> | 5 | 170 | 0.909 |
| <i>phzS</i> | 5 | 175 | 0.960 |
| <i>exoS</i> | 5 | 166 | 0.868 |
| <i>exoY</i> | 5 | 177 | 0.980 |
| <i>exoT</i> | 5 | 173 | 0.940 |
| <i>exoU</i> | 0 | 23 | 0.423 |
| Total (n) | 5 | 179 | |

Table D14: Comparison of virulence gene prevalence in NSU clinical isolates and all other isolates.

| | NSU Environment Isolates | All Other Isolates | χ^2 test |
|--------------------------|--------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 4 | 180 | 1.000 |
| <i>lasB</i> | 4 | 180 | 1.000 |
| <i>phzI</i> | 4 | 179 | 0.991 |
| <i>phzII</i> | 4 | 180 | 1.000 |
| <i>phzM</i> | 4 | 176 | 0.965 |
| <i>phzH</i> | 3 | 172 | 0.677 |
| <i>phzS</i> | 4 | 176 | 0.965 |
| <i>exoS</i> | 4 | 167 | 0.882 |
| <i>exoY</i> | 4 | 178 | 0.982 |
| <i>exoT</i> | 4 | 174 | 0.947 |
| <i>exoU</i> | 2 | 21 | 0.032 |
| Total (n) | 4 | 180 | |

Table D15: Comparison of virulence gene prevalence in NSU environmental isolates and all other isolates.

| | All Isolates From Other Wards | All Other Isolates | χ^2 test |
|--------------------------|-------------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 65 | 119 | 1.000 |
| <i>lasB</i> | 65 | 119 | 1.000 |
| <i>phzI</i> | 65 | 118 | 0.956 |
| <i>phzII</i> | 65 | 119 | 1.000 |
| <i>phzM</i> | 63 | 117 | 0.927 |
| <i>phzH</i> | 63 | 112 | 0.852 |
| <i>phzS</i> | 65 | 115 | 0.826 |
| <i>exoS</i> | 61 | 110 | 0.924 |
| <i>exoY</i> | 64 | 118 | 0.964 |
| <i>exoT</i> | 65 | 113 | 0.740 |
| <i>exoU</i> | 6 | 17 | 0.354 |
| Total (n) | 65 | 119 | |

Table D16: Comparison of virulence gene prevalence in isolates from other wards and all other isolates.

| | Other Ward Clinical Isolates | All Other Isolates | χ^2 test |
|--------------------------|------------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 52 | 132 | 1.000 |
| <i>lasB</i> | 52 | 132 | 1.000 |
| <i>phzI</i> | 52 | 131 | 0.963 |
| <i>phzII</i> | 52 | 132 | 1.000 |
| <i>phzM</i> | 52 | 128 | 0.852 |
| <i>phzH</i> | 50 | 125 | 0.927 |
| <i>phzS</i> | 52 | 128 | 0.852 |
| <i>exoS</i> | 48 | 123 | 0.956 |
| <i>exoY</i> | 52 | 130 | 0.926 |
| <i>exoT</i> | 52 | 126 | 0.778 |
| <i>exoU</i> | 5 | 18 | 0.487 |
| Total (n) | 52 | 132 | |

Table D17: Comparison of virulence gene prevalence in clinical isolates from other wards and all other isolates.

| | Other Ward Environment Isolates | All Other Isolates | χ^2 test |
|--------------------------|---------------------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 13 | 171 | 1.000 |
| <i>lasB</i> | 13 | 171 | 1.000 |
| <i>phzI</i> | 13 | 170 | 0.984 |
| <i>phzII</i> | 13 | 171 | 1.000 |
| <i>phzM</i> | 11 | 169 | 0.617 |
| <i>phzH</i> | 13 | 162 | 0.851 |
| <i>phzS</i> | 13 | 167 | 0.934 |
| <i>exoS</i> | 13 | 158 | 0.784 |
| <i>exoY</i> | 12 | 170 | 0.804 |
| <i>exoT</i> | 13 | 165 | 0.901 |
| <i>exoU</i> | 1 | 22 | 0.611 |
| Total (n) | 13 | 171 | |

Table D18: Comparison of virulence gene prevalence in environmental isolates from other wards and all other isolates.

| | Outpatient Isolates | All Other Isolates | χ^2 test |
|--------------------------|------------------------|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 80 | 104 | 0.654 |
| <i>lasB</i> | 80 | 104 | 0.654 |
| <i>phzI</i> | 79 | 104 | 0.717 |
| <i>phzII</i> | 80 | 104 | 0.654 |
| <i>phzM</i> | 79 | 101 | 0.579 |
| <i>phzH</i> | 76 | 99 | 0.672 |
| <i>phzS</i> | 77 | 103 | 0.800 |
| <i>exoS</i> | 78 | 93 | 0.318 |
| <i>exoY</i> | 79 | 103 | 0.670 |
| <i>exoT</i> | 75 | 103 | 0.938 |
| <i>exoU</i> | 2 | 21 | 0.001 |
| Total (n) | 77 | 107 | |

Table D19: Comparison of virulence gene prevalence in out-patient isolates and all other isolates.

| | Outpatient Isolates (CF excluded) | All Other Isolates | χ^2 test |
|--------------------------|---|-----------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value |
| <i>apr</i> | 13 | 104 | 0.92 |
| <i>lasB</i> | 13 | 104 | 0.92 |
| <i>phzI</i> | 13 | 104 | 0.92 |
| <i>phzII</i> | 13 | 104 | 0.92 |
| <i>phzM</i> | 13 | 101 | 0.84 |
| <i>phzH</i> | 13 | 99 | 0.79 |
| <i>phzS</i> | 12 | 103 | 0.89 |
| <i>exoS</i> | 12 | 93 | 0.84 |
| <i>exoY</i> | 13 | 103 | 0.90 |
| <i>exoT</i> | 13 | 103 | 0.90 |
| <i>exoU</i> | 4 | 21 | 0.41 |
| Total (n) | 13 | 104 | |

Table D20: Comparison of virulence gene prevalence in outpatient isolates and all other isolates (CF isolates excluded).

| | Community Clinical Isolates | All Other Isolates | χ^2 test | Community Environment Isolates | χ^2 test |
|--------------------------|-----------------------------------|-----------------------|---------------|--------------------------------------|---------------|
| Virulence Factor Gene | Present (n) | Present (n) | p value | Present (n) | p value |
| <i>apr</i> | 8 | 176 | 1.000 | 10 | 1.000 |
| <i>lasB</i> | 8 | 176 | 1.000 | 10 | 1.000 |
| <i>phzI</i> | 8 | 175 | 0.987 | 10 | 0.982 |
| <i>phzII</i> | 8 | 176 | 1.000 | 10 | 1.000 |
| <i>phzM</i> | 8 | 172 | 0.949 | 10 | 0.926 |
| <i>phzH</i> | 8 | 167 | 0.885 | 9 | 0.827 |
| <i>phzS</i> | 8 | 172 | 0.949 | 10 | 0.926 |
| <i>exoS</i> | 7 | 164 | 0.870 | 10 | 0.778 |
| <i>exoY</i> | 8 | 174 | 0.975 | 10 | 0.963 |
| <i>exoT</i> | 8 | 170 | 0.924 | 10 | 0.888 |
| <i>exoU</i> | 2 | 21 | 0.307 | 2 | 0.229 |
| Total (n) | 8 | 176 | | 10 | |

Table D21: Comparison of virulence gene prevalence in community clinical isolates, community environmental isolates and all other isolates.

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