THE PREVALENCE OF IS*6100* AND ITS ASSOCIATION WITH HYPERMUTABILITY IN CYSTIC FIBROSIS ISOLATES OF *PSEUDOMONAS AERUGINOSA* FROM LOCAL HOSPITALS

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Dedicated with love to my mother

DECLARATION

I, Joanna Evans, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Pseudomonas aeruginosa strains isolated from the sputa of patients with cystic fibrosis (CF) are commonly more resilient and resistant to antibiotics than their counterparts isolated from other sites of infection. Previous identification of an insertion sequence, IS*6100*, in *P. aeruginosa* isolated from CF patients suggested an association between the presence of this element and the ability of *P. aeruginosa* to adapt to the harsh environment of the CF lung. In this study, the presence of IS*6100* was investigated in *P. aeruginosa* isolated from CF and non-CF patients from Groote Schuur Hospital and Red Cross War Memorial Children's Hospital.

Of the 48 non-CF strains included in this study, 12 were shown, using PCR assays, to contain IS*6100*. Surprisingly, IS*6100* was not detected in all of the 32 CF *P. aeruginosa* isolates tested. These results were confirmed by DNA-DNA hybridization studies.

Hypermutability is a common feature of *P. aeruginosa* CF isolates, and is thought to contribute to the survival of *P. aeruginosa* in the CF lung. Genes associated with hypermutability and imipenem resistance were screened for IS*6100* insertions. The element was not detected in *mutS* or *oprD*. An IS*Psy2*-like element, however, was inserted into *oprD* of *P. aeruginosa* strain 8. Preliminary mutator assays using rifampicin were carried out on 3 CF and 4 non-CF, IS*6100*-containing *P. aeruginosa* strains. Two of 3 CF strains were hypermutable, whereas the non-CF strains did not have a significantly increased mutation rate. Taken together, these data suggest that IS*6100* is not associated with hypermutability in these strains.

ABBREVIATIONS

59-be	59 base element
%	percent
AAC	aminoglycoside acetyltransferase
AGE	agarose gel electrophoresis
bp	base pair(s)
°C	degrees Celsius
CTAB	Hexadecyltrimethylammonium bromide
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
g	gram(s)
GSH	Groote Schuur Hospital
IPTG	isopropyl-β-D-thio-galactosidase
IS	insertion sequence
kb	kilobase(s)
l	litres
M	molar
MCS	multiple cloning site
mg	milligram(s)
$MgCl_2$	magnesium chloride
M-H	Mueller-Hinton
MIC	minimum inhibitory concentration
ml	millilitre(s)
mM	millimolar
µ	micro
NaCl	sodium chloride

NCCLS	National Committee for Clinical Laboratory Standards
ng	nanogram(s)
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pmol	picomole(s)
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
rpm	revolutions per minute
RX	Red Cross War Memorial Childrens Hospital
SDS	sodium dodecyl sulphate
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
Tn	transposon
U	unit(s)
UV	ultraviolet
V	voltage
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
YT	yeast triptone

CONTENTS

FIGURES

TABLES

CHAPTER 1
INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) was first described in 1882 when a physician, Gessard, investigated the blue green colour observed on several wound bandages. Since then a great deal of research has been conducted upon this organism, which is an important nosocomial pathogen. Pyocyanin, the pigment that was responsible for the characteristic colouring of the bandages, is not produced by any other gram negative rods, making it a useful and rapid means of identifying *P. aeruginosa* (Daly *et al*, 1984; Ringen & Drake, 1952) [Fig. 1.1.1].

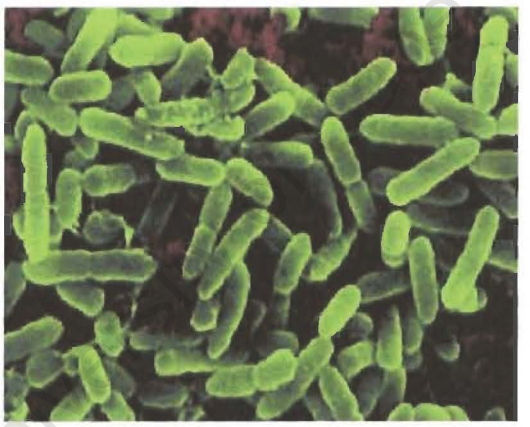


Figure 1.1.1: An electron micrograph of *P. aeruginosa* bacterial cells. *P. aeruginosa* produces the pigment pyocyanin, which gives it a characteristic green colour.
(http://www.fiu.edu/~matheek/researchgroup.htm)

P. aeruginosa is strictly aerobic and belongs to the family *Pseudomonadaceae* (Prescott *et al*, 1999). It possesses a single polar flagellum that facilitates movement through aqueous environments (Doyle *et al*, 2004). While motile bacteria usually encode one set of genes involved in motility (*motA* and *motB*) (Michel *et al*, 1998; Garza *et* al, 1995; Silverman *et* al, 1976), *P. aeruginosa* has two homologous sets, both of which have been shown to be functional in motility (Doyle *et al*, 2004). This suggests that swimming ability is an important feature of *P. aeruginosa*.

P. aeruginosa is a highly versatile bacterium, flourishing in environments such as soil (Huang *et al*, 2003; Green *et* al, 1974) and water (Römling *et al*, 1994; Trust & Bartlett, 1976; Ringen & Drake, 1952). It is best studied, however, for its role as a human pathogen, being associated with urinary tract infections (Astal, 2005), burns (Shahid & Malik, 2005) and pneumonia (Trouillet *et al*, 2002; Woods *et al*, 1986). Of importance to this study is that *P. aeruginosa* is a major pathogen in patients with the inherited disease, cystic fibrosis (CF) (Speert *et al*, 2002; Govan & Deretic, 1996).

1.2 Epidemiology of *P. aeruginosa*

CF is the most regularly occurring autosomal recessive disease worldwide, affecting approximately 1 in 2000-4000 Caucasians (Mateu *et al*, 1999). Over 1500 mutations resulting in CF have been described (Cystic Fibrosis Mutation Database). In 60% of CF patients, however, the disease occurs as a result of a three-base deletion encoding a phenylalanine amino acid residue in the CF gene (Hart & Winstanley, 2002; Riordan *et al*, 1989). This gene product, cystic fibrosis transmembrane conductance regulator (CFTR), is an ion channel that conducts chloride ions across cell membranes, and mutation of this gene results in defective sodium chloride transportation in epithelial tissues (Rich *et al*, 1990). A number of organs are affected, including the pancreas, digestive tract, salivary glands, sweat glands and liver, but the cause of death in approximately 95% of CF patients is respiratory disease [Fig. 1.1.2], usually caused by chronic bacterial infection (Welsh & Fick, 1987).

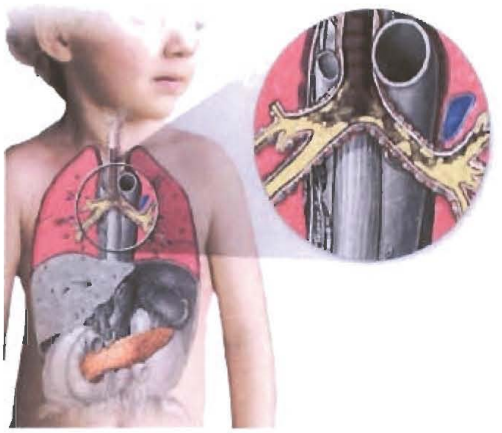


Figure 1.1.2: Respiratory disease is the major complication in the treatment of CF. Symptoms of CF include excess mucus secretion in the lungs, and reduced nutrient absorption by the pancreas.
(http://www.nlm.nih.gov/medlineplus/ency/imagepages/18135.htm)

Bacterial colonisation occurs in CF patients as a result of poor lung clearance (Robinson & Bye, 2002; Govan & Deretic, 1996). The reduced ion transport in the lungs of CF patients leads to the production of thick, viscous mucus (Govan & Deretic, 1996) and the mucociliary mechanisms that are responsible for the clearance of foreign particles from the lungs of unaffected individuals are believed to be impaired in these CF patients (Robinson & Bye, 2002). The presence of two sets of motility genes in *P. aeruginosa* (Doyle *et al*, 2004) and its ability to move through viscous substances (O' Toole *et al*, 1998) may provide a selective advantage in the initial colonisation of the mucus-filled CF lung.

The spread of non-CF *P. aeruginosa* strains that result in acute infection within the hospital setting is well documented (Thuong *et al*, 2003; Gibb *et al*, 2002; Dubois *et al*, 2001; Hsueh *et al*, 1998), although some reports do still indicate that dissemination does not occur (Gutiérrez *et al*, 2004). Until recently the spread of *P. aeruginosa* within a CF setting had not been considered a major risk (Speert *et al*, 2002; Oliver *et al*, 2000), however, outbreaks of antibiotic resistant *P. aeruginosa* in CF clinics are becoming increasingly documented (O'Carroll *et al*, 2004; Armstrong *et al*, 2003; Denton *et al*, 2002; Jones *et al*, 2001; Cheng *et al*, 1996). Colonisation of CF siblings with genotypically similar *P. aeruginosa* strains is recognised (Grothues *et al*, 1988), but whether this can be attributed to patient-to-patient transmission, or whether the organism was acquired from the same environmental source in these cases is not known. Evidence of co-infection in CF patients with two or more genotypically distinct *P. aeruginosa* strains (McCallum *et al*, 2001), and the transmission of *P. aeruginosa* from an adult CF carrier to her two non-CF parents (McCallum *et al*, 2002) suggest that the spread of *P. aeruginosa* between CF patients may be developing into a problem. Although no other reports of transmission of *P. aeruginosa* from CF to non-CF hosts have been documented to date, this has serious implications for the future care of CF sufferers, who rely heavily upon non-CF relatives and friends on an everyday basis (McCallum *et al*, 2002).

The possibility of epidemic spread is of great concern due to the difficulty in eradicating *P. aeruginosa* from the CF lung (Govan & Deretic, 1996), and it may soon become essential to isolate all CF patients infected with this organism, especially those harbouring multi-drug resistant (MDR) strains (Aloush *et al*, 2006).

A recent patient segregation trial resulted in a decrease in the prevalence of an epidemic strain, but not complete eradication (Griffiths *et al*, 2005), indicating that there are other factors to consider in CF *P. aeruginosa* infection other than patient-to-patient transmission. A better understanding of the mechanism of *P. aeruginosa* infection in CF will assist in the control of epidemic outbreaks and aid in reducing the severity of such infections.

1.3 Pathogenesis of *P. aeruginosa* in cystic fibrosis

The majority of CF patients succumb to infection by *P. aeruginosa* (Stover *et al*, 2000; Govan & Deretic, 1996), which has a remarkable ability to adapt to the harsh environment within the lungs of these patients, causing severe lung infection (Welsh & Fick, 1987; Woods *et al*, 1986). There are a number of phenotypic and genetic changes that occur within *P. aeruginosa* isolated from CF patients, and these changes are thought to provide a survival advantage in the lung. The prevalence of *P. aeruginosa* outside the human host suggests that initial infection in CF patients is with isolates displaying the wild-type phenotype, and that adaptation occurs within the lung, resulting in the CF phenotype (Speert *et al*, 2002). It is not only the versatility of *P. aeruginosa* that enables such chronic lung infection in CF patients, however, as experiments have shown that in the absence of immune and inflammatory cells, normal lung epithelia possess activity against *P. aeruginosa* whilst cells within CF lung epithelia fail to eradicate the organism (Smith *et al*, 1996). Thus a complex relationship between *P. aeruginosa* and the altered lung environment of CF patients seems to have developed that makes these infections very difficult to treat.

1.3.1 Biofilm formation by *P. aeruginosa* CF strains

One of the alterations that CF *P. aeruginosa* isolates undergo during chronic lung infection is that they grow within a biofilm, as opposed to their free-swimming, planktonic counterparts found in nature and in other infections (Hentzer *et al*, 2001). A biofilm is a thick, polysaccharide matrix that encases populations of bacterial cells, and is also a means of attachment to a solid surface (De Kievit *et al*, 2001).

P. aeruginosa growing in a biofilm are not exclusive to CF infections, and have been associated with, among others, contact lens surfaces (Lomholt & Kilian, 2003) and catheters in hospitals (Nickel *et al*, 1985). The high prevalence of *P. aeruginosa* infection in CF patients may, however, be as a result of its ability to readily form these biofilms (Bagge *et al*, 2004), which may act as a protective mechanism against the harsh environment of the CF lung (Watnick & Kolter, 1999).

Bacteria lacking flagellae and type IV pili are defective in biofilm formation, as shown by insertional inactivation experiments (Watnick & Kolter, 1999; O' Toole & Kolter, 1998). Contrary to biofilm formation in *Vibrio cholerae*, in which neither flagellae nor type IV pili are required for the initial attachment phase of biofilm production (Watnick & Kolter, 1999), in *P. aeruginosa* the flagellae are necessary for attachment to the abiotic surface, whilst type IV pili are only required at a later stage in the development of the biofilm (O'Toole & Kolter, 1998). The dependence upon flagellae for movement to the abiotic surface and attachment of *P. aeruginosa* during biofilm formation could account for the duplication of motility genes in this organism (Doyle *et al*, 2004). The type IV pili, which confer twitching motility, appear to be required for movement along the abiotic surface and the formation of microcolonies (O'Toole & Kolter, 1998). Mutants in exopolysaccharide (EPS) production, such as alginate, can still attach to a surface, but biofilm formation is inhibited, possibly as EPS may be required for stabilisation of the 3-dimensional biofilm (Watnick & Kolter, 1999).

P. aeruginosa release antigens when growing in biofilms within the lung, causing the production of high levels of antibodies against *Pseudomonas* (Costerton *et al*, 1999). The inability of these antibodies to access and react with their specific antigens on cells growing in the inner regions of the biofilm commonly results in an inflammatory response and extensive damage to the lung tissue (Costerton *et al*, 1999). In addition, therapy with antimicrobial agents fails to eradicate *P. aeruginosa* growing in biofilms in the CF lung due to the inability of the antibiotics to target the bacteria growing inside the biofilm (Costerton *et al*, 1999). The success of *P. aeruginosa* as a pathogen in CF is therefore greatly enhanced by their ability to exist in microbial communities within biofilms.

1.3.2 Conversion to mucoidy of *P. aeruginosa* in the CF lung

Alginate, an EPS that plays a key role in the formation of biofilms (Nivens *et al*, 2001), is also associated with the conversion of wild-type *P. aeruginosa* to a mucoid phenotype (Hentzer et al, 2001; Goldberg & Ohman, 1984), a common feature of *P. aeruginosa* CF strains (Govan & Deretic, 1996). This conversion to mucoidy occurs as a result of the overproduction of alginate (Hentzer et al, 2001; Goldberg & Ohman, 1984), the major virulence factor in *P. aeruginosa* CF lung infection (Pier et al, 2001). Conversion of mucoid *P. aeruginosa* isolates to the non-mucoid phenotype upon growth in nutrient rich media suggests that nutrient deficiency in a chronic infection selects for these mucoid strains (Govan & Deretic, 1996; Sokol *et al*, 1994; Goldberg & Ohman, 1984). The increased alginate in mucoid strains seems to aid in the production of a more structured biofilm when compared to non-mucoid strains of *P. aeruginosa* growing in a biofilm (Hentzer et al, 2001). Mucoid *P. aeruginosa* are usually non-motile and do not express fully developed O-antigen sidechains in their lipopolysaccharide cell walls (Hancock et al, 1983), nor do they produce the pigment pyocyanin (Govan & Deretic, 1996). Loss of flagellation alone is not sufficient for conversion to the mucoid phenotype (Luzar et al, 1985), suggesting that other factors play a role in the alteration of *P. aeruginosa* CF strains to mucoidy. Also, the isolation of mucoid *P. aeruginosa* from non-CF patients suggests that this phenotype is not specific to CF *P. aeruginosa* isolates, but occurs during chronic *P. aeruginosa* infection (Govan & Deretic, 1996), which is commonly observed in CF patients, as opposed to acute infections.

The over-production of alginate, and the resulting conversion to mucoidy, is due to an alteration of the transcriptional regulator of the alginate synthesis genes, AlgU (Martin *et al*, 1993). This protein is a stress sigma factor, σ^{22} (Wu *et al*, 2004), that regulates the *algD* operon, which encodes the genes involved in alginate production (De Vries & Ohman, 1994). The gene encoding AlgU is found in an operon consisting of *algU-mucABCD* and expression of *algU* activates the synthesis of alginate (Martin *et al*, 1993). The *mucA* gene encodes a transmembrane protein, an anti-σ factor, which binds to and represses the σ^{22} *algU* product (Mathee *et al*, 1997; Schurr *et al*, 1996). MucB is a periplasmic protein that negatively regulates AlgU

(Schurr *et al*, 1996; Flynn & Ohman, 1988). It is not completely understood how MucC negatively regulates alginate synthesis (Boucher *et al*, 1997a). MucD is thought to be involved in the removal of misfolded or otherwise damaged proteins (Wood & Ohman, 2006). Under environmental stress conditions, the complex formed by MucA-MucB-AlgU is thought to be disrupted, resulting in the release of bound σ^{22} from MucA, and the production of alginate (Mathee *et al*, 1997; Schnider-Keel *et al*, 2001). It follows, therefore, that mutations in *mucA* are the common cause of over-production of alginate in *P. aeruginosa* CF isolates, with some studies reporting up to at least 84% incidence of mutation in this gene in mucoid isolates (Boucher *et al*, 1997b). In the same study, complementation of wild-type *mucA* in mucoid isolates harbouring mutations in this gene resulted in a return to the non-mucoid phenotype, confirming the major role of *mucA* in the pathogenicity of *P. aeruginosa* in the CF lung.

1.3.3 Hypermutability

The continually changing CF lung seems to provide an ideal environment for the development of strains with an ability to acquire mutations in genes encoding DNA repair enzymes (Oliver *et al*, 2000). This leads to the production of strains with a higher rate of spontaneous mutation relative to wild-type strains by up to 1000-fold, and these strains are said to be hypermutable (Horst *et al*, 1999). Oliver *et al* (2000) demonstrated that hypermutable *P. aeruginosa* isolates were identified in 37% of 30 CF patients tested, while none were isolated from 75 acutely-infected, non-CF patients. A more recent study to determine the prevalence of these mutator strains in non-CF patients with chronic lung infection, however, revealed that 53% of isolates were hypermutable (Maciá *et al*, 2005). These results suggest that the mutator strains are not exclusive to CF patients, but that they seem to be associated with chronic infection rather than acute *P. aeruginosa* infection (Maciá *et al*, 2005). Mutator strains arise as a result of mutation(s) in the genes encoding DNA mismatch repair (MMR) proteins MutS, MutL, MutH and UvrD (Oliver *et al*, 2002). MutS is responsible for the recognition of errors made by DNA polymerase, such as nucleotides that are mismatched and insertions or deletions of up to four nucleotides (Smania *et al*, 2004; Oliver *et al*, 2002). Once a mutation has been identified, MutS

becomes complexed to MutL, resulting in the activation of an endonuclease, MutH (Oliver *et al*, 2002). The *uvrD* gene encodes a DNA helicase that facilitates the unwinding of the DNA strands, allowing the mismatch to be repaired (Oliver *et al*, 2002). In both CF and non-CF hypermutable *P. aeruginosa* isolates, as well as in other bacterial species (Watson *et al*, 2004), *mutS* is most frequently disrupted (Smania *et al*, 2004; Oliver *et al*, 2002).

The selective pressures encountered by *P. aeruginosa* in the CF lung include oxidative stress due to a heightened immune response (Wood *et al*, 2001) and reactive oxygen radicals (Ciofu *et al*, 2005). These conditions may play a role in the conversion to mucoidy and hypermutability (Ciofu *et al*, 2005; Mathee *et al*, 1999). In addition, mutations in genes that affect resistance to antibiotics often confer a survival advantage upon exposure to antibiotics (Oliver *et al*, 2000; Carmeli *et al*, 1999).

1.3.4 Multi-Drug Resistant *P. aeruginosa* in CF

P. aeruginosa displays intrinsic resistance to a number of antimicrobials, making infection with this organism very difficult to treat (Schweizer, 2003; Yoneyama & Nakae, 1993). Multi-drug resistant (MDR) *P. aeruginosa*, which can arise as a result of continuous treatment with antibiotics (Gutiérrez *et al*, 2004; Paramythiotou *et al*, 2004; Mouton *et al*, 1993), is predominantly associated with CF isolates (Aloush *et al*, 2006). This is, in part, due to the extensive time periods spent by CF patients in hospitals, or other health-care facilities, as resistance levels of isolates acquired within hospital environments tend to be higher than those that are acquired outside of the hospital setting (Aloush *et al*, 2006; Trouillet *et al*, 2002). *P. aeruginosa* isolates are considered MDR if they display resistance to piperacillin, ceftazidime, imipenem and ciprofloxacin, the most common antibiotics used in the treatment of pseudomonal infections (Paramythiotou *et al*, 2004).

Only a small percentage (10.2%) of non-CF strains develop MDR during treatment, when compared with *P. aeruginosa* from the CF lung (Carmeli *et al*, 1999). It is suggested that this elevated rate of resistance in CF strains is associated with biofilm

production by these isolates (De Kievit *et al*, 2001). Bacterial cells found within the biofilm, near the abiotic surface to which they are attached, are in an environment where nutrients and oxygen are scarce, and are therefore not metabolising actively (De Kievit *et al*, 2001). Since the mechanism of action of many antimicrobial agents involves the disruption of metabolic processes, only actively metabolising cells are affected (Walters III *et al*, 2003). Indeed, following detachment from the biofilm, planktonic *P. aeruginosa* isolates display reduced levels of resistance to antibiotics (Stewart & Costerton, 2001).

Alginate is a major component of the *P. aeruginosa* biofilm, and overproduction of this EPS not only provides a thick, protective physical barrier around the bacteria, but consequently results in increased resistance to numerous antimicrobial agents (Bagge *et al*, 2004; Hentzer *et* al, 2001). Increased expression of the genes involved in alginate synthesis in the presence of the β-lactam antibiotic, imipenem, suggests that the increased frequency of mucoid isolates in CF patients may be as a direct result of the large amounts of antibiotic therapy these patients receive (Bagge *et al*, 2004). The increase in alginate production upon exposure to imipenem resulted in a thicker biofilm, and therefore imipenem susceptibility of the organisms within the biofilm may be decreased due to impaired penetration of the antibiotic (Bagge *et al*, 2004).

Several studies have demonstrated a link between the development of MDR and hypermutability in *P. aeruginosa* (Maciá *et al*, 2005; Maciá *et al*, 2004; Oliver *et al*, 2000). Kresse *et al* (2003) presented the first evidence of hypermutability arising within the lung after antibiotic treatment, but shortly afterwards Gutiérrez *et al* (2004) found that there was no relationship between the two. It seems that one does not arise as a result of the other, but rather, in the case of any chronic *P. aeruginosa* infection it should be assumed that high numbers of hypermutable isolates will be resistant to at least one antipseudomonal agent, and that therapy with multiple antibiotics should be implemented (Oliver *et al*, 2004).

1.4 The Genetic Diversity of *P. aeruginosa*

The ability of *P. aeruginosa* to adapt so specifically to its niche in CF airways is, at least in part, due to its remarkable genetic versatility. Bacteria that are closely related to each other can usually be identified based on the similarity of gene arrangement and chromosomal organisation between them (Tillier & Collins, 2000). Also, pathogenic bacteria are often distinguishable from their environmental counterparts due to the production of virulence factors that aid in pathogenesis (Musser, 1996; Brenner *et al*, 1988). Interestingly, however, CF *P. aeruginosa* isolates appear to display decreased virulence following prolonged lung infection (Smith *et al*, 2006). Sequence diversity between bacteria of the same species can arise as a result of selective pressures due to a changing environment (Denamur & Matic, 2006). A greater understanding of exactly how these evolutionary changes come about may be of great significance in shaping the treatment regime currently employed to combat these pathogenic bacteria (Spratt & Maiden, 1999).

The *P. aeruginosa* genome, at 6.3 million base pairs in size, is the second largest bacterial genome to have been fully sequenced (Stover *et al*, 2000), the largest being that of *Burkholderia cenocepacia* at approximately 7.3 million base pairs (Copeland *et al*, 2006). The genome of *Escherichia coli* K-12 is relatively small by comparison, comprising only 4.6 million base pairs (Blattner *et al*, 1997). The large genome size of *P. aeruginosa* is a factor crucial to its complexity and diversity, and which contributes to its ecological versatility (Ruimy *et al*, 2001; Stover *et al*, 2000; Woods *et al*, 1986). The advance in molecular typing methods has led to the sub-division of *P. aeruginosa* into at least four clonal groups, clone C, J, K and M, all of which have been detected in CF patients (Römling *et al*, 1994). The isolation of the same major *P. aeruginosa* clones from the environment as well as in a disease setting suggests that the genetic variations between different clones are not selective for a particular habitat (Kiewitz & Tümmler, 2000; Römling *et* al, 1994). Conversely, recombinational events seem to occur in more than 10% of the *P. aeruginosa* clone C genome (Römling *et al*, 1997b) and this genomic versatility may explain the ability of *P. aeruginosa* to colonise such a diverse range of habitats.

1.4.1 *P. aeruginosa* Clone C

In 1992, following macrorestriction analysis of CF *P. aeruginosa* isolates, Römling *et al* detected a common *P. aeruginosa* clone, clone C, colonising 30% of CF patients in a clinic in Hannover. The predominance of this *P. aeruginosa* clone suggested a possible survival advantage in CF infections, and therefore analysis of its genetic composition would provide invaluable information about colonisation of the CF airway. Genomic typing methods performed on *P. aeruginosa* isolates from a variety of different sources revealed, however, that these clone C isolates occur with similar frequency in CF sputa (28%) and in the aquatic environment (21%), suggesting that there does not seem to be a particular predilection for infection within the CF airway (Römling *et al*, 1994). Similarly, clone J *P. aeruginosa* strains, the second most frequently identified clones in CF after clone C, were found in aquatic environments as well (Römling *et al*, 1994). Two other less frequently identifed *P. aeruginosa* clones are clone K and clone M (Römling *et al*, 1994). Clone K was isolated from both CF and non-CF infections, while clone M was identified in CF patients and on machinery in the clinical setting (Römling *et al*, 1994). The detection of a *P. aeruginosa* clone C isolate associated with an ear infection also indicates that the pathogenicity of clone C strains is not specific for CF infection (Römling *et al*, 1994).

P. aeruginosa clone C isolates have subsequently been further divided into four subgroups based on similarity of macrorestriction patterns with the reference strain from each subgroup (Römling *et al*, 1997b) [Fig. 1.1.3]. Subgroup C contains only CF isolates; subgroup C2 contains CF isolates as well as environmental isolates and isolates from other sites of infection; subgroup SG17M consists of clone C isolates from the environment and from the inanimate hospital setting; and subgroup C13 consists of isolates from a single CF patient (Römling *et al*, 1997b).

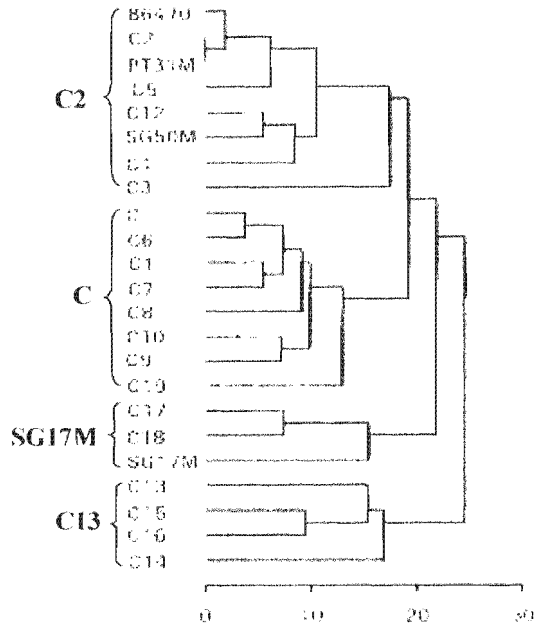


Figure 1.1.3: The *P. aeruginosa* clone C genotype. Sub-division of *P. aeruginosa* clone C isolates into four groups, C2, C, SG17M and C13, based on the similarities of their macrorestriction patterns.
(Adapted from Römling *et al*, 1997b)

The widespread detection of *P. aeruginosa* isolates of the same clone in such a broad range of environments serves to confirm the complexity of the organism, as well as to suggest that genetic diversity in this organism is driven by factors other than pathogenicity.

1.5. The role of mobile genetic elements in contributing to sequence diversity in *P. aeruginosa* clone C

Genetic diversity can arise in bacteria as a result of spontaneous mutations in existing genes, or due to the acquisition of foreign DNA from the external environment (Spratt & Maiden, 1999). Mobile genetic elements that can be acquired by bacteria include plasmids, transposons, integrons, insertion sequences and bacteriophages (Sørensen *et al*, 2005), and these elements often confer an advantage upon the recipient bacterium, such as decreased antibiotic susceptibility.

1.5.1. Plasmids

Bacteria can exchange genetic material through the transfer of plasmids, by a process known as conjugation (Sørensen *et al*, 2005). The precise mechanism by which plasmids in their episomal form are maintained within the bacterial cell is not known (Sørensen *et al*, 2005), but they can also be found reversibly or irreversibly integrated into the bacterial host chromosome (Zagaglia *et al*, 1991).

Comparative analysis of a physical and genetic map of *P. aeruginosa* PAO with *P. aeruginosa* C, a clone C strain (Römling *et al*, 1997b), revealed an increased genome size of 600kb in clone C as a result of a number of large insertions (Schmidt *et al*, 1996). These large regions of inserted DNA appeared to be derived from elements such as plasmids, phages or transposons due to their comparatively low G + C content of 50% as opposed to the 67% G + C content of the *P. aeruginosa* chromosome (Stover *et al*, 2000; Römling *et al*, 1997b). The structural backbone of the chromosomes of both *P. aeruginosa* PAO and clone C strains is conserved, as was shown by the gene order and the arrangement of the *rrn* operons, which encode ribosomal RNA (Schmidt *et al*, 1996). This means that by genotyping methods such as ribotyping, which makes use of probes derived from the 16S and 23S rRNA genes, these two strains would probably appear to be related. Indeed, one of the short-comings of ribotyping is the difficulty in differentiating between closely related strains due to the small number of signals produced (Olive & Bean, 1999). By macrorestriction analysis, however, they appear to be only distantly related (Römling *et al*, 1995), due to the additional restriction sites encoded by the inserted DNA (Schmidt *et al*, 1996). This suggests that although rearrangement of much of the genome of the clone C isolate compared with that of PAO has occurred, the rearrangements are somewhat ordered in that they only affect certain parts of the genome.

Pathogenicity islands (PAIs) are genomic islands carrying genes that encode virulence factors (Hentschel & Hacker, 2001). Blum *et al* (1994) first described large DNA insertions in the chromosome of *E. coli* carrying genes encoding hemolysin, *hly*, and and P-related fimbriae, *prf*. The tRNA genes were identified as the site of

integration of PAIs carrying virulence genes in *E. coli* and other Gram-negative bacteria (Blanc-Potard & Groisman, 1997; Blum *et al*, 1994). Similarly, 3 years later, a 95 kb plasmid, pKLC102, was found to be reversibly integrated into the chromosome of *P. aeruginosa* clone C isolates (Römling *et al*, 1997b). The plasmid integrates at the $tRNA^{Lys}$ site near to the *pilA* region (Klockgether *et al*, 2004), a region shown to be particularly suited to the insertion and excision of DNA (Kiewitz *et al*, 2000). The plasmid, pKLC102, was found in both the episomal and the chromosomally integrated form in *P. aeruginosa* clone C strains from environmental and disease habitats other than CF lungs (Klockgether *et al*, 2004; Römling *et al*, 1997b). Interestingly, in clone C isolates from patients with CF pKLC102 existed only in the integrated form (Klockgether *et al*, 2004; Römling *et al*, 1997b). The ability of pKLC102 to exist both chromosomally and episomally is as a result of its composition, which is partly phage-derived and partly plasmid-derived (Klockgether *et al*, 2004).

A 106 kb plasmid with high homology to pKLC102, pKLK106, can be reversibly integrated into the chromosome of *P. aeruginosa* clone K strains at both of the two $tRNA^{Lys}$ sites found in this organism, (Kiewitz *et al*, 2000), while pKLC102 is only ever found integrated into the $tRNA^{Lys}$ gene in the region of *pilA* (Kiewitz *et* al, 2000; Klockgether *et al*, 2004). This is because the $tRNA^{Lys}$ gene in the vicinity of *oprL-phnAB*, the second site at which pKLK106 integrates in clone K strains, contains the 23.4kb genome island PAGI-4(C) in *P. aeruginosa* strain C (Klockgether *et al*, 2004). The exact function of PAGI-4(C) in clone C is unknown, but it appears as though a transposon, with a stretch of DNA 90.4% homologous with a 239bp direct repeat found within pKLC102, underwent recombination, irreversibly integrating the plasmid into the host chromosome, and thereby rendering this $tRNA^{Lys}$ gene unavailable for the reversible integration of pKLC102 in *P. aeruginosa* clone C strains (Klockgether *et al*, 2004).

Plasmid pKLC102 encodes at least 18 genes involved in conjugation, recombination and repair, and it also includes a pilin gene cluster consisting of 10 *pil* genes and a *chvB* gene with sequence homology to a cyclic β-glucan synthase (Klockgether *et al*,

2004). Although the integration of plasmids into tRNA genes is not well documented (Kiewitz *et al*, 2000), the insertion of virulence genes into tRNAs in *E. coli* (Blum *et al*, 1994) and *Salmonella typhimurium* (Blanc-Potard & Groisman, 1997) suggests that pKLC102 and pKLK106 in *P. aeruginosa* clone C and clone K strains, respectively, may be required for pathogenicity. The irreversible integration of pKLC102 into *P. aeruginosa* strain C isolates from chronic CF infections suggests that the plasmid induces virulence in its integrated form. The presence of a gene on pKLC102 with high sequence homology to that encoding a virulence-associated protein, cyclic β-glucan synthase, in *Brucella abortus* (Roset *et al*, 2004) and *Agrobacterium tumefaciens* (Douglas *et al*, 1982), suggests that pKLC102 may have been initially acquired, and then maintained, due to its association with virulence in subclone C *P. aeruginosa* strains.

1.5.2 Transposons

Genetic elements that can be excised from one site in a DNA molecule and inserted into another site are known as transposons (Holmes & Jobling, 1996). Each transposon encodes a transposase enzyme, necessary for its transposition, and is flanked by nucleotide repeats, 15-25bp in length, due to duplication of short nucleotide sequences upon transposition (Holmes & Jobling, 1996). Of the three groups of transposons [Fig. 1.1.4], Class 1 transposons include insertion sequences (IS), which contain only the genes necessary for transposition, and composite transposons that have IS elements flanking an internal region (Holmes & Jobling, 1996). The gene cassettes within the composite transposon often encode antibiotic resistance markers (Cabrera *et al*, 2006; Reyes *et al*, 2003; Gonzalez *et al*, 1998). Class 2 transposons have longer terminal nucleotide repeats of 35-40bp and lack the flanking IS elements (Holmes & Jobling, 1996). All members of this class encode both a transposase and a resolvase, as well as antibiotic resistance genes (Holmes & Jobling, 1996). The third class of transposon consists of the whole genome of bacteriophage Mu (Holmes & Jobling, 1996). Random integration of Mu at various sites in the genome can sometimes lead to deletions in the adjacent DNA sequence (Roy *et al*, 1995).

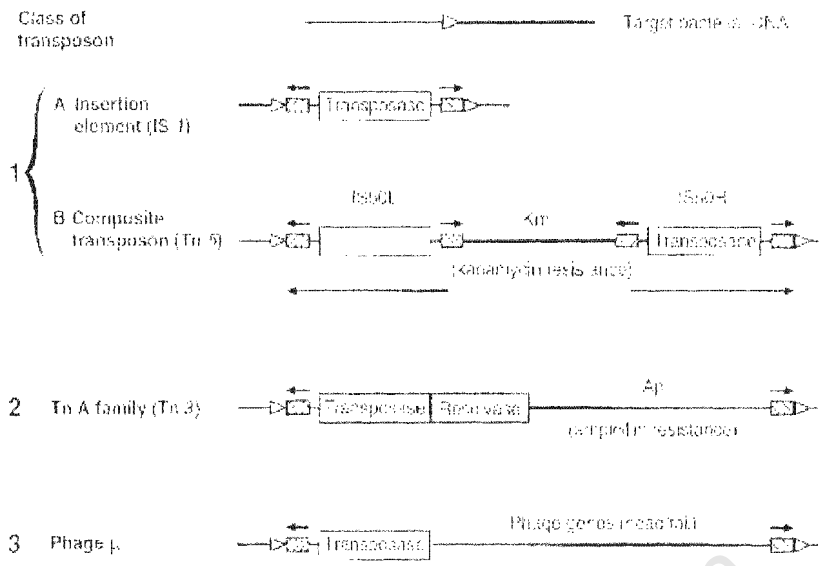


Figure 1.1.4: The classification of transposons. Transposons can be divided into three classes, based on their structure. Class 1 transposons include insertion sequences, which contain only a transposase gene, and composite transposons that can harbour gene cassettes; Class 2 transposons encode an additional resolvase gene that facilitates recombination, and Class 3 transposons are derived from bacteriophage Mu. (Adapted from Holmes & Jobling, 1996)

Transposons have been detected within the chromosome of *P. aeruginosa* since the early 1980s when Tn*2521*, encoding resistance to the antibiotics carbenicillin, streptomycin, spectinomycin and sulphanilamide, was discovered (Sinclair & Holloway, 1982) A transposon of particular interest in the CF-associated subgroup C *P. aeruginosa* strains is TNCP23 (Klockgether *et al*, 2004). This large class 1 transposon is inserted upstream of the *pil* operon, within the integrated pKLC102, and is flanked by an IS element, IS*6100* (Klockgether *et al*, 2004). The presence of TNCP23 exclusively in clone C, subgroup C strains of *P. aeruginosa*, also the only subgroup to contain only the chromosomal form of pKLC102, suggests that the transposon may be involved in the immobilisation of the plasmid within the chromosome (Römling *et al*, 1997b).

1.5.3 Integrons

Three classes of integrons harbouring antibiotic resistance markers have been described to date (Partridge *et al*, 2001), with class 1 integrons, carrying gene cassettes encoding resistance to a large number of antibiotics, most commonly found in *P. aeruginosa* (Fonseca *et al*, 2006; Yatsuyanagi *et al*, 2004; Poirel *et al*, 2001;

Naas *et al*, 1999a; Tribuddharat & Fennewald, 1999) [Fig. 1.1.5]. Class 1 integrons possess a recombination site, *attI*, at which one or more gene cassettes can be integrated due to a single site-specific recombination event with a 59-base element at the 3` end of the cassette (Collis & Hall, 1995). The integrated gene cassettes are flanked by two conserved sequences (CS) (Nesvera *et al*, 1998). The 3`-CS encodes three genes: *qacEΔ1* encodes antiseptics resistance, *sulI* confers sulfonamide resistance, *ORF5* encodes an acetyltransferase (Nesvera *et al*, 1998). The 5`-CS consists of the integrase gene, *int1*, transcribed from P_{int} on the antisense strand and two other promoters, P_{ant} and P2, which allow transcription of the integrated gene cassettes (Collis & Hall, 1995). Gene cassettes near P_{ant} are transcribed to a greater extent than those nearest the 3` CS, and in the presence of the secondary promoter P2, which is not present in all integrons, a further increase in the level of transcription is observed (Collis & Hall, 1995).

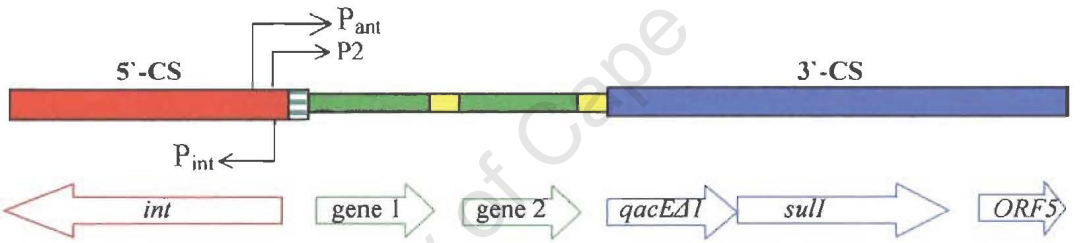


Figure 1.1.5: The general structure of class 1 integrons. The 5`-CS, shown in red, contains the gene for integrase (*int*); the 3`-CS, shown in blue, encodes antiseptics resistance (*qacEΔ1*), sulfonamide resistance (*sulI*) and an ORF of unknown function. Inserted are 2 gene cassettes, shown in green, and their 59-base elements are shown in yellow. The position of *attI* is shown by a block filled with horizontal stripes. The direction of transcription of all genes is shown by arrows. Promoter P_{ant} is responsible for gene cassette expression, while the divergently transcribed integrase gene is under the control of promoter P_{int}. P2 represents a secondary promoter, only present in some integrons.
(Adapted from Nesvera *et al*, 1998 and Collis & Hall, 1995)

The carbapenem group of β-lactam antibiotics, which includes imipenem and meropenem, are notorious for their antipseudomonal activity and their stability to hydrolysis by the enzymes produced by *P. aeruginosa* (Müller-Premru & Lejko-Zupanc, 2002; Livermore, 2001; Tausk *et al*, 1985). The emergence of resistance to this recently described group of antibiotics in *P. aeruginosa*, however, occurred rapidly after their introduction into the hospital setting (Sasaki *et al*, 2004; Torres *et al*, 2000; Caldwell *et al*, 1999). The genes encoding enzymes that display high levels of activity against the carbapanems, such as IMP- and VIM-type enzymes, are

frequently described worldwide in *P. aeruginosa* in association with mobile class 1 integrons (Patzer *et* al, 2004; Sardelic *et al*, 2003; Lee *et al*, 2002; Poirel *et al*, 2001; Poirel *et al*, 2000; Lauretti *et al*, 1999), which accounts for the rapid dissemination of these carbapenem-hydrolysing enzymes.

The region adjacent to the left copy of IS*6100*, flanking TNCP23, is homologous to a class 1 integron, In7 (Nesvera *et al*, 1998), that characteristically has a 3`-CS that ends 24bp after the *sul1* stop codon (Arduino *et al*, 2003). This integron is 4.8kb in size, with an intact 3'-CS and a truncated *int1* that is probably not functional (Klockgether *et al*, 2004). Inserted into the *att1* recombination site is a gene cassette encoding an aminoglycoside-adenyltransferase, *aadB*, which confers resistance to gentamicin and tobramycin (Klockgether *et al*, 2004). The genes on TNCP23 external to the integron encode products required for typical plasmid maintenance, and appear to confer no survival advantage on *P. aeruginosa* (Klockgether *et al*, 2004). This suggests that the immobilisation of TNCP23 within the genome of *P. aeuginosa* subclone C strains may have occurred in order to maintain this In7-like integron in the absence of its own functional integrase.

1.5.4 Insertion Sequences

IS elements are the simplest form of a class 1 transposon [Fig.1.1.4] in that they encode only the genes necessary for their transposition (Holmes & Jobling, 1996). The first IS detected within the *P. aeruginosa* chromosome was IS*22* (Nash & Krishnapillai, 1982). A number of IS elements have since been described in this organism, including IS*21* and IS*26*, both of which were associated with increased antibiotic resistance (Boutoille *et al*, 2004; Naas *et al*, 1999b).

IS elements are also known to cause spontaneous mutations (Horst *et al*, 1999), and result in changes in gene expression (Boutoille *et al*, 2004; Bagge *et al*, 2002). In addition, they have been shown to cause genetic rearrangements of bacterial chromsomes (Güneş *et al*, 1999). Inversion of up to 92% of the *P. aeruginosa* genome has been reported in CF isolates from three of the four clone C subgroups (Römling *et al*, 1997a), and the inversion endpoints seem to be located in regions of

the *P. aeruginosa* clone C genome that are absent in *P. aeruginosa* PAO (Römling *et al*, 1997a; Schmidt *et al*, 1996). Strains displaying large chromosomal inversions (LCIs) were all CF strains from subgroups C, C2 and C13, whilst the environmental SG17M group displayed no inversions (Römling *et al*, 1997a), suggesting that this phenomenon is not a random occurrence (Kresse *et al*, 2003). All *P. aeruginosa* clone C strains have three regions that are more susceptible to variation, termed hypervariable regions (Römling *et al*, 1997b), and all three regions are found within blocks of DNA absent from *P. aeruginosa* PAO (Römling *et al*, 1997b; Schmidt *et al*, 1996). They include plasmid pKLC102, the region between *lipA* and *phoA-I* and the region adjacent to *toxR* (Römling *et al*, 1997b). One common feature of the LCIs in *P. aeruginosa* clone C is that all inversions occurred in regions other than around *oriC* and the position of *Ter* was not altered significantly (Römling *et al*, 1997b).

The transposon TNCP23, located exclusively within the chromosomally integrated pKLC102 in subgroup C *P. aeruginosa* CF strains, is flanked by IS*6100* (Klockgether *et al*, 2004), an 880bp element flanked by 17bp inverted repeats (Klockgether *et al*, 2004; Sundin & Bender, 1995). IS*6100* has been shown to account for the LCIs occurring in *P. aeruginosa* clone C strains from subgroup C (Kresse *et al*, 2003). The absence of this element in subgroups C2 and C13 prompted investigation into the cause of the LCIs observed in CF isolates from these groups and a novel IS element of the IS*3* family, IS*Pa20*, was found to be associated with inversions in subgroup C13 only (Kresse *et al*, 2006). IS*Pa20* is approximately 1.2kb in size and consists of two ORFs, one of which has homology with the transposase gene of *Shigella flexneri* serotype 2a strain 2457T, flanked by 26bp inverted repeats (Kresse *et al*, 2006).

1.6 The aim of this study

The large genome size and complex genetic rearrangements of *P. aeruginosa* seem to confer a selective growth advantage in a wide range of habitats. Of particular concern is the ability of this pathogen to adapt so readily to the environment encountered in the lungs of CF patients. It has recently been documented that adaptation in *P. aeruginosa* CF isolates of the major CF-associated subclone C group occurs, at least in part, due to the presence of IS*6100* (Kresse *et al*, 2003). In addition, a large percentage of CF *P. aeruginosa* strains are hypermutable, which facilitates rapid adaptation to the CF lung environment (Oliver *et al*, 2000). The aim of this study was to determine the prevalence of IS*6100* in *P. aeruginosa* isolates from CF patients in two local hospitals, and to investigate their role in the conversion to the mutator phenotype.

CHAPTER 2

SCREENING FOR THE PRESENCE OF IS*6100* AND GENOTYPING OF *P. aeruginosa* ISOLATES

2.1 Introduction

P. aeruginosa is an important human pathogen, especially in the case of cystic fibrosis infections (Speert *et al*, 2002; Govan & Deretic, 1996). Of particular interest are the phenotypic changes that *P. aeruginosa* undergoes when infecting a CF patient. The major changes include biofilm production (Hentzer *et al*, 2001), conversion to mucoidy (Govan & Deretic, 1996) and hypermutability (Oliver *et al*, 2000), and these changes appear to confer a survival advantage on *P. aeruginosa* in the CF lung environment.

It has been reported that up to 50% of *P. aeruginosa* CF isolates display large chromosomal inversions (LCIs) (Schmidt *et al*, 1996) of up to 6kb (Römling *et al*, 1997a). An insertion sequence, IS*6100*, is associated with rearrangements of the bacterial genome (Güneş *et al*, 1999) and has recently been shown to be associated with such genomic rearrangements in *P. aeruginosa* CF isolates (Kresse *et al*, 2003). IS*6100* was first described in *Mycobacterium fortuitum* in 1990 (Martin *et al*), and has since been associated with genes involved in the degradation of nylon oligomers in *Flavobacterium* and *Pseudomonas* (Kato *et al*, 1994), and with increased streptomycin resistance in *Xanthomonas campestris* pv. vesicatoria (Sundin & Bender, 1995).

IS*6100*, which belongs to the IS*6* family of IS elements (Günes *et al*, 1999; Martin *et al*, 1990) is an 880bp element that is flanked by two 17bp imperfect inverted repeats with one mismatch (Klockgether *et al*, 2004; Sundin & Bender, 1995) [Fig. 2.1.1]. Integration of IS*6100* does not appear to be site-specific, as the regions both upstream and downstream of previously described insertion sites are not homologous (Kato *et al*, 1994; Martin *et al*, 1990) [Fig. 2.1.1].

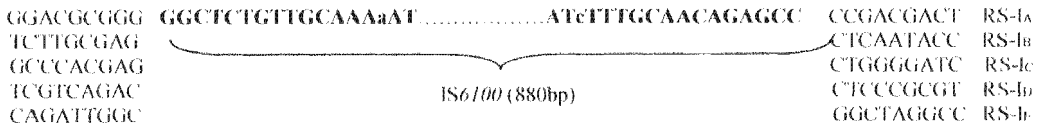


Figure 2.1.1: IS*6100* integration into random DNA sequences. IS*6100* integration is non-specific, as is demonstrated by the non-homology of surrounding sequences. This element is flanked by two 17 bp inverted repeats, shown in bold; the mismatched nucleotide is shown in lower case letters. Strains in which IS*6100* was located are indicated on the right of the flanking sequences. (Adapted from Kato *et al*, 1994).

The spread of a major *P. aeruginosa* clone within the CF setting could have devastating effects, due to the difficulty in eradicating this organism from patients with CF. For this reason, methods of genotyping *P. aeruginosa* clinical isolates have been extensively investigated in order to facilitate accurate monitoring of the epidemiology of this organism, and to aid in identifying the source of such an outbreak should one occur.

Two methods that are popularly used in typing *P. aeruginosa* are random amplified polymorphic DNA (RAPD) typing (Menon *et al*, 2003; Elaichouni *et al*, 1994; Bingen *et al*, 1993) and pulsed-field gel electrophoresis (PFGE) (Grothues *et al*, 1988; Jung *et al*, 2002). RAPD typing makes use of a single primer that binds to random sequences within the bacterial genomic DNA to generate specific banding patterns, following amplification by a polymerase chain reaction (PCR) assay. More discriminatory results can be obtained by using primers designed specifically to bind to the genome of the organism under investigation (Mahenthiralingam *et al*, 1996; van Belkum, 1994). PFGE involves the *in situ* lysis of bacterial cells and digestion of genomic DNA with an enzyme that cuts the genome infrequently. The application of an alternately pulsing electric field allows the large genomic DNA fragments generated to be resolved, producing a unique banding pattern for each strain.

Comparitive genotyping of *P. aeruginosa* by these two methods has revealed RAPD to be as discriminatory as PFGE (Renders *et al*, 1996; Kersulyte *et al*, 1995), although single-band differences in RAPD profiles may sometimes need to be overlooked in order for complete correlation with PFGE-derived genotypes (Renders

et al, 1996). As RAPD is a much less labour-intensive and more rapid procedure, it has been recommended that this method be used for preliminary screening of large numbers of isolates (Mahenthiralingam *et al*, 1996), but that genotyping by PFGE be performed in order to confirm results (Renders *et al*, 1996).

To investigate the prevalence of IS*6100* in CF *P. aeruginosa* isolates from local hospitals, PCR assays were performed using IS*6100*-specific primers. DNA-DNA hybridisation studies were then carried out to confirm the presence of IS*6100* in the *P. aeruginosa* strains investigated. Genotyping of *P. aeruginosa* strains harbouring IS*6100* was performed using RAPD typing and PFGE analysis to establish whether IS*6100*-containing isolates in local hospitals are related and may descend from a common dominant clone.

2.2 Experimental Procedures

2.2.1 Bacterial strains and growth conditions

P. aeruginosa strains were isolated from patients at Groote Schuur Hospital (GSH) and Red Cross War Memorial Children's Hospital (RX) in Cape Town over a period of 6 years, ranging from 2000 to 2006 [Table 2.3.1]. A total of 32 *P. aeruginosa* isolates were obtained from sputa of CF patients and 48 strains were isolated from various sites of infection [Table 2.3.1]. *P. aeruginosa* PAO1 (Stover *et al*, 2000) was included as a control strain when necessary. All strains were grown aerobically on Mueller-Hinton (M-H) agar [Oxoid] or in M-H broth [Oxoid] with shaking at 37°C.

2.2.2 Antibiotic disc susceptibility testing

The antibiotic susceptibilities of the *P. aeruginosa* strains included in this study were determined by the Kirby Bauer disc diffusion test on M-H agar at 37°C overnight. Zones of growth inhibition caused by diffusion of antibiotic from discs into the agar indicate levels of resistance. The disc susceptibilities were interpreted according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS, 1997).

2.2.3 Genomic DNA preparation

Two methods were used to extract genomic DNA from all strains.

2.2.3.1 CTAB DNA Extraction

Genomic DNA was obtained from each strain by phenol:chloroform extraction and ethanol precipitation according to the method of Ausubel *et al* (1987). From a single colony on M-H agar, *P. aeruginosa* cells were grown to saturation in 5ml M-H broth for ± 18 hours. An aliquot of 1.5ml of culture was microfuged [Eppendorf Centrifuge 5417C] at 14000 rpm for 2 minutes and the resulting bacterial pellet was resuspended in 567µl Tris-EDTA (TE) buffer (Appendix A). Following resuspension of the pellet by repeated pipetting, the cells were lysed in 0.5% (w/v) Sodium Dodecyl Sulphate (SDS). Proteinase K was added at a concentration of 100mg/ml to denature the cellular proteins released following cell lysis. The suspension was mixed thoroughly and incubated at 37°C for 1 hour to allow cell lysis and protein digestion to occur. Following lysis, 100µl of 5M sodium chloride (NaCl) was added to increase the salt concentration prior to the addition of 80µl of hexadecyl trimethylammonium bromide (CTAB)/NaCl (Appendix A) and incubation at 65°C for 10 minutes. Under conditions of high salt concentration CTAB does not complex with nucleic acids, but becomes complexed to denatured proteins and polysaccharides, causing them to precipitate out of solution. An equal volume of chloroform:isoamyl alcohol (24:1) was added to extract CTAB-complexes, and the solution was microfuged at 14000 rpm for 5 minutes. Following centrifugation, the aqueous solution located above a white interface was removed and the DNA contained within this aqueous phase was further extracted by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The solution was centrifuged for 5 minutes at 14000 rpm and the DNA was precipitated by addition of 0.6 volumes isopropanol to the recovered upper aqueous phase. DNA was collected by centrifugation at 14000 rpm for 5 minutes, and the resulting DNA pellet was washed with 70% ethanol to remove any residual CTAB and centrifuged as before. After removal of the supernatant, the DNA pellet was allowed to air-dry for 10-15 minutes at room temperature and then resuspended in 50µl TE buffer (Appendix A) at 4°C overnight.

2.2.3.2 Genomic DNA Extraction Kit

The Wizard® Genomic DNA Purification Kit [Promega] was used to extract total genomic DNA. A 1.5ml aliquot of *P. aeruginosa* grown overnight in M-H broth was pelleted by centrifugation and resuspended in 600µl Nuclei Lysis Solution [Promega]. Lysis occurred during incubation at 80°C for 5 minutes, after which the suspension was cooled to room temperature. A volume of 3µl RNase solution [Promega] was added and the mixture was incubated for 1 hour at 37°C. Once samples had cooled to room temperature, 200µl of Protein Precipitation Solution [Promega] was added and mixed with the cell lysate by vortexing for 20 seconds. Samples were then held on ice for 5 minutes and centrifuged for 3 minutes at 14 000 rpm to remove precipitated protein from the suspension. The recovered supernatant containing the DNA was transferred to a tube containing 600µl of room temperature isopropanol. Solutions were gently mixed by inversion until DNA became visible, due to precipitation by the isopropanol, and then centrifuged for 2 minutes at 14 000 rpm to pellet the precipitated DNA. Following centrifugation the supernatant was removed and 600µl of room temperature 70% ethanol was added to wash the DNA. The samples were centrifuged again for 2 minutes at 14 000 rpm and after removal of the supernatant and air-drying of the DNA pellet, DNA was resuspended in 100µl DNA Rehydration Solution (10mM Tris-HCl, 1mM EDTA) [Promega] and stored at 4°C to resuspend until required.

2.2.4 Agarose Gel Electrophoresis and Nucleic Acid Visualisation

Agarose gel electrophoresis resolves DNA fragments on the basis of size due to differing migration rates in agarose gel (Ausubel *et al*, 1987). DNA carries a negative charge and therefore, upon application of an electric field, smaller DNA fragments will migrate through the pores in the agarose gel toward the cathode more rapidly than larger fragments. Separation and visualisation of nucleic acid products was carried out by electrophoresis on gels consisting of 1% (w/v) agarose dissolved in 1X Tris-acetate EDTA (TAE) buffer (Appendix A) by heating in a microwave oven. Ethidium bromide (EtBr) is a compound that intercalates between DNA bases and fluoresces upon exposure to ultra-violet (UV) light, thus allowing visualisation of the nucleic acids after agarose gel electrophoretic separation. EtBr was added to the

agarose to a final concentration of 10ng/µl. Gel tracking dye (Appendix A) was added to all DNA samples prior to loading into the wells, which serves three purposes; the bromophenol blue allows the migration of the samples to be monitored, the sucrose increases the density of the sample, ensuring that it drops evenly into the well and remains there, and the EDTA chelates Mg^{2+} ions that are required for enzymatic activity, thereby inhibiting further enzymatic activity within the sample. Molecular weight marker (MWM) Hyperladder I [Bioline] (Appendix B) was loaded alongside samples to enable DNA fragment size and concentration determination. Visualisation of nucleic acid products was carried out using a Fotodyne Inc. UV light box (302nm) and the results were captured using a Kodak EDAS 290 camera.

2.2.5 DNA Purification and Quantification

2.2.5.1 DNA in aqueous solution

The DNA solution to be purified was diluted to a final volume of 100µl and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was vortexed for 10 seconds and then centrifuged [Eppendorf Centrifuge 5417C] briefly for about 30 seconds at room temperature. The upper aqueous phase containing the DNA was transferred to a clean tube, and the steps described above were repeated until no white protein precipitate was visible at the aqueous/organic interface. One tenth of the volume of the supernatant of 3M sodium acetate, pH 5.2, was added and the solution was mixed by flicking the tube. Three volumes of ice-cold 100% ethanol, calculated after the addition of the sodium acetate, were added and the solution was incubated at -70°C for 1 hour. Following centrifugation for 5 minutes at 14 000 rpm and removal of the supernatant, 1 ml of room temperature 70% ethanol was added to wash the DNA. Following centrifugation as before, the air-dried DNA pellets were resuspended in 10µl dH_20. Purified DNA was stored at 4°C.

2.2.5.2 DNA in agarose gels

DNA fragments were recovered from agarose gels and purified using a MinElute Gel Extraction Kit [Qiagen]. Fragments were excised from the gel and 3 volumes Buffer

QG (Solubilisation and Binding Buffer) [Qiagen] were added to 1 volume of gel slice. The samples were incubated at 50°C for 10 minutes, or until the gel slice had

dissolved, then 1 gel volume of isopropanol was added to precipitate the DNA. Each sample was applied to a MinElute column [Qiagen] and centrifuged for 1 minute at 14000 rpm to bind the DNA to the matrix within the columns. The flow-through was discarded and a further 500µl Buffer QG was added to the column and this was centrifuged for 1 minute at 14000 rpm. Once again the flow-through was discarded and 750µl of Buffer PE (5X Wash Buffer) [Qiagen] was applied to the column. Centrifugation at 14000 rpm for 1 minute served to wash the bound DNA and the flow-through was discarded and the column centrifuged as before to remove any residual traces of ethanol. Bound DNA was then eluted by the addition of 10µl of distilled water to the centre of the column, incubation at room temperature for 1 minute, and centrifugation for 1 minute at 14000 rpm. The purified DNA samples were stored at -20°C.

Quantification of DNA samples purified from agarose gels was carried out by electrophoresis of the purified products on 1% agarose gels alongside the MWM Hyperladder I [Bioline] (Appendix B). Hyperladder I is both a molecular weight and quantitative marker, with each band containing a known amount of DNA. Thus, the concentration of purified PCR products was estimated by comparison with the bands produced by Hyperladder I. Genomic DNA preparations were quantified both by electrophoresis alongside Hyperladder I [Bioline] and by spectrophotometry at a wavelength of 280nm. The amount of protein contamination in the genomic DNA preparations was determined by measuring the ratio 260nm/280nm. A ratio of greater than 1.8 indicated good quality DNA with minimal protein contamination.

2.2.6 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an enzymatic reaction that is used to amplify small quantities of specific DNA fragments to provide sufficient product for subsequent analysis (Marx, 1988). Primers that are homologous to the regions surrounding the target DNA sequence are designed. Following denaturation of the double stranded DNA template, the primers bind to their homologous sequences

during the annealing phase, and extension of the primer sequence occurs by a thermostable DNA polymerase from *Thermus aquaticus*, known as *Taq* polymerase. Thermostable *Taq* polymerase is used in PCR as it is able to withstand the high temperatures required during the denaturation step. Repeated cycles of denaturation, annealing and extension result in an exponential increase in the number of copies of the target DNA [Fig. 2.2.1].

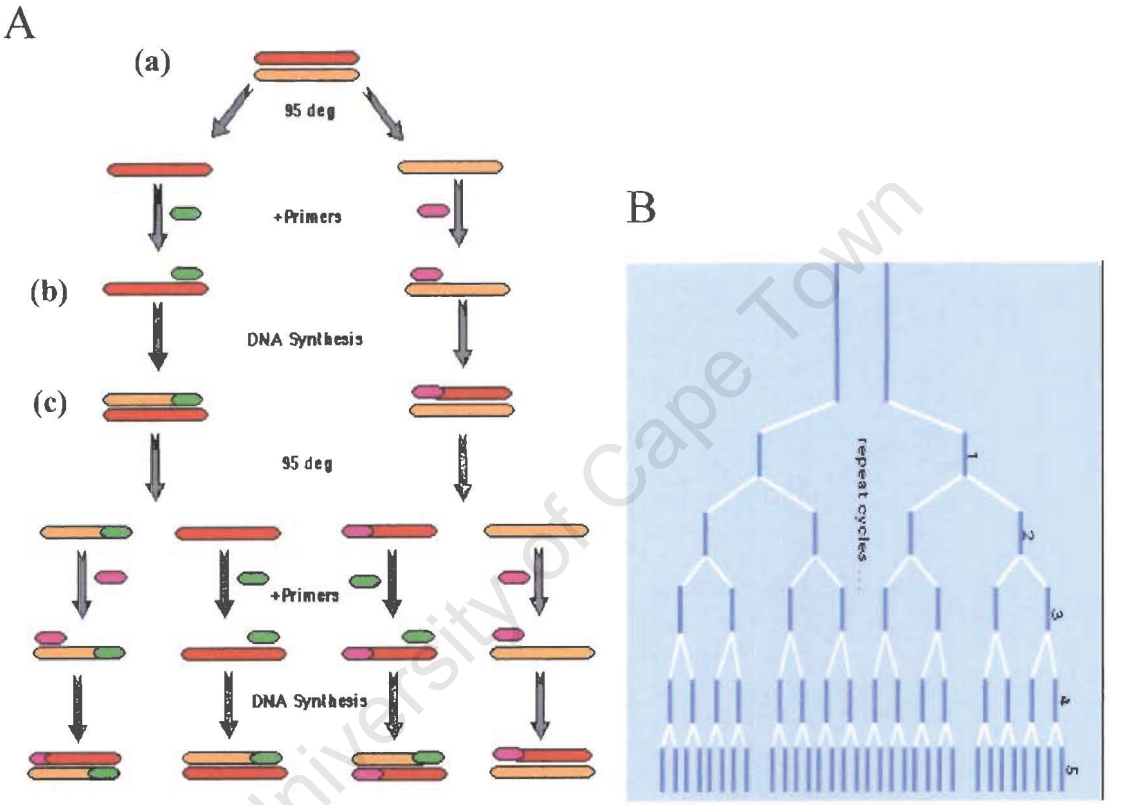
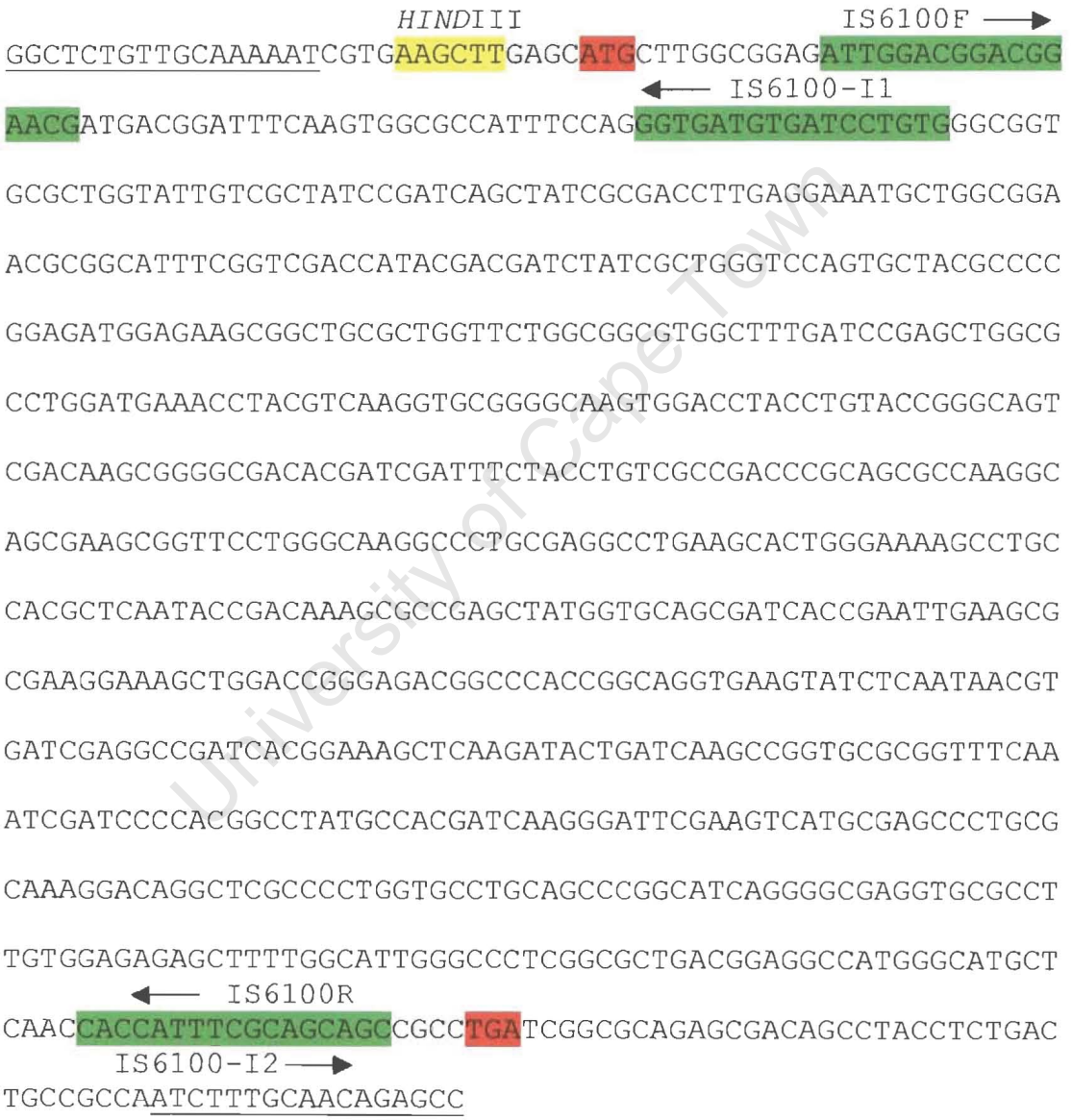


Figure 2.2.1: Schematic representation of the polymerase chain reaction.
A. The amplification reaction consists of repeated cycles of double-stranded DNA denaturation (a), primer annealing (b) and primer elongation (c).
(http://members.aol.com/BearFlag45/Biology1A/LectureNotes/lec24.html)
B. The polymerase chain reaction results in the exponential amplification of specific segments of DNA. (http://www.roche.com/pages/facets/pcr_e.pdf)

All PCR assays were to a final volume of 50µl and contained 0.2mM of each dNTP [Fermentas], 3mM $MgCl_2$ [Promega], 1X GoTaq PCR buffer [Promega], 20 pmoles of each primer [Fig. 2.2.2], 1.25U GoTaq® DNA polymerase [Promega] and approximately 200ng template genomic DNA. All primers used in this study were synthesised at the Synthetic DNA Laboratory of the University of Cape Town, using a Beckman 1000M DNA synthesiser on the high purity program. Typically,

following an initial denaturation step at 95°C for 5 minutes, the PCR reactions consisted of 35 cycles of denaturation at 95°C for 45 seconds, primer annealing at 56°C for 45 seconds and primer extension at 72°C for 1 minute. A final elongation step at 72°C for 5 minutes was performed to complete elongation of all unfinished or incomplete products. PCR assays were carried out using a GeneAmp PCR System 2400 [Perkin Elmer] thermocycler and all PCR products were visualised by agarose gel electrophoresis (2.2.4).



GGCTCTGTTGCAAAAATCGTGAAGCTTGAGCATGCTTGGCGGAGATTGGACGGACGG

AACGATGACGGATTTCAAGTGGCGCCATTTCCAGGGTGATGTGATCCTGTGGGCGGT

GCGCTGGTATTGTCGCTATCCGATCAGCTATCGCGACCTTGAGGAAATGCTGGCGGA

ACGCGGCATTTCGGTCGACCATACGACGATCTATCGCTGGGTCCAGTGCTACGCCCC

GGAGATGGAGAAGCGGCTGCGCTGGTTCTGGCGGCGTGGCTTTGATCCGAGCTGGCG

CCTGGATGAAACCTACGTCAAGGTGCGGGGCAAGTGGACCTACCTGTACCGGGCAGT

CGACAAGCGGGGCGACACGATCGATTTCTACCTGTCGCCGACCCGCAGCGCCAAGGC

AGCGAAGCGGTTCCTGGGCAAGGCCCTGCGAGGCCTGAAGCACTGGGAAAAGCCTGC

CACGCTCAATACCGACAAAGCGCCGAGCTATGGTGCAGCGATCACCGAATTGAAGCG

CGAAGGAAAGCTGGACCGGGAGACGGCCCACCGGCAGGTGAAGTATCTCAATAACGT

GATCGAGGCCGATCACGGAAAGCTCAAGATACTGATCAAGCCGGTGCGCGGTTTCAA

ATCGATCCCCACGGCCTATGCCACGATCAAGGGATTCGAAGTCATGCGAGCCCTGCG

CAAAGGACAGGCTCGCCCCTGGTGCCTGCAGCCCGGCATCAGGGGCGAGGTGCGCCT

TGTGGAGAGAGCTTTTGGCATTGGGCCCTCGGCGCTGACGGAGGCCATGGGCATGCT

CAACCACCATTTCGCAGCAGCCGCCTGATCGGCGCAGAGCGACAGCCTACCTCTGAC

TGCCGCCAATCTTTGCAACAGAGCC

Figure 2.2.2: Nucleotide sequence of IS*6100* (880bp). The 17bp inverted repeats are underlined; primers used in IS*6100* amplification (IS6100F & IS6100R) and inverse PCR (IS6100-I1 & IS6100-I2) are shaded green; arrows indicate primer orientation (IS6100R and IS6100-I2 primers are identical in sequence, but orientated in the opposite direction); the start (ATG) and stop (TGA) codons of the transposase gene, *tnpA*, are shaded red; the *Hind*III site, used in cloning IS*6100*, is shaded yellow.

2.2.7 DNA Sequencing and Analysis

Cycle sequencing of PCR amplified products was performed using the ABI Prism® BigDye™ Primer Cycle Sequencing Ready Reaction Kit [Applied Biosystems]. A sequencing enzyme derived from *Thermus aquaticus* DNA polymerase is used in the cycle sequencing reaction. This AmpliTaq® DNA polymerase contains a mutation in the active site that results in less discrimination against dideoxynucleotides, and another mutation in the amino terminal domain that almost entirely eliminates the 5`→3` nuclease activity. Dideoxynucleotides that are labelled with dyes with different emission spectra are sequentially added to the 3' terminus of the growing chain, terminating the chain elongation, and the sequence of the product can then be determined based on the emission spectra. This procedure is based on the automated Sanger dideoxy sequencing reaction protocol (Zimmermann *et al*, 1988). The results were obtained in the form of an electropherogram [Fig. 2.2.3], which contains the sequence of the product, and were viewed using Chromas 2.31 [Technelysium Pty Ltd].

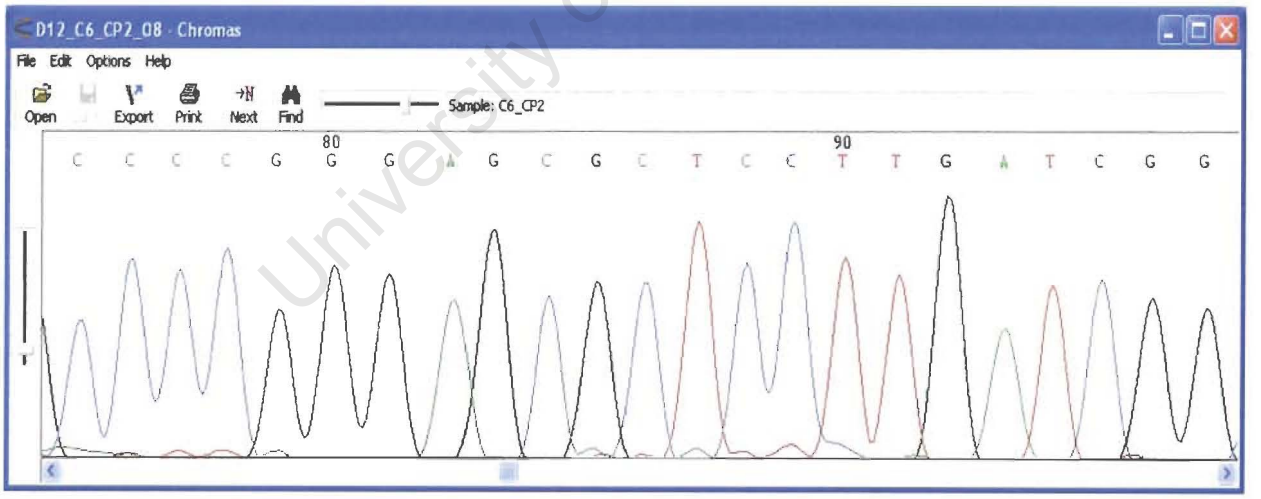


Figure 2.2.3: An electropherogram displaying a region of DNA sequence. The termination of chain elongation by the sequential addition of differently labelled dideoxynucleotides (ddNTPs) allows the sequence to be determined. Each ddNTP has a unique emission spectra and is therefore identified by the colour it emits. C- cytosine; G – guanine; A – adenosine; T – thymidine.

Automated sequencing of purified PCR products was carried out at the GeneCare Molecular Genetics Sequencing Division using an ABI 3130 Genetic Analyser. DNA sequences were analysed using DNAMAN [version 4.0, Lynnon Biosoft], and sequences obtained after analysis were compared with sequences in the database using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*, 1997).

2.2.8 DNA-DNA Hybridisations

2.2.8.1 Transfer of DNA

a) Slot blot

Five micrograms of genomic DNA extracted from the *P. aeruginosa* isolates included in this study was transferred onto positive Hybond^{TM}-N^{+} membrane by a vacuum, using the Minifold II Slot-Blot Apparatus [Schleicher and Schuell; Germany]. Distilled water was added to each sample to a volume of 50µl, and the DNA was denatured by heating at 95°C for 10 minutes. Samples were immediately placed on ice to prevent renaturation. An equal volume of transfer buffer, 20X SSC (Appendix A), was added to the DNA. The transfer buffer is required to provide the ionic strength that is required to bind the negatively charged DNA to the positively charged nylon membrane. A sheet of Whatman 3MM paper and the Hybond^{TM}-N^{+} membrane were prewet with 10X SSC. The slot-blot apparatus was assembled by placing the prewet membrane on top of the filter paper and sealing the apparatus as described by the manufacturers. Samples were loaded into individual slots, and a vacuum was applied until all samples had been transferred onto the membrane. The apparatus was disassembled and the DNA bound to the membrane was denatured by placing the membrane on top of a piece of filter paper soaked in a solution containing 1.5M NaCl and 0.5M NaOH for 5 minutes. The membrane was then soaked in a solution of 0.5M Tris pH 7.2, 1.5M NaCl and 1mM EDTA for 5 minutes to neutralise the alkalinity of the denaturing buffer. The transferred DNA was cross-linked to the membrane by UV light for 30 seconds at a wavelength of 254nm [Hoefer Scientific Instruments; California, USA], and the membrane was stored at 4°C in a sealed plastic sleeve.

b) Southern blot

Southern blotting was first described in 1975 by Edward Southern. The process involves the transfer of digested genomic DNA onto a positively charged membrane in order to detect specific sequences within the DNA by hybridisation with a labelled homologous probe. The process is illustrated in Figure 2.2.4.

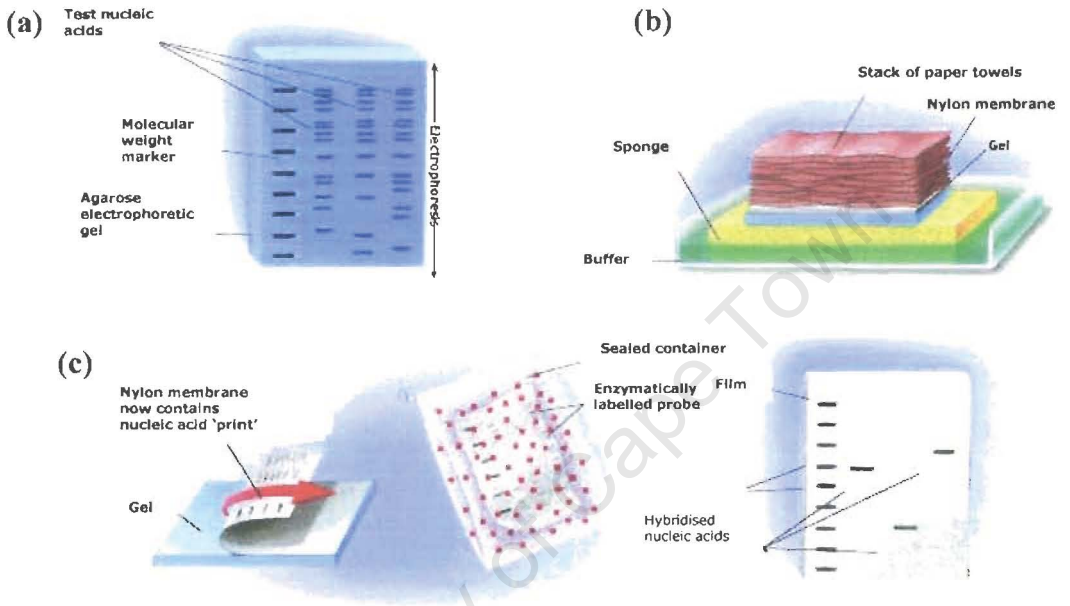


Figure 2.2.4: The process of DNA-DNA hybridisation by the Southern technique. (a) Genomic DNA is digested with a restriction enzyme that does not cut within the sequence of interest and electrophoresed on agarose gel; (b) negatively charged DNA binds to a positively charged membrane following transfer via capillary action; (c) an enzymatically labelled probe is allowed to hybridise to complementary DNA bound to the membrane and the presence of such complementary sequences is detected by autoradiography.

Genomic DNA (5µg) was digested with 10U of restriction enzyme (RE) *Bam*HI in a volume of 30µl for 5 hours at 37°C. *Bam*HI was used as it does not cut within the IS*6100* probe sequence and therefore would not generate two DNA fragments containing portions of the same copy of IS*6100*, which would both produce a signal and give the impression of an inflated number of copies of IS*6100*. The digested genomic DNA was electrophoresed on 1% agarose at low voltage for approximately 20 hours to ensure sufficient separation of fragments. Following electrophoresis, the agarose gel was washed in 0.25M HCl (Appendix A) for 30 minutes at room temperature with gentle agitation. This process, known as depurination, serves to

remove purines (A & G) from the separated DNA fragments, making the transfer of larger fragments onto the membrane more efficient. The gel was then placed into denaturing buffer (Appendix A) and incubated for 20 minutes at room temperature with agitation. This denaturing step facilitates the conversion of the DNA from the double-stranded form to the transferable single-stranded form, and was repeated twice. A further 20 minute wash is performed with agitation at room temperature in neutralising buffer (Appendix A), which is essential to neutralise the alkalinity of the denaturing buffer. This wash was also repeated twice.

The digested genomic DNA was transferred onto a positive Hybond^{TM}-N^{+} membrane [Amersham Biosciences] by capillary transfer for approximately 20 hours. The agarose gel was placed on top of three sheets of Whatman 3MM paper that had been soaked in 20X SSC (Appendix A), and that had ends protruding into a container containing 20X SSC. A positively charged nylon membrane, which had been pre-soaked in 5X SSC, was placed on top of the gel and a further three sheets of Whatman 3MM paper soaked in 20X SSC were placed on top of the membrane. A layer of absorbent towel approximately 5cm thick was placed above the Whatman 3MM paper, and a weight of 0.5-1kg was placed on top of this. The whole apparatus was covered in Saran Wrap in order to prevent evaporation of the transfer 20X SSC and left overnight. The DNA was cross-linked to the membrane after transfer by placing the membrane under UV light for approximately 30 seconds at a wavelength of 254nm in a Hoefer Scientific Instruments (California, USA) UV Cross-linker. The membrane was wrapped in Saran Wrap and stored at 4°C until required.

2.2.8.2 Preparation of IS*6100* probe

A 758bp fragment encoding the transposase gene of IS*6100* was amplified by PCR (2.2.6) using the primers IS6100F and IS6100R [Fig. 2.2.2]. The amplified product was electrophoresed on 1% agarose (2.2.4) and subsequently purified and quantified (2.2.5.2). Labelling of the IS*6100* probe was performed according to the protocol of the ECL Direct Nucleic Acid Labelling and Detection System [Amersham Biosciences] [Fig. 2.2.5]. The purified IS*6100* probe was diluted to a final concentration of 10ng/µl. A total of 100ng of probe DNA was denatured by placing in a boiling waterbath for 10 minutes and then cooled immediately on ice for

5 minutes to prevent renaturation. An equivalent volume of the labelling reagent, containing positively charged horseradish peroxidase complexes, was added and this binds loosely to the negatively charged probe. The addition of gluteraldehyde at a volume equivalent to that of the labelling reagent and incubation at 37°C for

10 minutes results in the covalent cross-linking of the peroxidase enzyme to the probe DNA. The labelled probe was used in hybridisation experiments to determine the number of copies of IS*6100* present in the *P. aeruginosa* strains.

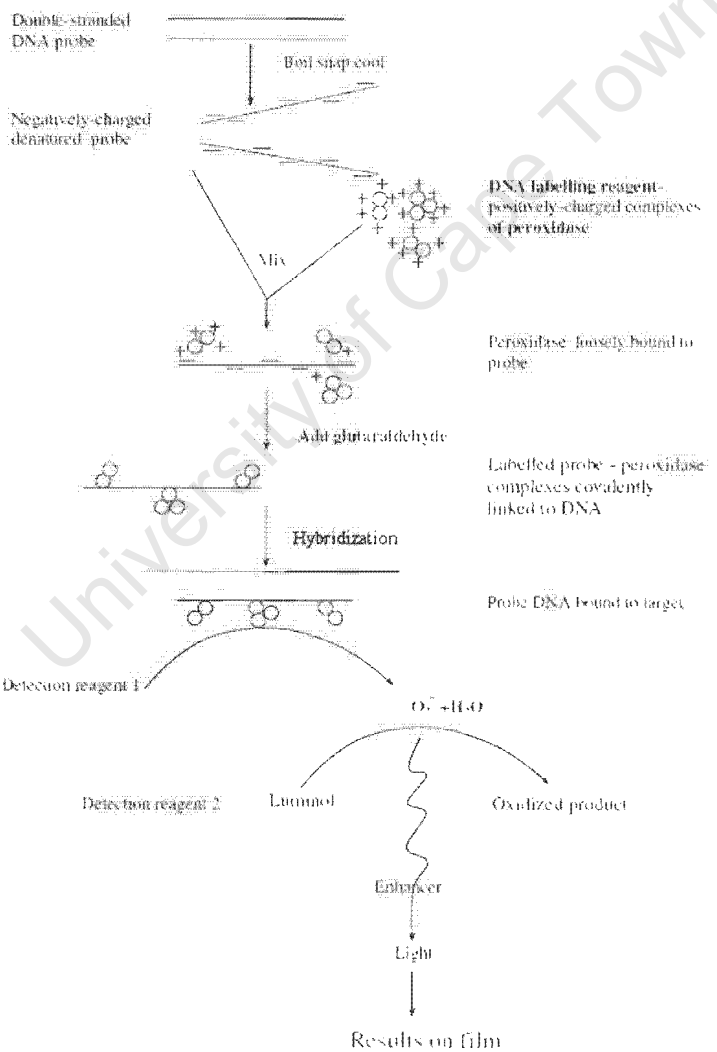


Figure 2.2.5: The principles of the ECL Direct Nucleic Acid Labelling Kit.
(adapted from the manual of the ECL Labelling Kit)

2.2.8.3 DNA-DNA Hybridisation

The hybridisation procedure used to immobilise the labelled IS*6100* probe to the genomic DNA bound to the nylon membrane was performed according to the protocol of the ECL direct nucleic acid labelling and detection system [Amersham Biosciences]. Hybridisation buffer (Appendix A) at a volume equivalent to 0.125ml/cm^2 of membrane area was prepared. A blocking agent supplied by the manufacturers was added to a final concentration of 5% (w/v) in order to block sites on the membrane where the probe may bind non-specifically. NaCl was added to the hybridisation buffer to a final concentration of 0.5M, which is the recommended salt concentration for effective probe hybridisation. The solution was mixed for 1 hour at room temperature on a magnetic stirrer to ensure complete suspension of the blocking agent, and the buffer was then heated to 42°C for 1 hour, the temperature at which the hybridisation was performed. Prehybridisation, during which the hybridisation buffer is applied to the membrane to allow blocking of non-specific binding sites was performed in a Hybridiser HB-1D oven [Techne, Cambridge, UK] for 1 hour at 42°C. Following prehybridisation, the labelled IS*6100* probe was added to the prehybridisation buffer and hybridisation was allowed to proceed overnight at 42°C.

Once hybridisation had occurred, the membrane was washed in order to remove any traces of unbound labelled probe that would interfere with signal detection. The membrane was first rinsed in 5X SSC for 5 minutes at 42°C, then washed with primary wash buffer (Appendix A) that had been pre-heated to 42°C. Washing proceeded for 20 minutes at 42°C, after which the buffer was discarded and replaced with an equivalent volume for a further 10 minute wash. This second wash was repeated twice. The membrane was then washed in secondary wash buffer (2X SSC) for 5 minutes at room temperature with gentle agitation, and this wash was repeated twice. The membrane was covered with Saran wrap and stored at 4°C to maintain moisture.

2.2.8.4 Signal Generation and Detection

Signal generation and detection were performed according to the protocol of the ECL labelling system. Detection reagents 1 and 2 were mixed in equal volumes to give a final volume of approximately 0.125ml/cm^2 of membrane area. Detection reagent 1 decays to hydrogen peroxide, which is the substrate for the enzyme peroxidase, with which the IS*6100* probe was labelled [Fig. 2.2.5]. Detection reagent 2 contains luminol, which produces light upon oxidation, and is coupled to the reduction of hydrogen peroxide by peroxidase. The membrane was covered with the detection reagents and incubated for 1 minute at room temperature, after which excess detection reagent was drained off. The membrane was sealed between two pieces of Saran wrap with care to avoid the formation of any air pockets, and exposed to X-ray film [Amersham Biosciences] in a developing cassette for time periods ranging between 1 minute and 24 hours. Following exposure, the X-ray film was developed and the image was recorded using a Kodak EDAS 290 camera.

2.2.9 Random Amplified Polymorphic DNA Typing

Random amplified polymorphic DNA (RAPD) typing was performed in order to determine the genetic relatedness of *P. aeruginosa* isolates included in this study. Amplification of the genomic DNA template was performed using the previously described primer 272 (5`-AGCGGGCCAA-3`) (Mahenthiralingam *et al*, 1996) in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. RAPD reactions consisted of 0.25mM of each dNTP [Fermentas], 1.5mM $MgCl_2$ [Promega], 1X GoTaq PCR buffer [Promega], 40 pmoles primer 272, 1U GoTaq® DNA Polymerase [Promega] and approximately 200ng template genomic DNA in a final volume of 25µl. The amplification reaction consisted of an initial 4 cycles of denaturation at 94°C for 5 minutes, primer annealing at 36°C for 5 minutes and primer extension at 72°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 36°C for 1 minute and primer extension at 72°C for 2 minutes. A final primer extension at 72°C for 10 minutes was included to ensure complete elongation of any unfinished products. RAPD products were electrophoresed on 1.5% agarose at 9V/cm and visualised (2.2.4).

2.2.10 Pulsed-Field Gel Electrophoresis

Following incubation overnight at 37°C on M-H agar, *P. aeruginosa* isolates were resuspended to an optical density (OD) of 0.7 at a wavelength of 600nm in 1ml SE buffer [75mM NaCl; 25mM EDTA]. A volume of 350µl of the suspension was mixed with an equal volume of 2% Seakem® Gold low melting point (LMP) agarose [Canbrex] dissolved in TE buffer [10mM Tris-HCl pH 8.0, 10mM EDTA; Appendix A] and transferred into plug moulds where plugs were allowed to set at 4°C. Plugs were removed from moulds and cells were lysed and proteins digested by incubation for 19.5 hours at 55°C in 3ml gram negative lysis buffer [1% (w/v) N-lauroyl sarcosine; 500mM EDTA, pH 8.0; 500 µg proteinase K]. Following lysis, plugs were washed 3 times for 30 minutes at 4°C in 3ml TE buffer and once for 60 minutes at 4°C in 3ml TE buffer. A 2mm portion was cut from the plug, covered with 100µl of 1X RE buffer and incubated at 4°C for 1 hour. Following incubation the RE buffer was replaced with 100µl of fresh 1X RE buffer, and 30U of RE *Spe*I was added. Plugs were digested for 20 hours at 37°C. The remaining plugs were stored at 4°C in TE buffer until required. Digested plugs were electrophoresed on 1.2% PFGE agarose [Bio-Rad] dissolved in 1X Tris Borate EDTA (TBE) buffer [Appendix A]. Electrophoresis was at 200V for 22 hours in 0.5X TBE running buffer with an initial pulse time of 1 second and a final pulse time of 50 seconds.

2.3 Results

2.3.1 Antibiotic disc susceptibilities of *P. aeruginosa* strains

The disc susceptibilities for selected antibiotics of the 80 *P. aeruginosa* isolates included in this study are shown in Table 2.3.1.

Table 2.3.1: Disc susceptibility profiles for selected aminoglycoside and β-lactam antibiotics of CF and non-CF *P. aeruginosa* clinical isolates from patients at Groote Schuur Hospital and Red Cross War Memorial Children's Hospital.

Strain	Date of isolation	Hospital	Site of Isolation	Antibiotics						
				Gm	Ak	Tob	Caz	Fep	Imp	Mem
CF:										
277	2004/07/12	GSH	CF sputum	S	S	S	S	S	S	S
281	2004/07/13	RX	CF sputum	**R**	**R**	**R**	S	S	S	S
297	2004/07/26	GSH	CF sputum	**R**	**R**	**R**	S	S	**R**	S
312	2004/08/03	RX	CF sputum	**R**	**R**	S	S	S	S	S
324	2004/08/17	RX	CF sputum	**R**	**R**	S	S	S	S	S
327	2004/08/16	GSH	CF sputum	S	S	S	S	S	S	S
333	2004/08/19	GSH	CF sputum	S	S	S	**R**	**R**	**R**	**I**
503	2006/01/31	RX	CF sputum	S	S	S	S	S	S	S
504	2006/02/07	RX	CF sputum	S	S	S	S	S	S	S
505	2006/02/07	RX	CF sputum	**R**	**I**	S	S	S	S	S
506	2006/02/07	RX	CF sputum	**I**	**R**	S	S	**R**	**R**	S
507	2006/02/07	RX	CF sputum	S	**R**	S	S	S	S	S
508	2006/02/07	RX	CF sputum	**R**	**R**	S	S	S	S	S
509	2006/01/21	RX	CF sputum	S	S	S	S	S	S	S
510	2006/02/28	RX	CF sputum	S	S	S	S	**I**	S	S
511	2006/02/28	RX	CF sputum	S	S	S	S	S	S	S
512	2006/03/14	RX	CF sputum	S	S	S	S	S	S	S
513	2006/03/24	RX	CF sputum	S	S	S	S	S	S	S
527	2006/04/04	RX	CF sputum	S	S	S	S	S	S	S
528	2006/04/04	RX	CF sputum	S	S	S	**I**	**R**	S	S
529	2006/04/04	RX	CF sputum	S	S	S	S	S	S	S
530	2006/04/04	RX	CF sputum	S	**R**	S	S	S	S	S

545	2006/04/06	RX	CF sputum	S	S	S	**R**	**R**	S	S
546	2006/04/11	RX	CF sputum	S	S	S	S	S	S	S
548	2006/05/02	RX	CF sputum	S	S	S	S	S	S	S
549	2006/05/02	RX	CF sputum	S	S	S	S	S	S	S
561	2006/05/09	RX	CF sputum	S	S	S	S	S	S	S
562	2006/05/09	RX	CF sputum	**R**	**R**	S	S	S	**R**	S
563	2006/05/09	RX	CF sputum	S	S	S	S	S	S	S
564	2006/05/09	RX	CF sputum	S	S	S	S	S	S	S
565	2006/05/09	RX	CF sputum	S	S	S	S	S	S	S
566	2006/05/09	RX	CF Sputum	**R**	**R**	S	S	S	S	S
Non-CF:										
1	2000/04/02	GSH	Blood	**R**	**R**	**R**	S	**I**	**R**	**R**
2	2000/05/18	GSH	Tracheal	**R**	**R**	**R**	**R**	**R**	**R**	**R**
3	2000/05/25	GSH	Tracheal	**R**	**R**	**R**	**R**	**R**	**R**	**R**
4	2000/11/15	GSH	Pus	**R**	**R**	**R**	S	**R**	**R**	**R**
5	2000/11/16	GSH	Tracheal	S	S	S	S	S	S	S
6	2000/06/22	GSH	Pus	**R**	**R**	**R**	S	**R**	**R**	**R**
7	2000/06/26	GSH	Tracheal	**R**	**R**	**R**	S	R	**R**	**R**
8	2000/09/28	GSH	Tracheal	**R**	**R**	**R**	S	R	**R**	**R**
9	2000/06/08	GSH	Tracheal	S	S	S	S	S	**R**	**R**
11	2000/06/26	GSH	Tracheal	**R**	**I**	**R**	S	S	**I**	S
PACT 7	2000/05/18	GSH	Tracheal	**R**	**I**	**R**	S	I	S	S
278	2004/07/12	GSH	Sputum	S	S	nd	S	S	S	S
280	2004/07/13	GSH	Sputum	**R**	**R**	nd	S	S	**R**	S
283	2004/07/15	GSH	Tracheal	S	S	S	S	S	S	S
284	2004/07/19	GSH	Tracheal	S	S	S	S	S	S	S
285	2004/07/19	GSH	Tracheal	S	S	S	S	S	S	S
286	2004/07/20	GSH	Sputum	**R**	**R**	**R**	S	**I**	**R**	**R**
287	2004/07/22	GSH	Tracheal	S	S	**R**	S	S	S	S
288	2004/07/22	GSH	Tracheal	S	S	**R**	S	S	S	S
289	2004/07/22	GSH	Tracheal	**R**	S	**R**	S	S	**R**	S

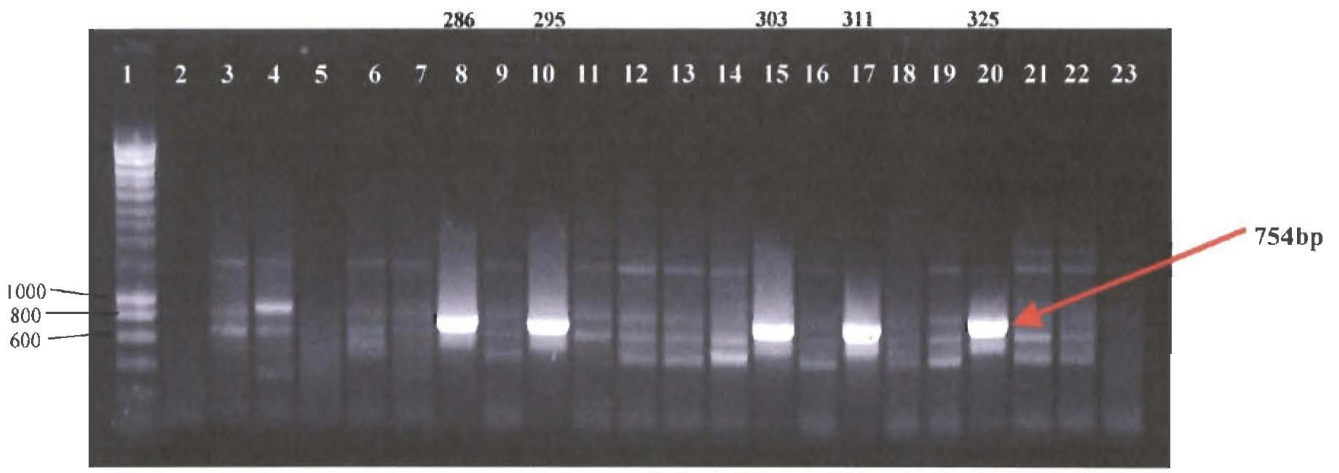
290	2004/07/26	GSH	Tracheal	S	S	S	S	S	S	S
292	2004/07/28	RX	Pus	**R**	S	**R**	S	S	S	S
293	2004/07/27	RX	Pus	**R**	S	**R**	S	S	S	S
295	2004/07/27	RX	Pus	**R**	S	**R**	S	S	**R**	**R**
298	2004/08/03	GSH	Sputum	S	S	S	S	S	S	S
299	2004/08/10	GSH	Tracheal	S	S	nd	S	S	**R**	**R**
300	2004/08/02	GSH	Tracheal	S	S	S	S	S	**R**	**R**
301	2004/08/08	GSH	Tracheal	S	S	S	S	S	S	S
302	2004/08/04	GSH	Blood	**R**	**R**	**R**	**R**	**I**	**R**	**R**
303	2004/07/28	RX	Burn	**R**	S	**R**	S	S	S	S
305	2004/07/28	RX	Tracheal	**R**	S	**R**	S	S	S	S
306	2004/07/26	GSH	Tracheal	S	S	S	S	S	**R**	**R**
307	2004/08/02	GSH	Sputum	S	S	S	S	S	S	S
308	2004/08/10	GSH	Sputum	S	S	S	S	S	S	S
309	2004/07/28	GSH	Pus	S	S	S	S	S	S	S
310	2004/07/29	GSH	Tracheal	S	S	S	S	S	S	S
311	2004/07/28	RX	Sputum	**R**	S	**R**	S	S	**R**	**R**
313	2004/08/02	GSH	Tracheal	S	S	S	S	S	S	S
323	2004/08/18	GSH	Tracheal	S	S	S	S	S	S	S
325	2004/08/23	GSH	Tracheal	S	S	S	S	S	**R**	**R**
326	2004/08/19	GSH	Tracheal	S	S	S	S	S	S	S
328	2004/08/18	RX	Tracheal	S	S	S	S	S	S	S
329	2004/08/23	RX	Tracheal	S	S	S	S	S	S	S
330	2004/08/16	RX	Tracheal	S	S	nd	S	S	S	S
331	2004/08/16	GSH	Tracheal	S	S	nd	S	S	S	S
332	2004/08/23	GSH	Tracheal	S	S	S	S	S	S	S
334	2004/08/15	GSH	Sputum	S	S	nd	S	S	S	S
335	2004/08/16	GSH	Tracheal	S	**R**	nd	S	S	**R**	**R**

Gm – gentamicin; Ak – amikacin; Tob – tobramycin; Caz – cefatzidime; Fep – cefepime; Imp – imipenem; Mem – meropenem.
R – resistant; **I**- intermediately resistant; S – susceptible; nd – not determined
GSH – Groote Schuur Hospital; RX – Red Cross War Memorial Children's Hospital

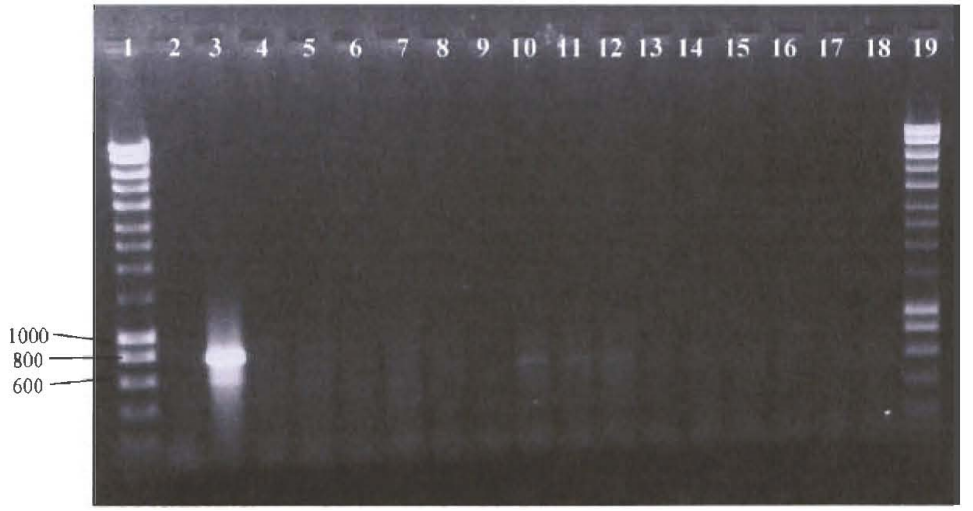
2.3.2 PCR amplification and identification of IS*6100*

The insertion sequence IS*6100* has been implicated in conferring a survival advantage on *P. aeruginosa* when growing in the CF lung (Kresse *et al*, 2003). Preliminary PCR assays (2.2.6) were carried out on a random selection of CF and non-CF *P. aeruginosa* strains, to screen for the presence of IS*6100*. Following agarose gel electrophoresis (2.2.4), products of the correct size (758bp) were obtained from strains 286, 295, 303, 311 and 325 [Fig. 2.3.1 A]. These strains are all non-CF *P. aeruginosa* isolates. The PCR product obtained from strain 311 was purified (2.2.5.2) and sequenced (2.2.7), and analysis of the sequencing data obtained revealed 100% homology with the published IS*6100* sequence [Fig. 2.3.2], confirming that the amplified product is IS*6100*. A number of spurious bands were observed, but the PCR was not optimised further as the presence of IS*6100* was later confirmed by DNA-DNA hybridisation [Fig. 2.3.3 & Fig. 2.3.4]. The PCR assay was repeated to screen for the presence of IS*6100* in the remaining strains, using 311 as a positive control [Fig. 2.3.1 B, C, D, E]. Again, strains 292, 293, 6, 7, 8, 11 and 12, which generated a product of the expected size, were non-CF, and IS*6100* was not detected in any of the CF isolates included in this study. A product of approximately 600bp was obtained from *P. aeruginosa* 329 [Fig. 2.3.1 C, lane 24]. This product was purified (2.2.5.2) and sequence analysis (2.2.7) of the product revealed no homology with any known sequences, and therefore the product is thought to have been amplified due to the non-specific binding of the primers.

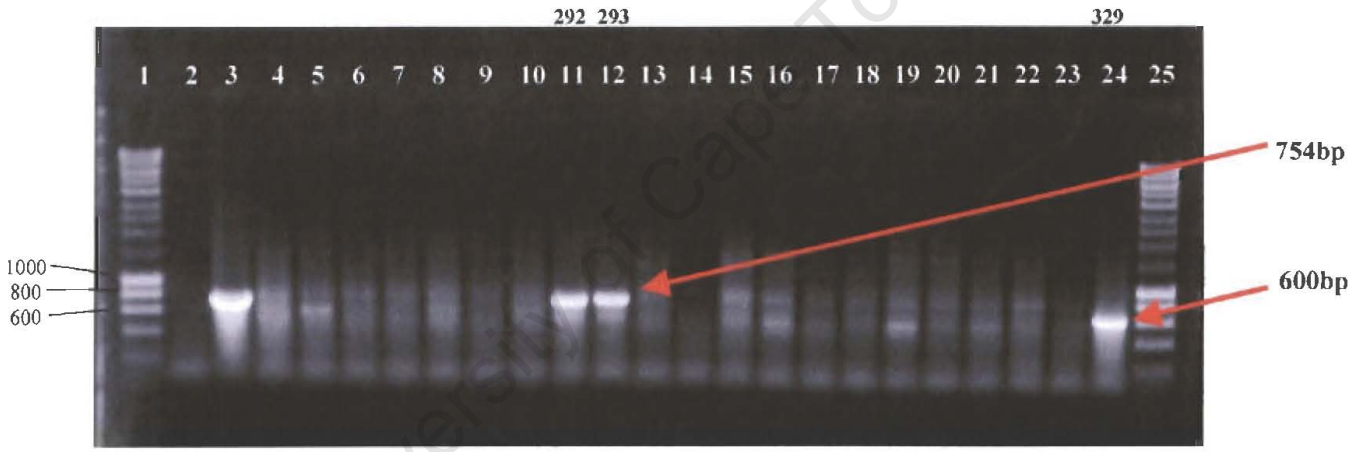
A



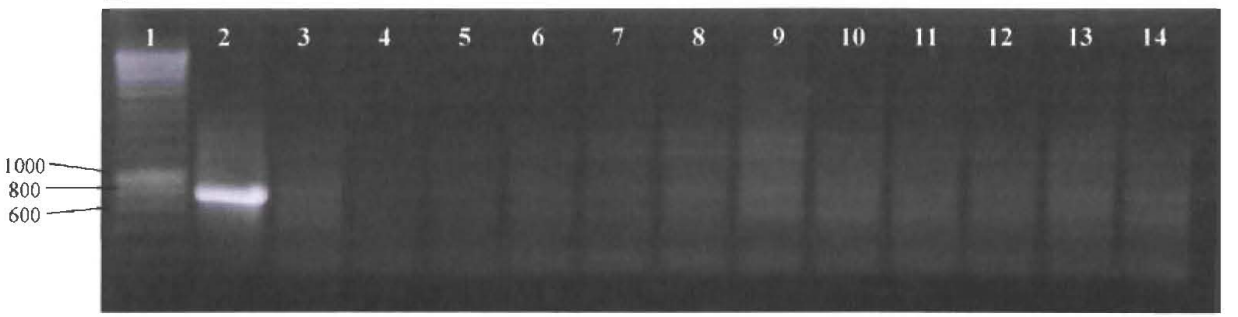
B



C



D



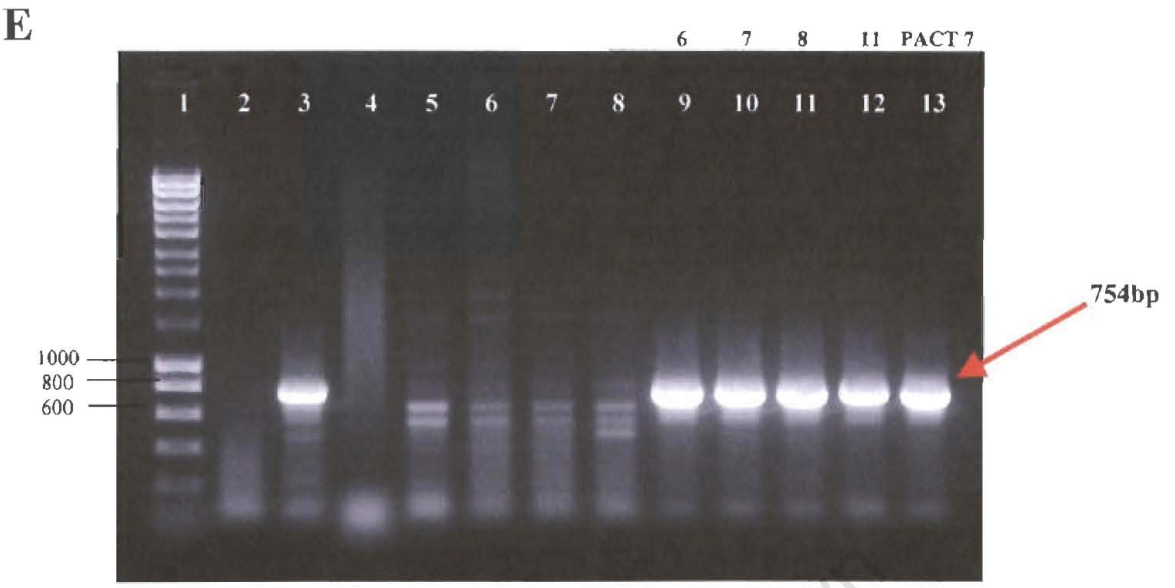


Figure 2.3.1: PCR amplification to detect the presence of IS*6100* in *P. aeruginosa* strains.
Numbers indicated above selected lanes represent strains that generated a PCR product of the correct size (754bp)

A. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, PAO1; lane 4, PACT5; lane 5, 277; lane 6, 280; lane 7, 281; lane 8, 286; lane 9, 289; lane 10, 295; lane 11, 297; lane 12, 299; lane 13, 300; lane 14, 302; lane 15, 303; lane 16, 306; lane 17, 311; lane 18, 312; lane 19, 324; lane 20, 325; lane 21, 327; lane 22, 333; lane 23, 335.

B. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, 311 positive control; lane 4, 503; lane 5, 504; lane 6, 505; lane 7, 506; lane 8, 507; lane 9, 508; lane 10, 509; lane 11, 510; lane 12, 511; lane 13, 512; lane 14, 513; lane 15, 527; lane 16, 528; lane 17, 529; lane 18, 530; lane 19, molecular weight marker Hyperladder I [Bioline].

C. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, 311 positive control; lane 4, 278; lane 5, 283; lane 6, 284; lane 7, 285; lane 8, 287; lane 9, 288; lane 10, 290; lane 11, 292; lane 12, 293; lane 13, 298; lane 14, 301; lane 15, 305; lane 16, 307; lane 17, 308; lane 18, 309; lane 19, 310; lane 20, 313; lane 21, 323; lane 22, 326; lane 23, 328; lane 24, 329; lane 25, molecular weight marker Hyperladder I [Bioline].

D. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, 311 positive control; lane 3, 330; lane 4, 331; lane 5, 332; lane 6, 334; lane 7, P16; lane 8, P17; lane 9, P18m; lane 10, P19n; lane 11, P19m; lane 12, P20; lane 13, P21; lane 14, P22.

E. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, 311 positive control; lane 4, 1; lane 5, 2; lane 6, 3; lane 7, 4; lane 8, 5; lane 9, 6; lane 10, 7; lane 11, 8; lane 12, 11; lane 13, PACT 7.

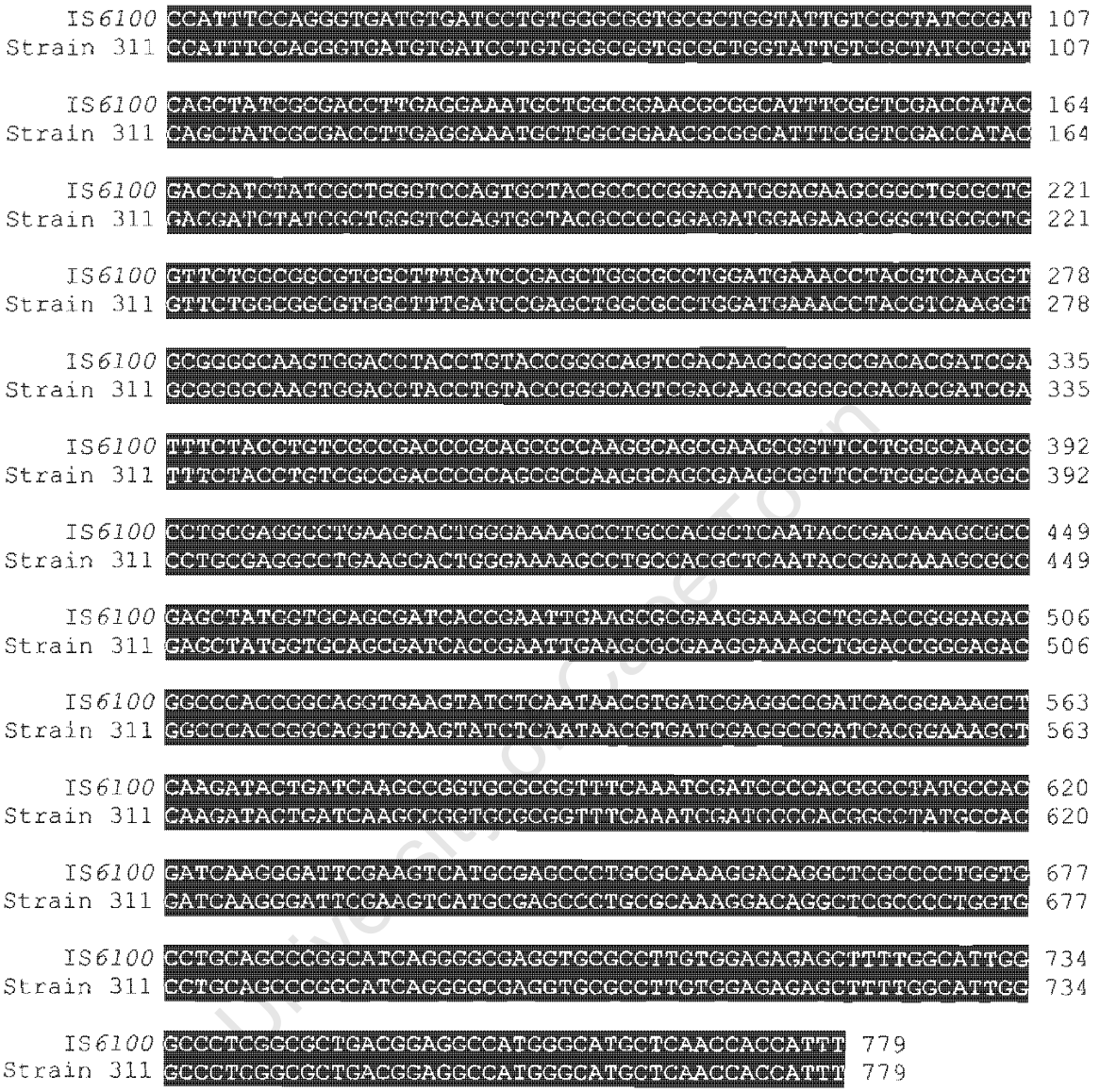


Figure 2.3.2: Nucleotide sequence alignment of 729bp of the IS*6100* PCR product from *P. aeruginosa* 311 with the corresponding region of IS*6100*. Regions shaded in black represent regions of 100% homology. The numbering on the right indicates the nucleotide position relative to the start codon of IS*6100*.

2.3.3 DNA-DNA Hybridisation to confirm the presence of IS*6100*

Five micrograms of genomic DNA from 68 of the 80 *P. aeruginosa* strains was transferred to a Hybond-N^+ membrane using a slot blot apparatus (2.2.8.1 a). The IS*6100* probe was prepared using the corresponding PCR product obtained from strain 311. All 32 CF isolates and 36 non-CF isolates, including 11 of the 12 strains positively identified as containing IS*6100* by PCR were loaded as indicated in Table 2.3.2. Strain 329, which generated a 600bp PCR product, was also included. Following DNA-DNA hybridisation, washing and detection, the x-ray film was exposed to the membrane for 15 minutes and then developed. The signals that were obtained after 15 minutes (A) and an extended exposure of 20 hours (B) are indicated in Fig 2.3.3.

As expected, the IS*6100* PCR product, which was transferred to the membrane as a positive control, produced strong signals [Fig.2.3.3], indicating that hybridisation was indeed specific. The autoradiograph confirmed the presence of IS*6100* in 9 of the 11 (286, 295, 303, 311, 325, 6, 7, 8 and 11) strains that were identified as positive by the PCR assay, while the remaining two strains, 292 and 293, produced no signal. No signal was detected from strain 329, which produced the 600bp PCR product. Following overnight exposure, signals were detected for a further 6 of the 68 strains (278, 283, 289, 300, 306, 312). Unexpectedly, signal was observed from the genomic DNA of *E. coli* JM109.

Table 2.3.2: Layout of samples on the slot blot to confirm IS*6100* presence in *P. aeruginosa* CF and non-CF strains. The water control (A1) served to confirm that neither the membrane nor the slot blot apparatus contained any contaminating DNA; the positive controls consisted of PCR amplified and purified IS*6100* from *P. aeruginosa* 311 to demonstrate that binding is specific; *E. coli* JM109 genomic DNA was included as a non-*Pseudomonas* control.

	A	B	C
1	water	9	+ve control
2	13	280	281
3	286	289	295
4	297	299	300
5	302	303	306
6	311	312	324
7	325	327	333
8	335	1	2
9	3	4	5
10	6	7	8
11	11	503	504
12	505	506	507
13	508	509	510
14	511	+ve control	513
15	527	528	530
16	278	283	284
17	288	290	292
18	293	298	305
19	308	309	310
20	313	323	326
21	328	329	330
22	332	334	P15
23	P16	P19m	P19n
24	P22	P20	*E. coli* JM109

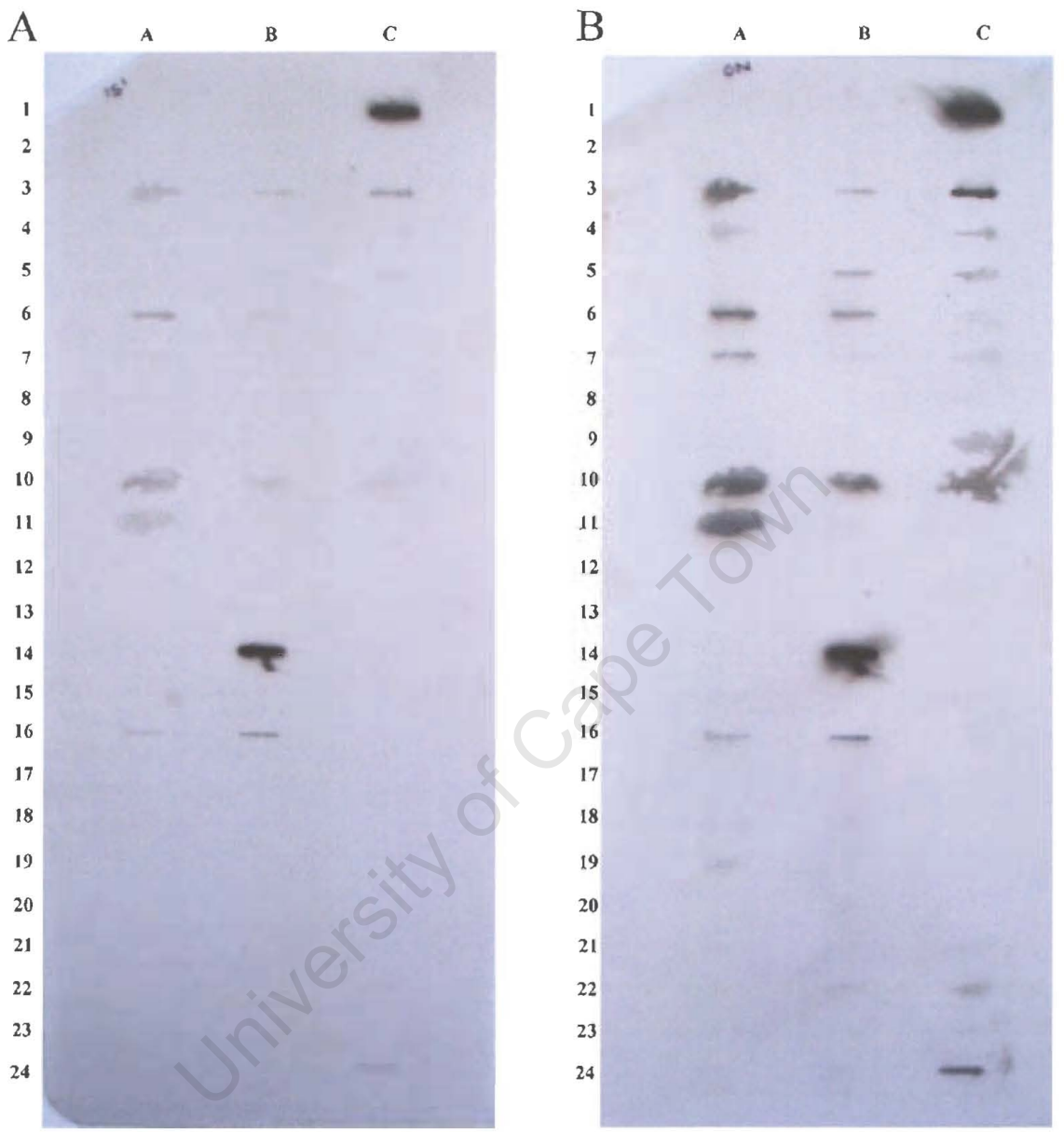


Figure 2.3.3: Autoradiograph of the slot blot performed using genomic DNA from the clinical *P. aeruginosa* isolates hybridised with an IS*6100* probe.
The probe itself was bound to the membrane as a positive control and *P. aeruginosa* 311, from which the probe was derived, was also included as another positive control. Genomic DNA from *E. coli* JM109 was included as a control. A. Autoradiograph exposure for 15 minutes. B. Autoradiograph exposure after approximately 20 hours.

2.3.4 DNA-DNA Hybridisation to determine IS*6100* copy number

To determine the number of copies of IS*6100* in strains positively identified by PCR and DNA-DNA hybridisation as containing IS*6100*, Southern hybridisation experiments were carried out [Fig. 2.3.4]. The IS*6100* PCR product, corresponding to the probe, generated a strong signal. No signal was detected for *P. aeruginosa* PAO1 and *E. coli* JM109 genomic DNA. Despite quantification of genomic DNA (2.2.5.2) to ensure that equivalent amounts of DNA were used for each strain, visualisation of the digested DNA used in the Southern hybridisation [Fig. 2.3.5] indicates that there is more *E. coli* JM109 DNA present relative to the other samples. As IS*6100* has not previously been described in *E. coli*, it is thought that the signal produced on the slot blot [Fig. 2.3.3] was as a result of non-specific binding to excess template DNA and prolonged autoradiograph exposure.

All 12 strains identified as containing IS*6100* by PCR generated a signal after Southern hybridisation of *Bam*HI-digested genomic DNA from these strains [Fig. 2.3.4]. In strains 286, 295, 303, 311, 7, 8, 11, 12, 292 and 293 two bands generated signals, suggesting that these strains harbour at least two copies of IS*6100* as more than one copy may be present on a particular band(s). The agarose gel of digested genomic DNA [Fig. 2.3.5] suggests that the weak hybridisation signals obtained from strains 11, 292 and 293 may be due to insufficient target DNA as a result of inaccurate DNA quantification. This would explain the absence of signal from strains 292 and 293 on the slot blot [Fig. 2.3.3]. In strain 286 the sizes of the fragments containing IS*6100* are approximately 6kb and >10kb and in strain 295 they are 2.2kb and >10kb. Strains 303, 292 and 293 contain IS*6100* on DNA fragments of 2.2kb and 10kb and strains 311, 7, 8, 11 and 12 all harbour IS*6100* on DNA fragments of 2.2kb and >10kb. Only one signal was generated from strain 325, and this was in the region of 4kb, suggesting the presence of at least one copy of IS*6100* in this strain. Three bands generated signals in strain 6, suggesting that this strain harbours at least three copies of IS*6100* on a DNA fragment of 2.2kb and on two fragments of >10kb.

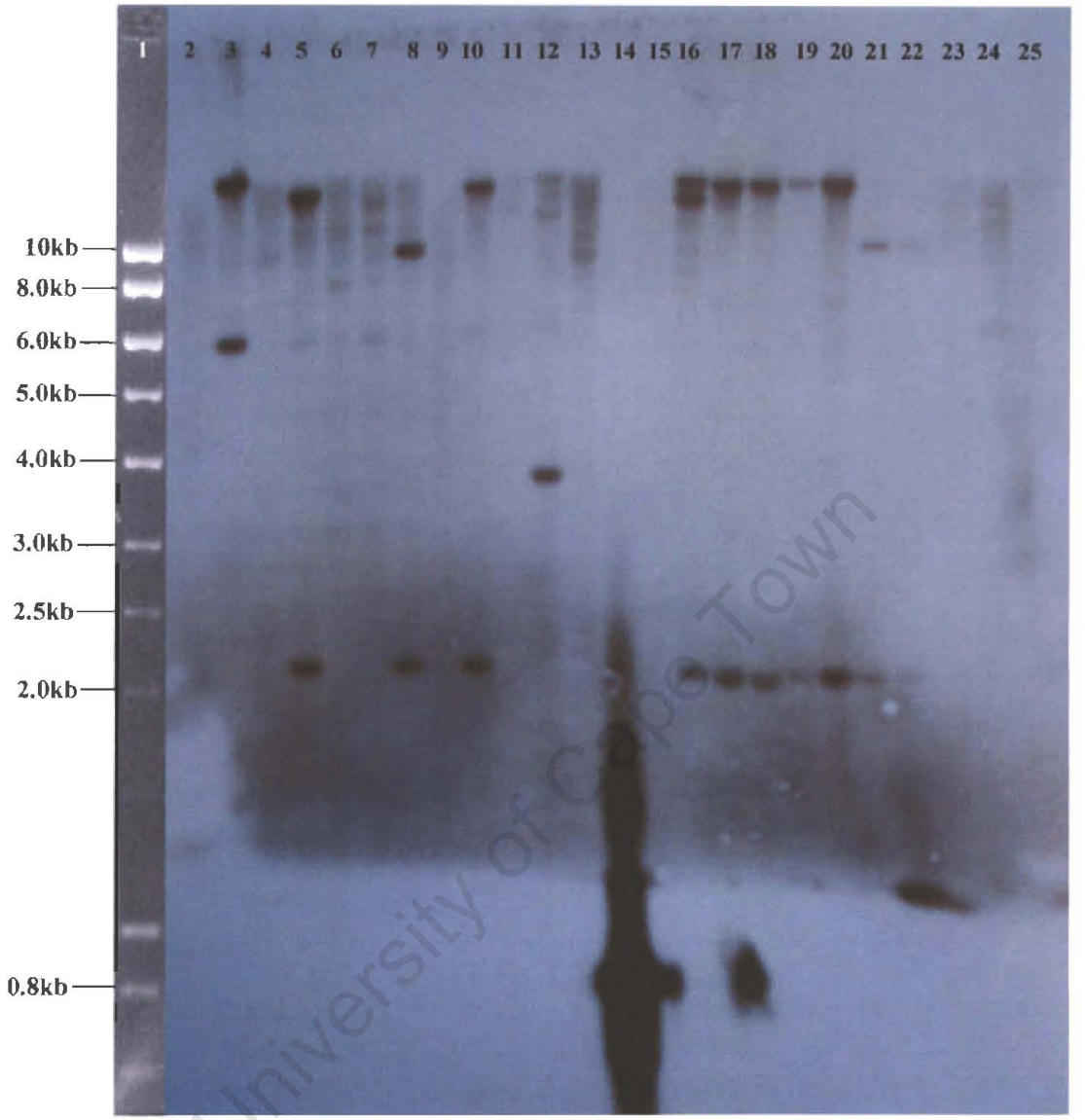


Figure 2.3.4: Autoradiograph of Southern hybridisation of Bam*HI*-digested genomic DNA from selected *P. aeruginosa* isolates using IS*6100* as a probe.
Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, PAO1; lane 3, 286; lane 4, 289; lane 5, 295; lane 6, 297; lane 7, 300; lane 8, 303; lane 9, 306; lane 10, 311; lane 11, 312; lane 12, 325; lane 13, 327; lane 14, positive IS*6100* control; lane 15, 5; lane 16, 6; lane 17, 7; lane 18, 8; lane 19, 11; lane 20, 12; lane 21, 292; lane 22, 293; lane 23, 329; lane 24, 333; lane 25; *E. coli* JM109.
The IS*6100* probe itself was transferred to the membrane as a positive hybridisation control.
Genomic DNA from *E. coli* JM109 and genomic DNA from *P. aeruginosa* PAO1 were included as controls.
Autoradiograph exposure was for approximately 20 hours.

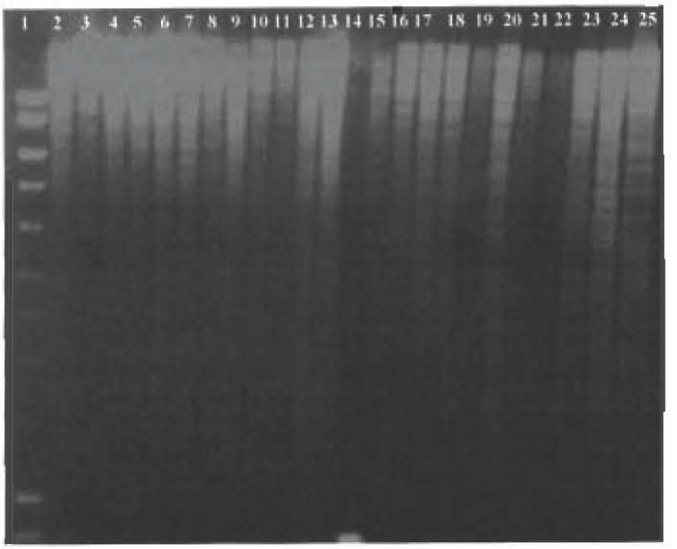


Figure 2.3.5: *Bam*HI-digested *P. aeruginosa* genomic DNA for Southern hybridisation. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, PAO1; lane 3, 286; lane 4, 289; lane 5, 295; lane 6, 297; lane 7, 300; lane 8, 303; lane 9, 306; lane 10, 311; lane 11, 312; lane 12, 325; lane 13, 327; lane 14, positive IS*6100* control; lane 15, 5; lane 16, 6; lane 17, 7; lane 18, 8; lane 19, 11; lane 20, 12; lane 21, 292; lane 22, 293; lane 23, 329; lane 24, 333; lane 25; *E. coli* JM109.

2.3 4 Genetic relatedness of *P. aeruginosa* strains

In order to establish whether a dominant clone of *P. aeruginosa* exisits within local hospitals, and to determine whether the isolates harbouring IS*6100* are descendant of a common clone, genotyping of the *P. aeruginosa* strains included in this study was carried out by RAPD analysis and PFGE. RAPD is a rapid means of genotyping bacteria, but it is recommended that this method be used in conjunction with another genotyping method, as RAPD results are often not as discriminatory as results obtained from methods such as PFGE (Mahenthiralingham *et al*, 1996; Renders *et al*, 1996). These two genotyping methods were used in order to determine whether the IS*6100*-containing, non-CF *P. aeruginosa* strains identified in this study are genetically related. RAPD analysis, using primer 272 (Mahenthiralingham *et al*, 1996), showed that strain 295 and strain 311, both of which harbour IS*6100*, are genetically related, but that these strains do not appear to be related to any other *P. aeruginosa* strains that were typed by this method [Fig. 2.3.6. A]. Strain 7 and strain 8 also produced identical RAPD banding patterns [Fig. 2.3.6. A], and strain 11

and PACT 7 differed from these strains in only one DNA fragment [Fig. 2.3.6.A & B], suggesting that these four IS*6100*-containing isolates are genetically related. RAPD typing also established a genetic relationship between strain 303, strain 292 and strain 293, as well as between strain 527, strain 506 and strain 562. The remaining 16 *P. aeruginosa* strains genotyped by this method appeared to be genetically distinct from each other and the strains mentioned above.

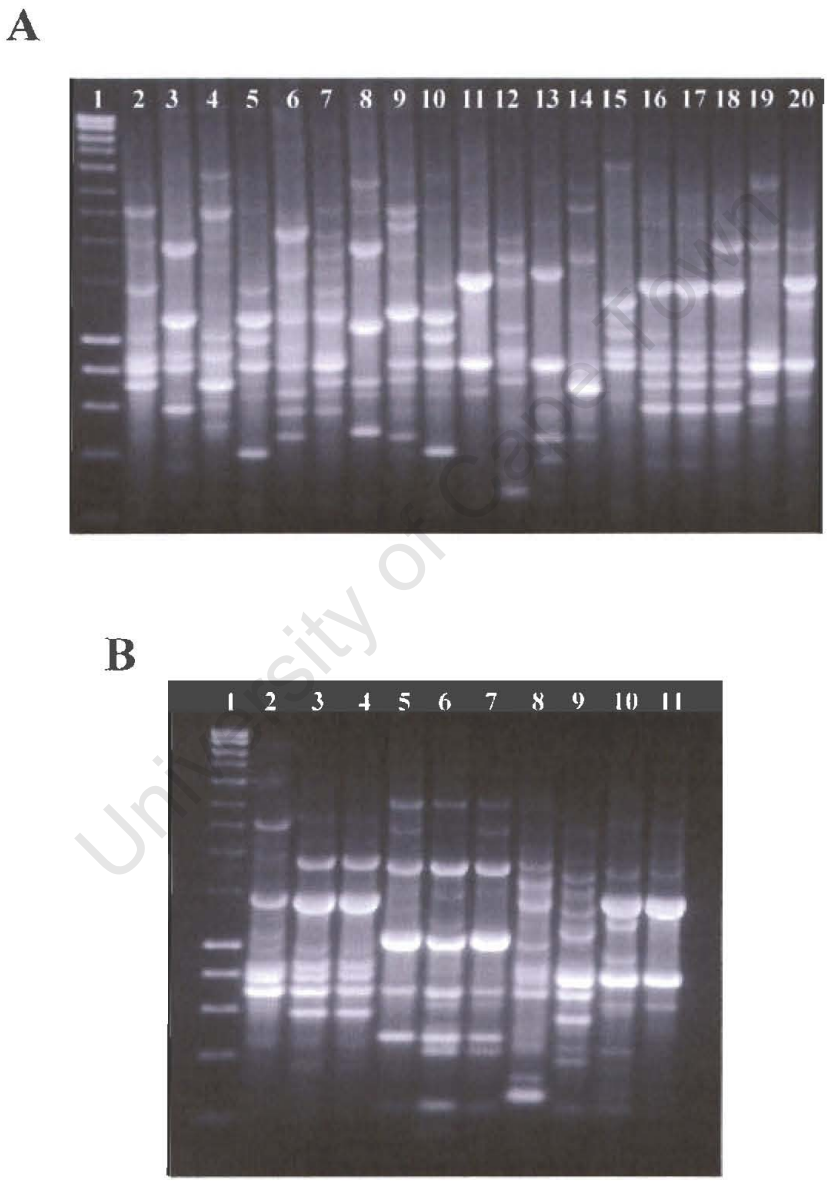


Figure 2.3.6: RAPD typing profiles of *P. aeruginosa* strains. A. Lane 1, molecular weight marker, Hyperladder I [Bioline]; lane 2, PAO1; lane 3, 286; lane 4, 289; lane 5, 295; lane 6, 297; lane 7, 300; lane 8, 303; lane 9, 306; lane 10, 311; lane 11, 312; lane 12, 325; lane 13, 327; lane 14, 5; lane 15, 6; lane 16, 7; lane 17, 8; lane 18, 11; lane 19, 333; lane 20, 527. **B.** Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, PAO1; lane 3, 11; lane 4, PACT 7; lane 5, 303; lane 6, 292; lane 7, 293; lane 8; 325; lane 9, 335; lane 10, 506; lane 11, 562.

As previously mentioned, PFGE is considered a more discriminatory method of genotyping than RAPD (Renders *et al*, 1996). The genetic profiles of *P. aeruginosa* strains obtained by RAPD analysis were therefore confirmed by PFGE typing. Following staining with EtBr, gels were visualised (2.2.4) [Fig.2.3.7].

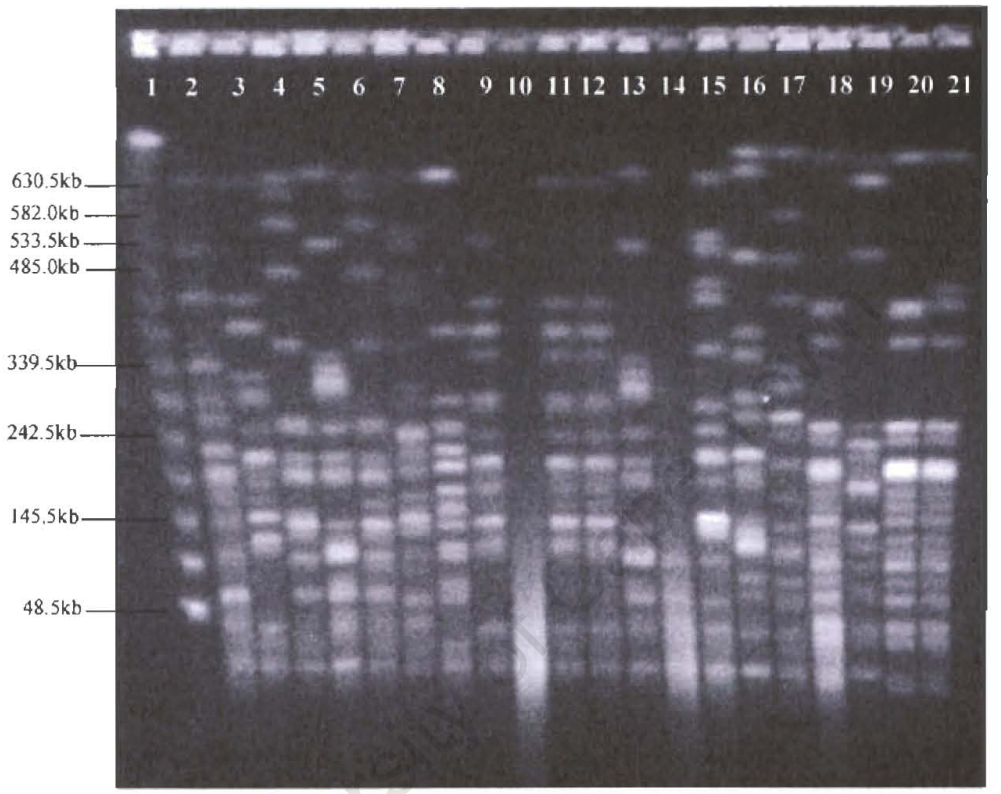


Figure 2.3.7: PFGE profiles of *Spe*I-digested genomic DNA from *P. aeruginosa*. Lane 1, λDNA-PFGE molecular weight marker [Amersham Biosciences]; lane 2, PAO1, lane 3, 286; lane 4, 295; lane 5, 303; lane 6, 311; lane 7, 325; lane 8, 6; lane 9, 7; lane 10, 8; lane 11, 11; lane 12, PACT 7; lane 13, 292; lane 14, 293; lane 15, 335; lane 16, 333; lane 17, 297; lane 18, 527; lane 19, 302; lane 20, 506; lane 21, 562.

PFGE typing confirmed that strain 295 and strain 311 are genotypically indistinguishable. Strain 11 and PACT 7 also produced identical restriction patterns, that differed from that of strain 7 by one DNA fragment. A DNA fragment of approximately 534kb in strain 7 was replaced by a larger fragment of 631kb in both strain 11 and PACT 7, indicating that these two strains had acquired additional genomic DNA relative to strain 7. PFGE analysis showed that strain 303 and strain 292 are genetically indistinct, and the DNA banding patterns produced by strain 527, strain 506 and strain 19n indicate that these three isolates are also all related.

A difference of 2 bands was observed between strain 325 and strain 335, which, according to the criteria of Tenover *et al* (1995) makes these two strains closely related. Restriction profiles could not be obtained for strain 8 and strain 293 as a result of DNA degradation. According to the PFGE typing results, strain 286, strain 6, strain 333, strain 297 and strain 302 do not appear to be genetically related to any of the other strains investigated. The genotypic profiles obtained for *P. aeruginosa* by RAPD analysis were confirmed by PFGE typing methods, suggesting that, in this case, RAPD typing was adequate for determining the relatedness of strains.

2.4 Discussion

Previously, IS*6100* has been associated with *P. aeruginosa* strains belonging to subgroup C, which comprises exclusively CF isolates (Klockgether *et al*, 2004; Kresse *et al*, 2003). This insertion sequence has been shown to cause large structural rearrangements in genomic DNA organisation in these subgroup C isolates, and is associated with the transposon TNCP23, also exclusively found in *P. aeruginosa* subgroup C CF isolates (Klockgether *et al*, 2004). It was therefore surprising that none of the 32 *P. aeruginosa* strains from CF patients that were included in this study contained IS*6100*, whereas 12 of 48 non-CF *P. aeruginosa* isolates included in this study generated an IS*6100*-specific PCR product [Fig. 2.3.1]. Nine of 12 strains were confirmed for the presence of IS*6100* following DNA-DNA hybridisation studies using a slot blot apparatus [Fig. 2.3.3]. The remaining three strains produced a positive hybridisation signal following Southern hybridisation [Fig. 2.3.4]. After prolonged autoradiograph exposure of the slot blot hybridisation, signals were observed for an additional 6 strains. These strains, however, did not generate an IS*6100* PCR product. It is possible that these strains were not identified as IS*6100*-containing strains by PCR due to nucleotide sequence mutations in the primer binding region. Alternatively, the signals obtained may be due to non-specific DNA binding and prolonged autoradiograph exposure, as seen for *E. coli* JM109.

In CF *P. aeruginosa* isolates, IS*6100* flanks TNCP23, a large 23kb class I transposon, which, in turn, is located within the 100kb chromosomally-integrated form of plasmid pKLC102 (Klockgether *et al*, 2004). Upon IS*6100*-induced LCIs, a

copy of IS*6100* is inserted within the bacterial chromosome at the breakpoint of inversion (Kresse *et al*, 2003). There are two *Bam*HI restriction sites within the TNCP23 sequence, and they are both within the first 2kb of the 23kb sequence. Therefore, if IS*6100* was present in *P. aeruginosa* flanking TNCP23, a Southern hybridisation of *Bam*HI-digested DNA with IS*6100* would be expected to produce at least two signals, corresponding to DNA fragments sufficiently different in size to be distinguishable. Reports of IS*6100* in non-CF strains in association with Tn*801* and Tn*5041* have only detected IS*6100* in single-copy (Yamane *et al*, 2004; Dubois *et al*, 2002), although the presence of more than one copy cannot be excluded as Southern hybridisation with an IS*6100*-specific probe was not carried out.

At least two copies of IS*6100* were detected in non-CF *P. aeruginosa* strains 286, 295, 303, 311, 7, 8, 11, PACT 7, 292 and 293, suggesting that IS*6100* may be present in these strains flanking a TNCP23-like transposable element. Only one signal was generated in strain 325, suggesting a possible arrangement similar to that of Tn*801* and Tn*5041* in this strain. Of particular interest was that at least three copies of IS*6100* were detected in strain 6 on two fragments >10kb and a 2kb fragment. Since rearrangement of the bacterial genome by IS*6100* is associated with the production of an additional copy of this IS element (Kresse *et al*, 2003; Boyd *et al*, 2002), it may be that the presence of at least three copies of IS*6100* in strain 6 is more than likely indicative that some form of LCI may have occurred in the genome of this strain.

Interestingly, IS*6100* is located on DNA fragments of >10kb and 2.2kb in strains 303, 292 and 293. This may indicate a similar genome arrangement in these strains, possibly suggesting that these strains are genetically related. Similarly, two fragments of the same size generated signals in each of strains 311, 7, 8, 11 and 12. The remaining strains, 286, 295, 325 and 6, generated unique signal banding patterns on the autoradiograph, suggesting that they may be genetically distinct from each other and the other IS*6100*-containing strains. Genotyping of a selection of the *P. aeruginosa* isolates included in this study was therefore carried out to determine if these strains were in fact related.

Previous reports have suggested that genotyping by RAPD analysis alone is not adequate for producing conclusive genotyping results, and that this preliminary

method of genotyping should be performed in conjunction with another genotyping method, such as PFGE (Mahenthiralingham *et al*, 1996; Renders *et al*, 1996). Strain 295 and strain 311 were genetically indistinguishable by both RAPD analysis [Fig. 2.3.6] and PFGE typing [Fig. 2.3.7]. This is not surprising as these two strains were isolated, one day apart, from the same patient, display identical antibiotic resistance profiles, and may therefore be considered as the same strain.

The combined RAPD and PFGE results show that strain 11 and PACT 7 are genetically indistinguishable, and that they are closely related to strain 7 and strain 8, differing by only one band. It is interesting to note that in the PFGE typing a band of approximately 534kb in strain 7 is replaced by a band of approximately 631kb in strain 11 and PACT 7, while RAPD analysis shows only an additional band in strain 11 and PACT 7. This is indicative of how much more discriminatory PFGE typing is than RAPD typing. One may conclude further, that the replacement of the approximately 534kb band in strain 7 with the approximately 631kb band in strain 11 and PACT 7 indicates insertion or duplication of genomic DNA in these strains. Interestingly, Southern hybridisation showed that strain 7, strain 8, strain 11 and PACT 7 harbour IS*6100* on DNA fragments of the same sizes. The four strains were isolated from four patients at GSH, within four months of each other. Unfortunately, strain 8 could not be typed by PFGE as a result of DNA degradation. Strains refractory to PFGE typing due to nuclease activity have previously been described (Mahenthiralingham *et al*, 1996). However, the RAPD profile obtained for strain 8 was similar to that of strain 7, strain 11 and PACT 7. The antibiotic resistance profiles of these strains, together with the genotyping results, suggest that these strains are indeed related. Strain 6 was isolated during the same period as strain 7, strain 8, strain 11 and PACT 7, yet both its RAPD profile and its PFGE profile are markedly different from that of strains 7, 8, 11 and PACT 7. Either strain 6 is not related to these strains, or a subsequent major genetic rearrangement(s) within the chromosome of this strain resulted in it appearing genetically distinct from its progenitor. The presence of at least three copies of IS*6100* in strain 6 and the role of the third copy of this element in creating LCIs suggests that the unique genetic profile of strain 6 may be as a result of the latter.

Strain 303 and strain 292 were isolated from two patients at RX on the same day. Strain 293 was isolated from the same patient as strain 292, one day prior to the isolation of strain 292. Identical profiles were obtained by PFGE analysis [Fig. 2.3.7] for strain 303 and strain 292, and strain 293 was also shown to be related to these two strains by RAPD typing [Fig. 2.3.6]. Further evidence supporting the relatedness of these strains comes from the identical banding patterns observed following IS*6100* hybridisation experiments [Fig. 2.3.4]. In addition, these strains displayed identical antibiotic resistance profiles, suggesting that these strains are related.

Strain 335, which was shown not to harbour IS*6100*, was isolated from the same patient one week prior to strain 325, which does contain IS*6100*. It was therefore not too surprising that strain 325 and strain 335 displayed different genotypic profiles by RAPD analysis. PFGE analysis, however, indicated that a three band difference in the profiles of these strains would suggest that they should be considered closely related according to the criteria of Tenover *et al* (1995). As with strain 6, it is possible that this patient was simultaneously infected by two different *P. aeruginosa* strains. It is also possible, however, that selective pressure due to antibiotic treatment received by the patient during the week had resulted in the acquisition of IS*6100*, possibly as part of a larger mobile genetic element from a co-infecting organism. Class 1 composite transposons consist of IS elements flanking one or more gene cassettes (Holmes & Jobling, 1996) and these gene cassettes commonly encode antibiotic resistance markers (Cabrera *et al*, 2006; Reyes *et al*, 2003; Gonzalez *et al*, 1998). The similarity in the antibiotic resistance profiles of strain 325 and strain 335 suggest that acquisition of such an element may not have occurred. The possibility cannot be excluded, however, that the acquired gene cassette(s) confer resistance to antibiotics other than those examined here. Alternatively, IS*6100* may have been acquired by strain 325 in association with selective pressures other than those imposed by antimicrobial therapy. The identification of the insertion point of IS*6100* in strain 325, as well as in the other IS*6100*-conatining *P. aeruginosa* strains identified in this study, may provide some useful information in this regard. The insertion of additional DNA containing IS*6100* would result in alteration of the genotypic profile of strain 335, perhaps to a profile resembling that of strain 325. These results clearly demonstrate the need for more than one method of genotyping in order to obtain conclusive genotypic profiles.

The absence of IS*6100* in *P. aeruginosa* CF strains from local hospitals is encouraging, as this element has been identified as a contributor to the phenotypic changes associated with poor outcome in CF patients infected with *P. aeruginosa* (Kresse *et al*, 2003). The contribution of IS*6100* to the increased resilience of CF isolates, specifically their increased resistance to antibiotics (Oliver *et al*, 2004), makes the dissemination of this element highly unfavourable. The detection of IS*6100* in 25% of non-CF isolates was, however, unexpected. Although 7 of 12 of the IS*6100*-containing isolates could be assigned to one of two genotypic profiles, the remaining 5 isolates appeared to be genetically distinct. These preliminary results suggest that IS*6100*-containing, non-CF *P. aeruginosa* strains from local hospitals are not descendant of one clone, but that IS*6100* was acquired independently in these strains. The presence of IS*6100* on fragments of the same sizes in strain 7, strain 8, strain 11, strain 311 and PACT 7 may indicate that the IS element is located within the same genetic environment in these strains, whether this be chromosomally located, or as part of a larger mobile genetic element, such as a plasmid or transposon. The distinct genetic profile of strain 311, in comparison to that of strain 7, strain 8, strain 11 and PACT 7, suggests that an association with a mobile genetic element is likely. If IS*6100* was located chromosomally, it would be expected that the element would be present on different sized DNA fragments in strain 311 in comparison with strain 7, strain 8, strain 11 and PACT 7, due to the lack of similarity in chromosomal structure observed between these strains. If IS*6100* were located upon a mobile genetic element in these strains, however, the location of the *Bam*HI restriction sites flanking IS*6100* would be the same, and therefore the element would be detected upon the same size DNA fragments by Southern hybridisation. Similarly, that strain 311 and strain 295 are genetically identical, yet harbour IS*6100* on different *Bam*HI DNA fragments, confirms this type of arrangement. To establish whether IS*6100* is associated with a mobile genetic element or is inserted directly into the bacterial chromosome, investigation into the location of IS*6100* in non-CF *P. aeruginosa* strains was carried out.

CHAPTER 3

GENETIC ENVIRONMENT OF IS*6100* AND ITS ROLE IN CONVERSION TO HYPERMUTABILITY

3.1 Introduction

Genotyping studies have revealed a *P. aeruginosa* clone, clone C, that occurs at a very high frequency in both the disease setting and in the environment (Römling *et al*, 1992). *P. aeruginosa* clone C isolates can be divided into four subgroups, C, C2, C13 and SG17M, and strains belonging to subgroup C, which consists only of CF strains, can be identified by the irreversible integration of a 95kb plasmid, pKLC102, into the bacterial chromosome (Klockgether *et al*, 2004; Kiewitz *et al*, 2000; Römling *et al*, 1997b). Also unique to subgroup C isolates is the presence of a large class 1 transposon, TNCP23, which is flanked by IS*6100*, and is inserted within the integrated pKLC102 sequence (Klockgether *et al*, 2004; Römling *et al*, 1997b).

Upon integration of the IS*6100*-flanked TNCP23 into pKLC102, an 8bp insertion site duplication occurs (Klockgether *et al*, 2004), a characteristic feature of IS elements belonging to the IS*6* family (Mahillon & Chandler, 1998). Of the two IS*6100* elements flanking TNCP23, IS*6100*-L encodes a functional transposase, while *tnpA* of IS*6100*-R is inactivated due to a frameshift mutation (Klockgether *et al*, 2004). Upon transposition of IS*6100*, a copy of IS*6100*-L, IS*6100*-t, is deposited within the interrupted gene immediately downstream of the breakpoint, and the LCI occurs between IS*6100*-L and IS*6100*-t (Kresse *et al*, 2003) [Fig. 3.1.1]. Following this inversion event, pKLC102 sequence is found 3' of IS*6100*-t, and adjacent to pKLC102 is the inverted region of the target gene, as well as TNCP23 flanked by IS*6100*-L and IS*6100*-R (Kresse *et al*, 2003) [Fig.3.1.1].

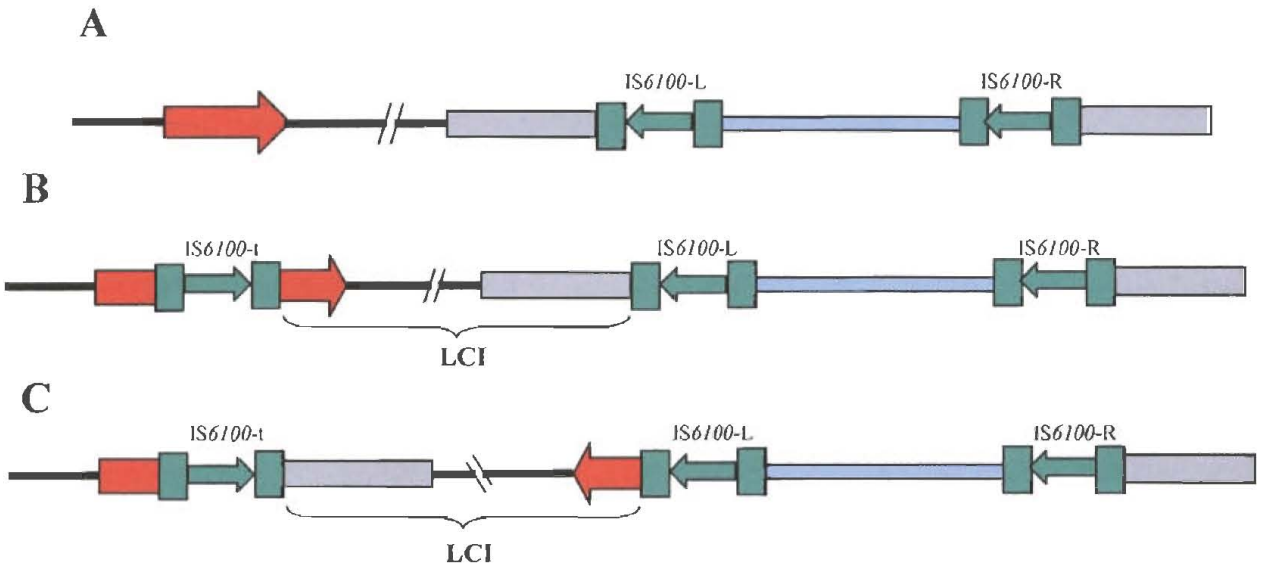


Figure 3.1.1: The mechanism of transposition of IS*6100*. **A**. The genetic environment of IS*6100* and TNCP23 prior to chromosomal inversion. The red arrow represents the target gene for inversion; the thick black lines represent *P. aeruginosa* chromosomal DNA, dashes indicate that chromosomal regions are not drawn to scale; The blocks shaded grey represent pKLC102 sequence; green blocks represent the 17bp repeats of IS*6100* and green arrows represent the transposase gene of IS*6100*, pointing in the direction of transcription of the gene; the region shaded light blue represents TNCP23 sequence. **B**. Following transposition of IS*6100*, a copy of IS*6100*-L, IS*6100*-t, disrupts the target gene, 3` of the breakpoint of inversion. **C**. The LCI occurs between IS*6100*-t and IS*6100*-L.
(Adapted from Kresse *et al*, 2003).

To date, IS*6100*-induced LCIs have been identified in four regions of the chromosome in CF *P. aeruginosa* strains (Kresse *et al*, 2003). Interestingly, in all four cases, the breakpoints of inversion were identified within genes involved in processes that are altered in CF strains relative to *P. aeruginosa* strains that are associated with other sites of infection (Kresse *et al*, 2003). Genes affected are *wbpM*, involved in the synthesis of O-specific antigen, *pilB*, required for the production of type IV pili, *mutS*, the DNA mismatch repair protein and PA4029, a hypothetical membrane transport protein (Kresse *et al*, 2003). Thus, it seems that IS*6100*-induced LCIs promote the selection of *P. aeruginosa* strains with an altered phenotype as seen in CF isolates. Interestingly, IS*Pa20*, the element responsible for the LCIs observed in subgroup 13 CF *P. aeruginosa* isolates, was shown to be located within *pilM* and between ORF13 and ORF14 of the LPS O1-antigen gene cluster in these strains (Kresse *et al*, 2006). Thus, as was the case with *P. aeruginosa* subgroup C isolates containing IS*6100* (Kresse *et al*, 2003), IS*Pa20* disrupted genes involved in the altered phenotype of CF *P. aeruginosa* isolates (Kresse *et al*, 2006). The identification of an outwardly directed putative strong promoter at the 3` end of

IS*Pa20*, together with the finding that up to 25 copies of this element may be present in some strains indicates that it may be involved in activation of gene expression as well as LCIs in *P. aeruginosa* clone C, subgroup C13 strains (Kresse *et al*, 2006).

There have been two reports of IS*6100* in non-CF *P. aeruginosa* isolates. The element has been described in non-CF strains in association with a transposon, Tn*801*, resulting in the immobilisation of an extended-spectrum β-lactamase (ESBL), TEM-21 (Dubois *et al*, 2002) [Fig. 3.1.2]. TEM-21 mediates resistance to several antipseudomonal β-lactams, excluding the carbapenems (Dubois *et al*, 2002). IS*6100* was located within the resolvase gene of Tn*801* and a gene encoding a 3-*N*-aminoglycoside acetyltransferase, *aac-(3)-II*, was identified 3` of Tn*801* (Dubois *et al*, 2002). The acetyltransferase encoded by this gene mediates resistance to gentamicin, tobramycin and netilmicin (Dubois *et al*, 2002). Putative promoter sequences located within Tn*801* may contribute to enhanced *aac(3)-II* transcription (Dubois *et al*, 2002). In a later study this exact arrangement was identified in 24 *P. aeruginosa* strains isolated from patients in a nursing home (Dubois *et al*, 2005). As the Tn*801* resolvase gene is interrupted by IS*6100*, and *aac(3)-II* is not located within Tn*801*, it is probable that all three genes are present as part of a larger mobile element, which would allow them to be co-transferred (Dubois *et al*, 2005).

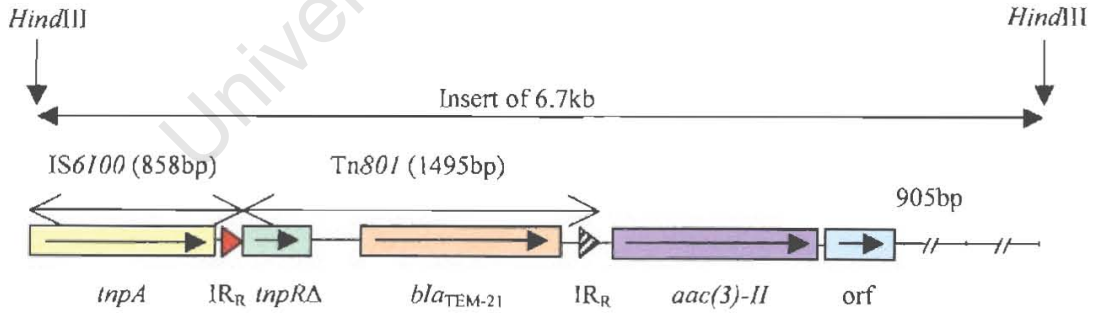


Figure 3.1.2: The genetic environment of IS*6100* in association with Tn*801* carrying a bla_{TEM-21} gene in non-CF *P. aeruginosa* strains. Hind*III* sites indicate the ends of the cloned DNA fragment of 6.7kb; the open arrowhead represents the right inverted repeat of IS*6100* and the striped arrowhead indicates the position of the inverted repeat of Tn*801*; *tnpRΔ* is the truncated Tn*801* resolvase gene; the bla_{TEM-21} gene is located upon Tn*801*; the orientation of translation is shown by horizontal arrows; an ORF located 3` of *aac(3)-II* showed homology with a protein previously associated with this gene; a 905kb region at the 3` end of the cloned DNA fragment showed no homology to previously described sequences. (Adapted from Dubois *et al*, 2002).

IS*6100* has also been described in non-CF *P. aeruginosa* isolates in upstream of the recently described *rmtA* aminoglycoside resistance gene, encoding a 16S rRNA methylase (Yamane *et al*, 2004; Yokoyama *et al*, 2003) [Fig. 3.1.3]. RmtA mediates resistance to a number of aminoglycosides, including amikacin, tobramycin, gentamicin and kanamycin (Yokoyama *et al*, 2003). In a non-CF *P. aeruginosa* isolate, the *rmtA* locus, composed of IS*6100*, truncated *orfA'* and sequence 2 [Fig. 3.1.3], was flanked by regions homologous to Tn*5401* (Yamane *et al*, 2004). The 5` end of the *rmtA* locus, *orfA*, was disrupted by IS*6100*, and a duplication of the region containing *orfI* and *orfQ* had occurred (Yamane *et al*, 2004) [Fig. 3.1.3]. This duplication was not present in another *P. aeruginosa* isolate lacking IS*6100*, but harbouring *rmtA* in a similar arrangement (Yamane *et al*, 2004). IS*6100*-induced LCIs have been reported in *Salmonella* strains (Boyd *et al*, 2002), but not in non-CF *P. aeruginosa* strains. The association of IS*6100* with genomic rearrangments (Güneş *et al*, 1999), however, suggests that this element may have been involved in duplication of the *orfI*/*orfQ* region (Yamane *et al*, 2004).

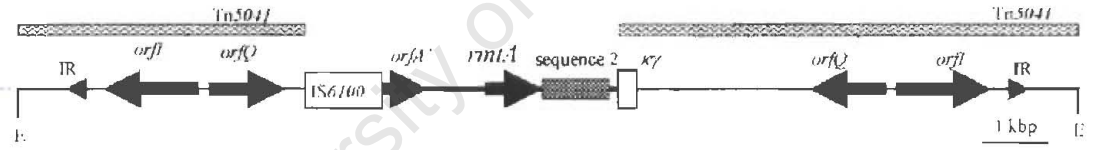


Figure 3.1.3: The genetic environment of IS*6100* in association with Tn*5041* in non-CF *P. aeruginosa* strains. E denotes the Eco*RI* sites used to clone the DNA fragment; sequences with homology to regions of Tn*5041* are shown by rectangles filled with wavy lines; solid arrowheads represent terminal inverted repeats; sequence 2 shows homology to a Na^+/H^+ antiporter; *orfA'* represents a truncated *orfA* gene that encodes a probable tRNA ribosyltransferase, located adjacent to IS*6100*; the κγ element, previously identified in Tn*5041*, is thought to have originated as part of a mobile genetic element. (Adapted from Yamane *et al*, 2004).

Since IS*6100* seems to be located within larger mobile elements in non-CF *P. aeruginosa*, it may be that IS*6100* itself did not mediate integration of these elements into the host chromosome. Rather, IS*6100* was fortuitously acquired due to integration mediated by another element. To gain further insight into the role of IS*6100* in the non-CF *P. aeruginosa* strains identified in this study, the genetic environment of IS*6100* was investigated.

3.2 Experimental Procedures

3.2.1 Inverse polymerase chain reaction

Whereas PCR involves the exponential amplification of DNA sequences located between two convergent primers, inverse PCR uses two divergent primers to amplify DNA sequences flanking known regions of DNA (Ochman *et al*, 1988). Genomic DNA is digested with a restriction enzyme that cuts external to the sequence of interest, and DNA fragments are then ligated to themselves to form circular molecules [Fig. 3.2.1]. Outwardly directed primers are designed within regions of interest [Fig. 2.2.2] and sequence analysis of amplified PCR products allows determination of flanking regions.

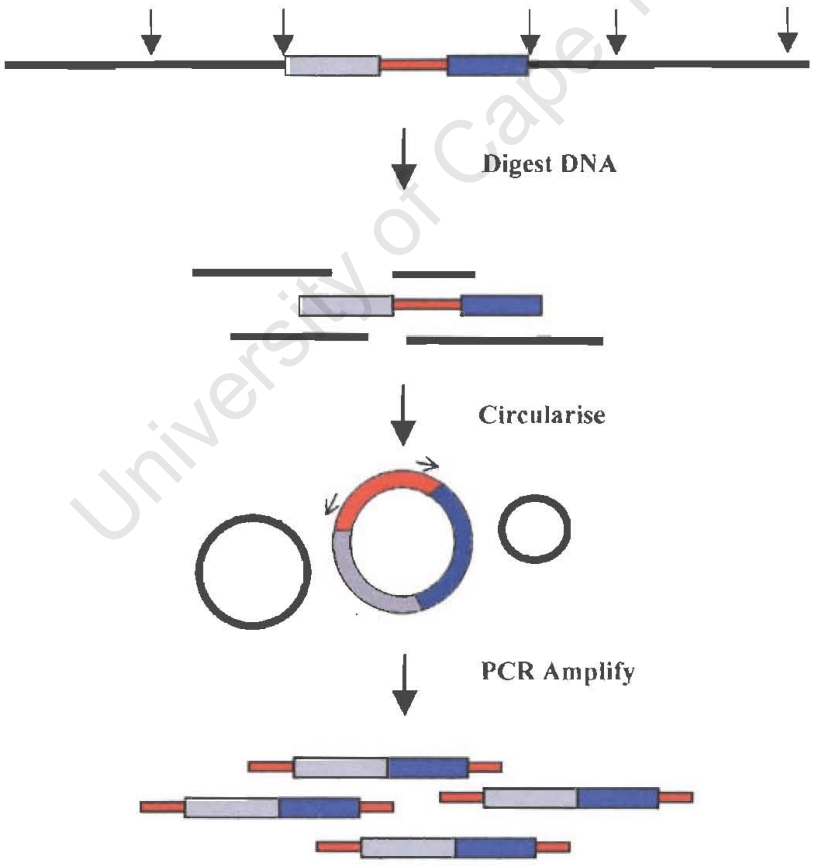


Figure 3.2.1: A schematic representation of the processes involved in inverse PCR.
The solid red box represents the known DNA fragment, IS*6100*; the solid grey and blue boxes represent the regions flanking the DNA sequence of interest; black arrows represent RE sites. The digested DNA is circularised and outwardly-directed primers designed within IS*6100*, shown as arrows on the circularised molecule, are used to amplify the flanking regions.
(Adapted from Ochman *et al*, 1988).

3.2.1.1 Cloning of IS*6100* into the vector pGEM-T Easy

PCR amplified IS*6100* was cloned into the vector pGEM-T Easy in order to provide a positive control in inverse PCR assays. The pGEM-T Easy vector [Promega] (Appendix C) is specifically modified to enhance the cloning of PCR amplified products. The thermostable polymerases used in the replication of DNA commonly add a deoxyadenosine residue to the 3`-end of the amplified product (Mole *et al*, 1989), and therefore pGEM-T Easy vectors are cut with *Eco*RV, within the multiple cloning site (MCS), and a thymidine is added to the 3` terminus at both ends of the vector. These thymidine residues not only provide compatible "sticky ends" for ligation of the PCR product, but also prevent recircularisation of the vector.

a) Insert preparation and ligation into pGEM-T Easy

DNA ligase is an enzyme that facilitates the ligation of double-stranded DNA by the formation of bonds between 5` phosphate residues and 3` hydroxyl moieties (Timson *et al*, 2000). IS*6100* was amplified by PCR (2.2.6) and the product was purified (2.2.5.2). A 3:1 insert:vector ratio was used in the ligation reaction, which consisted of 3 Weiss units of T4 DNA ligase [Promega], 1X Rapid Ligation Buffer [Promega] and 50ng pGEMT-Easy vector [Promega] in a final volume of 10µl. Ligation proceeded overnight at 4°C.

b) Transformation

Chemically competent One Shot® TOP10 cells [Invitrogen] were used for transformation of recombinant DNA molecules. A 50µl vial of One Shot® cells was thawed on ice. A volume of 5µl of ligation mix was added to the cells and mixed gently by tapping the tube. The mixture was incubated on ice for 30 minutes, and then placed in a 42°C waterbath for exactly 30 seconds, to facilitate the uptake of DNA. The cells were placed on ice, and 250µl of S.O.C medium [Invitrogen], pre-warmed to room temperature, was added. S.O.C is a rich medium, and therefore sterile technique was practised to avoid contamination. The cells were incubated at 37°C for exactly 1 hour with shaking, to allow for expression of the resistance marker, after which 100µl, 200µl and 300µl volumes of cells were plated out on

2X YT agar (Appendix A) containing 100µg/ml ampicillin and with 40µl of X-gal (40mg/ml) spread onto the plate surface, as per the manufacturer's protocol [Invitrogen].

c) Screening of *E. coli* transformants

Transformants were selected by the addition of ampicillin at 100µg/ml to the growth medium, 2 X YT agar (Appendix A). As pGEM-T Easy encodes resistance to this antibiotic, only cells containing the plasmid will be able to grow. Selection of transformants containing recombinant plasmids was carried out by blue-white colony screening, based on the presence of a functional β-galactosidase (β-gal) gene. pGEM-T Easy carries the *lacZ* gene, which encodes the amino-terminal (α fragment) of β-gal. Bacteria such as *E. coli* JM109 contain the carboxy-terminal (β fragment) of β-gal, and association of the two portions, by a process known as α complementation, results in the formation of a functional β-gal enzyme in transformants. The active β-gal enzyme hydrolyses X-gal to produce a blue dye that gives rise to the production of blue colonies. Upon ligation of insert DNA into the MCS of pGEM-T Easy, the α fragment of β-gal is disrupted and α complementation cannot occur, resulting in the formation of white colonies. Thus, blue colonies contain only vector, whilst white colonies contain vector plus insert DNA (Sambrook *et al*, 1989). When transforming with *E. coli* cells that are not commercially prepared, IPTG is included for induction of *lacZ* transcription. One Shot® TOP10 cells [Invitrogen], however, do not require IPTG for induction of transcription.

3.2.1.2 Small-scale plasmid preparation

Plasmid DNA was extracted from recombinant white colonies by the method of Sambrook *et al* (1989) with modifications. Single recombinant colonies were inoculated into 5ml 2X YT broth (Appendix A) with ampicillin (100µg/ml) selection and incubated overnight at 37°C. Cells from 2ml of overnight culture were harvested by centrifugation for 2 minutes at 14 000 rpm and pellets were resuspended in 0.2ml of Solution 1 [25mM Tris-HCl pH 8.0, 50mM EDTA, 1% w/v Glucose; Appendix A]. Resuspension in Solution 1 maintains salt and pH conditions that are compatible with biomolecules, and the EDTA chelates metal ions that are required by DNases.

Following resuspension, 0.4ml of Solution 2 [0.2N NaOH, 1% w/v SDS; Appendix A] was added and the solution was mixed by shaking and incubated on ice for 5 minutes. The SDS in Solution 2 causes the lysis of the bacterial cells, while the high alkalinity of Solution 2 aids in denaturing proteins and causes the cleavage of phosphate bonds in RNA, eliminating intereference from high molecular weight RNA molecules. The high pH of Solution 2 also causes the separation of the two strands of non-supercoiled DNA, leaving only intact supercoiled plasmids in solution. A volume of 0.3ml of pre-cooled Solution 3 [5M potassium actetate, 3M glacial acetic acid; Appendix A] was added to the now viscous solution and held on ice for 10 minutes. The acetic acid in Solution 3 neutralises the alkaline conditions caused by the sodium hydroxide in Solution 2, while the potassium acetate causes the precipitation of SDS and its associated membrane fragments and proteins. Following incubation on ice the solution was centrifuged for 10 minutes at 12 000 rpm in order to pellet the cellular protein, chromosomal DNA and membrane precipitate that had formed. The supernatant was transferred to a fresh tube containing 0.6 volumes of isopropanol, and the mixture was incubated at room temperature for 10 minutes to precipitate the plasmid DNA. Precipitated plasmid DNA was collected by centrifugation for 15 minutes at 12 000 rpm. The supernatant was discarded and the pellet was washed in 1ml 80% ethanol. Following centrifugation and removal of the ethanol, the plasmid DNA was resuspended in 50µl Tris-EDTA (TE) buffer [Appendix A] and stored at 4°C.

3.2.1.3 Inverse PCR

Inverse PCR assays were to a final volume of 50µl and contained 500µM dNTPs [Fermentas], 3mM $MgCl_2$ [Promega], 1X GoTaq PCR buffer [Promega], 50 pmoles of each primer, IS6100-I1 and IS6100-I2 (Fig. 2.2.2), 2.5U GoTaq® DNA polymerase [Promega] and approximately 0.1µg of circularised DNA was used as the template. The inverse PCR cycle consisted of an initial denaturation step at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, primer annealing at 54°C for 1 minute and primer extension at 72°C for 10 minutes. A final elongation step of 10 minutes at 72°C was performed to complete elongation of any unfinished or incomplete products. The inverse PCR assays were carried out using a

GeneAmp PCR System 2400 [Perkin Elmer] thermocycler and inverse PCR products were visualised by AGE (2.4).

3.2.2 Mutator Assays

To investigate hypermutability in *P. aeruginosa*, mutator assays were performed to determine the frequency of mutation to rifampicin resistance. Rifampicin binds to and inhibits RNA polymerase, thereby preventing the initiation of RNA synthesis. Hypermutable strains accumulate mutations at a much faster rate than their non-hypermutable counterparts, and therefore develop resistance to rifampicin through mutation more rapidly, allowing the differences in rates of mutation to be calculated (Loh *et al*, 2001).

Mutator assay were carried out according to the method of Oliver *et al* (2000), with modifications. Overnight cultures of *P. aeruginosa* strains, in a volume of 20ml, were pelleted by centrifugation at 5000rpm for 5 minutes. The pelleted cells were washed in 5ml M-H broth and collected by centrifugation as before. Pellets were then resuspended in 1ml M-H broth. Dilutions of 0, 10^{-1} and 10^{-2} were plated onto M-H agar containing rifampicin at 300µg/ml, and dilutions of 10^{-6}, 10^{-7} and 10^{-8} or 10^{-7}, 10^{-8} and 10^{-9} were plated on M-H agar only. All dilutions were plated in triplicate. Colony forming units on M-H plates were counted after incubation for 20 hours, and on M-H plates containing rifampicin after incubation for 40 hours. Mutation rates were then calculated relative to the control strain, *P. aeruginosa* PAO1 (Stover *et al*, 2000).

3.3 Results

3.3.1 Investigation of the genetic environment of IS*6100* in *P. aeruginosa* strains

3.3.1.1 Inverse PCR of IS*6100* from strain 6

a) Construction of pBT05 containing IS*6100*

PCR amplified IS*6100* (2.2.6) was cloned into the 3015bp vector pGEM-T Easy (3.2.1.1) as a positive control for subsequent IS*6100* inverse PCR assays. Following

transformation of *E. coli* One Shot® TOP10 cells, more than 1000 transformants were obtained. Twelve transformants were randomly selected and small-scale plasmid preparations were carried out on these clones. RE digestion of the plasmid DNA with *Pst*I [CTGCA/G], which cuts once within the IS*6100* sequence, released insert DNA fragments, as visualised by AGE (2.2.4) [Fig. 3.3.1]. Following digestion with *Pst*I, eight of the putative recombinants contained an insert corresponding to IS*6100*. This was shown by the presence of a 700bp DNA fragment in 3 digests, and a 100bp fragment produced when IS*6100* had been inserted in the other orientation. Four white colonies were shown to harbour non-recombinant plasmids and probably appeared white as a result of impeded α-complementation due to point mutations in the MCS of pGEMT-Easy.

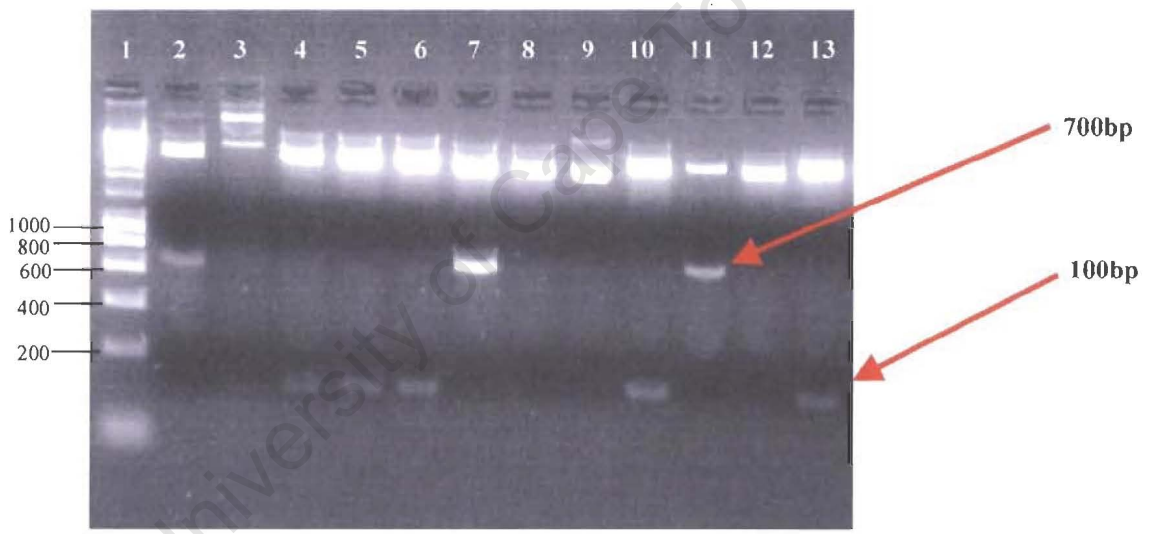


Figure 3.3.1: Analysis of putative recombinant plasmids by restriction enzyme digestion analysis with the enzyme *Pst*I.
Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, clone 1; lane 3, clone 2; lane 4, clone 3; lane 5, clone 4; lane 6, clone 5; lane 7, clone 6; lane 8, clone 7; lane 9, clone 8; lane 10, clone 9; lane 11, clone 10; lane 12, clone 11; lane 13, clone 12.

Recombinant plasmid obtained from clone 10, designated pBT05, was used in the optimisation of the inverse PCR assay and, subsequently, in inverse PCR assays as a positive control.

b) Optimisation of the inverse PCR assay

Optimisation of the inverse PCR assay, using pBT05 as a template, was performed by titrating the dNTP concentration and using Taq polymerase from two different suppliers (Takara and GoTaq). dNTP concentrations at 0.2mM, 0.3mM, 0.4mM, 0.5mM and 0.6mM were tested using both Taq polymerases. Products of the expected size (3.8kb) were obtained in all cases, with Takara DNA polymerase producing the more discrete amplified products. Products obtained following inverse PCR could be greater than 10kb in size, and therefore sufficient dNTPs must be present in the reaction mixture to amplify products of this size. For this reason, the Takara DNA polymerase and a dNTP concentration of 0.6mM [Fig. 3.3.2 A, lane 13] were used in further reactions. When the annealing temperature was increased from 52°C to 54°C to increase the specificity of primer binding, a more discrete amplification product was obtained [Fig. 3.3.2 B]. The additional spurious bands of decreased intensity and the 'smeared' effect observed are as a result of the excess dNTPs in the reaction.

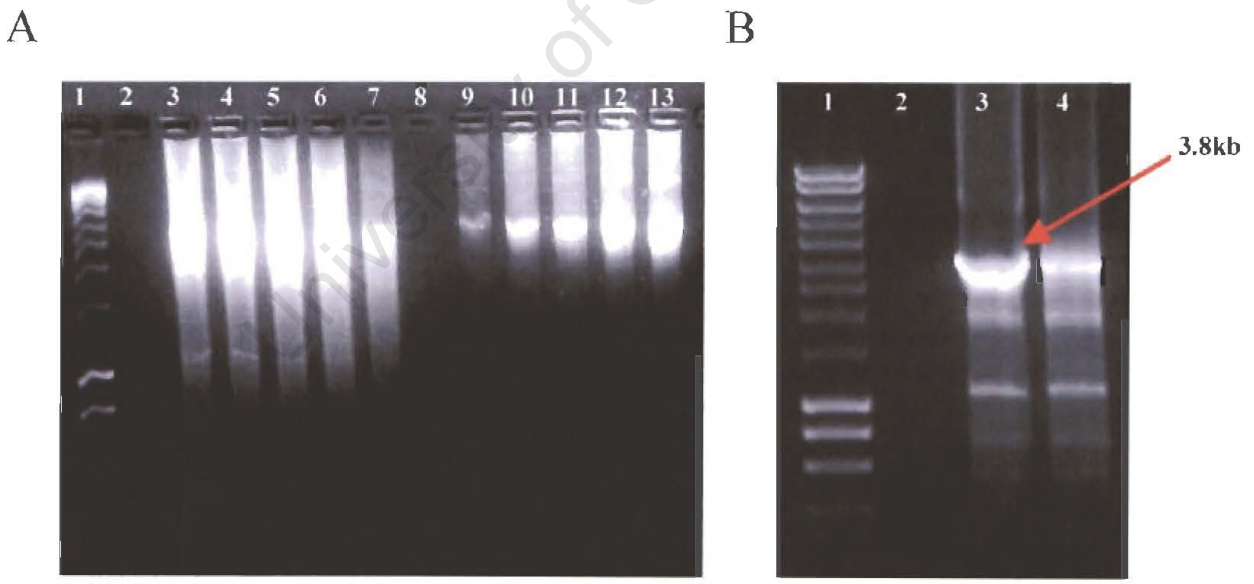


Figure 3.3.2: Optimisation of the inverse PCR assay. **A**. Lane 1, molecular weight marker Hyperladder 1 [Bioline]; lane 2, GoTaq, no DNA control; lane 3, GoTaq, 0.2mM dNTPs; lane 4, GoTaq, 0.3mM dNTPs; lane 5, GoTaq, 0.4mM dNTPs; lane 6, GoTaq, 0.5mM dNTPs; lane 7, GoTaq, 0.6mM dNTPs; lane 8, Takara, no DNA control; lane 9, Takara, 0.2mM dNTPs; lane 10, Takara, 0.3mM dNTPs; lane 11, Takara, 0.4mM dNTPs; lane 12, Takara, 0.5mM dNTPs; lane 13, Takara, 0.6mM dNTPs. **B.** Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, 100ng pBT05 template; lane 4, 20ng pBT05 template.

c) Inverse PCR to determine IS*6100* point of insertion in *P. aeruginosa* strains

Southern hybridisation (2.2.8.1 b) resulted in the identification of IS*6100* in strain 6 on two *Bam*HI fragments >10kb in size and one *Bam*HI fragment of 2kb [Fig. 2.3.4]. Following *Bam*HI digestion of strain 6 genomic DNA, the DNA in agarose slices in the region of these fragments was purified (2.2.5.1). Ligation reactions (3.2.1.1 a)) consisted of 3µl, 1µl or 0.2µl purified *Bam*HI-digested genomic DNA. The ligated products were purified (2.2.5.1) and 2µl of each was used as a template in inverse PCR assays (3.2.1.3), with pBT05 as a positive control [Fig. 3.3.3].

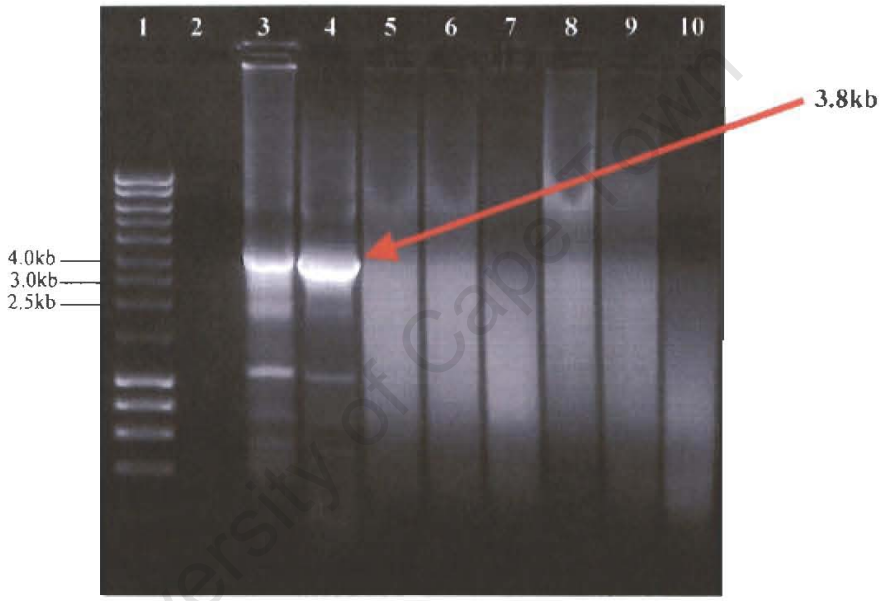


Figure 3.3.3: Inverse PCR to identify the regions flanking IS*6100* in non-CF *P. aeruginosa* strains. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, 100ng pBT05; lane 4, 20ng pBT05; lane 5, >10kb region, 3µl ligation; lane 6, >10kb region, 1µl ligation; lane 7, >10kb region, 0.2µl ligation; lane 8, 2kb region, 3µl ligation; lane 9, 2kb region, 1µl ligation; lane 10, 2kb region, 0.2µl ligation.

Both concentrations of pBT05 produced products of the expected size (3.8kb) when used as templates [Fig. 3.3.3]. Some DNA amplification was observed in three of the reactions, but this amplification was not specific and therefore did not produce any discrete bands that could be investigated further.

3.3.2 Determination of the presence of IS*6100* in association with Tn*801* in non-CF *P. aeruginosa* strains

IS*6100* has been described in non-CF *P. aeruginosa* isolates in association with Tn*801* (Dubois *et al*, 2002) and Tn*5041* (Yamane *et al*, 2004), and in CF isolates flanking TNCP23 (Klockgether *et al*, 2004). All IS*6100*-containing isolates identified in this study were isolated from non-CF patients, and therefore the association of this element with TNCP23 was not investigated further. In the absence of a positive control, the association of IS*6100* with Tn*5041* could not be investigated.

IS*6100* has been found inserted within the resolvase gene of transposon Tn*801*, which harbours a bla_{TEM-21} gene, and contains putative promoters for transcription of the adjacent *aac(3)-II* gene (Dubois *et al*, 2002). To determine whether IS*6100* is associated with Tn*801* in the 12 IS*6100*-containing, non-CF strains identified in this study, PCR assays (2.2.6) to screen for bla_{TEM-21} and *aac(3)-II* were carried out. *Acinetobacter baumannii* (*A. baumannii*) RAN (Segal *et al*, 2004) and *A. baumannii* SAK (Elisha & Steyn, 1991) were used as positive controls for the presence of bla_{TEM} and *aac(3)-II*, respectively. Primers used for amplification are indicated in Table 3.3.1. PCR cycling conditions were as previously described (2.2.6), but with an annealing temperature of 55°C for amplification of bla_{TEM} and 53°C for *aac(3)-II*. The elongation times were increased to 90 seconds in both reactions.

Table 3.3.1: Primers used in the amplification of genes associated with the Tn*801* arrangement of IS*6100*.

Amplified Gene	Primer Name	Primer Sequence (5` - 3`)	Product Size (bp)	Reference
bla_{TEM}	**F**: 3061 TEM	AGGAAGCAAAGCTGAAAGGAGT CAAATTTGG	1232	Nelson *et al*, 2003
	R: DEB	ATG AGT AAA CTT GGT CTG AC		Caniça *et al*, 1997
aac(3)-II	**F**:AACC2F	CGC GGA AGG CAA TAA C	786	Provided by H. Segal
	R:AACC2R	GCT TCT CAA GAT AGG TG		

F – forward primer; **R** – reverse primer

The PCR assay to detect *bla*$_{TEM}$ in IS*6100*-containing, non-CF *P. aeruginosa* strains produced a product of the expected size (1.232kb) from *A. baumannii* RAN [Fig. 3.3.4. A]. A product of the same size was obtained from *P. aeruginosa* strain 325 [Fig. 3.3.4. A], suggesting that this strain contains a gene that encodes a TEM β-lactamase. As expected, a 786bp fragment corresponding to a portion of *aac(3)-II* was obtained from *A. baumannii* SAK [Fig. 3.3.4. B]. A product of the same size was obtained from *P. aeruginosa* 325 [Fig. 3.3.4. B]. This suggests that only strain 325 contains IS*6100* as well as a *bla*$_{TEM}$ gene and an *aac(3)-II* gene. Following sequence analysis of the *bla*$_{TEM}$ product, the TEM variant present in strain 325 will be identified. It may be that in strain 325 IS*6100* is associated with *bla*$_{TEM-21}$ and *aac(3)-II* on Tn*801*.

A

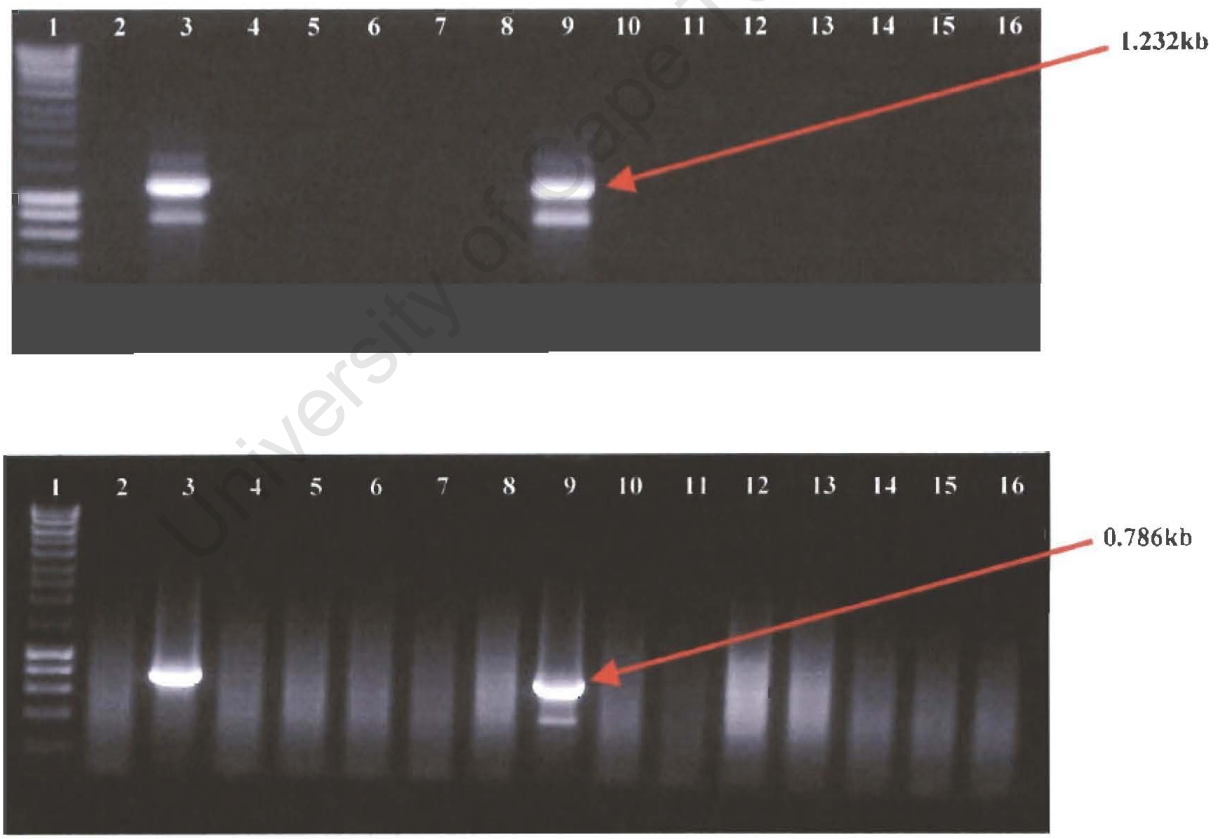


Figure 3.3.4: PCR amplification reactions to determine the presence of (A) *bla*$_{TEM}$ and (B) *aac(3)-II* in non-CF *P. aeruginosa* strains.
A & B: Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, positive controls; lane 4; PAO1; lane 5, 286; lane 6, 295; lane 7, 303; lane 8, 311; lane 9, 325; lane 10, 6; lane 11, 7; lane 12, 8; lane 13, 11; lane 14, 12; lane 15, 292; lane 16, 293.

3.3.3 The association of IS*6100* with hypermutability in *P. aeruginosa*

Genes continually acquire mutations, and those that are essential for the survival of the bacterium are not exempt. In such cases strain proliferation is prevented. Mutation(s) in non-essential genes, however, can often confer a survival advantage upon the bacterium, and as such, these strains dominate under selective pressure. Bacteria can acquire mutations in the genes encoding the DNA repair proteins and these strains, known as hypermutators, display a much higher rate of spontaneous mutation relative to the wild-type strains (Horst *et al*, 1999). Hypermutable *P. aeruginosa* strains are frequently isolated from both CF and non-CF patients with chronic infections (Maciá *et al*, 2005; Oliver *et al*, 2000), and the gene most commonly affected is *mutS* (Oliver *et al*, 2002). This gene has been identified as a breakpoint of inversion in IS*6100*-induced LCIs in CF *P. aeruginosa* subgroup C strains (Kresse *et al*, 2003).

The *mutS* gene in the 12 IS*6100*-containing strains was screened for the presence of insertions, that may correspond to IS*6100*, using PCR. *P. aeruginosa* PAO1 was included as a positive control, and PCR was carried out using primers MUTSF (5`-TTT GCA CCG TCC AAA GC-3`) and MUTSR (5`-CGA AGG TCA TTT TCT AG-3`). PCR conditions were as described (2.2.6), except denaturation was carried out for 1 minute, primer annealing at 50°C for 1 minute and elongation for 2 minutes. Products of the expected size (2.679kb), corresponding to an intact *mutS* gene, were obtained from all IS*6100*-containing strains [Fig. 3.3.5], suggesting that *mutS* was not interrupted by an insertion, particularly IS*6100*, in these strains.

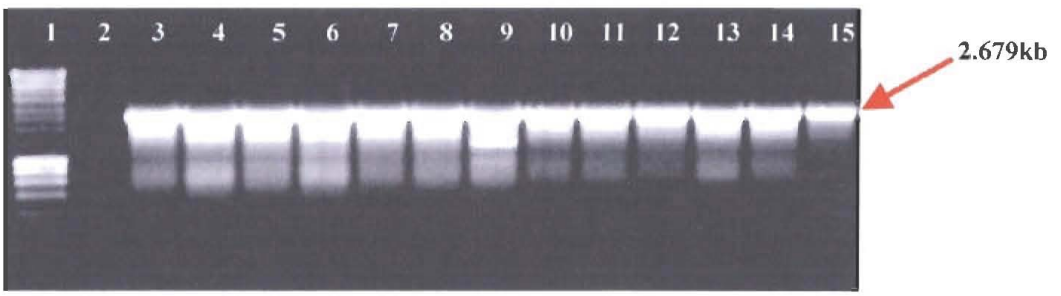


Figure 3.3.5: PCR amplification of *mutS* in IS*6100*-containing non-CF *P. aeruginosa* strains. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, PAO1; lane 4, 286; lane 5, 295; lane 6, 303; lane 7, 311; lane 8, 325; lane 9, 6; lane 10, 7; lane 11, 8; lane 12, 11; lane 13, 12; lane 14, 292, lane 15, 293.

The prevalence of hypermutable strains in chronically infected patients suggests that, even prior to treatment, mutants resistant to virtually all effective antipseudomonal antibiotics will be present (Oliver *et al*, 2004; Oliver *et al*, 2000). Thus, it follows, that as hypermutable *P. aeruginosa* strains evolve at the site of infection, multi-drug resistance develops (Maciá *et al*, 2005; Maciá *et al*, 2004; Oliver *et al*, 2000).

To determine directly whether IS*6100* may be associated with conversion to hypermutability, mutator assays (3.2.1) were carried out on selected *P. aeruginosa* strains. Both *P. aeruginosa* PAO1 and strain 302 were included as non-CF isolates that do not contain IS*6100*, with PAO1 susceptible to the carbapenem, imipenem, and strain 302 resistant to imipenem. Three CF isolates, 297, 333 and 527, all of which do not contain IS*6100*, were included. Strain 527 is an imipenem susceptible CF isolate and is non-mucoid, strain 297 is a non-mucoid, impenem resistant strain and strain 333 is both resistant to imipenem and displays the mucoid phenotype. Strains 311, 6 and 303 were selected as non-CF IS*6100*-containing isolates, with strains 311 and 6 resistant to imipenem and strain 303 susceptible to imipenem.

The differences in the mean number of colony forming units present for each strain grown in the presence and absence of rifampicin were compared with the control strain, *P. aeruginosa* PAO1 [Table 3.3.2]. Strains displaying mutation rates of more than 20 times that of PAO1 were considered to be hypermutable (Oliver *et al*, 2000).

Strain 302 displayed exactly the same mutation rate as PAO1, suggesting that the imipenem resistance observed in this strain is not associated with a hypermutable phenotype. Strain 527 had a 3.15-fold increase in mutation rate relative to PAO1, suggesting that this strain is a non-hypermutable CF strain. Strains 297 and 333 had a 33.65-fold and 233.5-fold increase in mutation rate, respectively, indicating that these two strains are hypermutable. Strains 311, 6 and 286 displayed 2.38-, 0.31- and 0.38-fold increases in mutation rates relative to PAO1, respectively, suggesting that IS*6100* does not play a role in hypermutability in these isolates.

Table 3.3.2: Mutation rates of selected *P. aeruginosa* strains grown in the presence of rifampicin.

P. aeruginosa strain		Mucoid phenotype	Imipenem susceptibility	IS*6100* presence	Colony forming units		Rate of mutation	[c] Fold increase in mutation rate relative to PAO1
					[a] M-H agar - rifampicin	[b] M-H agar + rifampicin		
Non-CF	PAO1	N	S	No	1.49×10^{10}	387	2.6×10^{-8}	0
	311	N	R	Yes	1.47×10^{10}	915	6.2×10^{-8}	2.38
	286	N	R	Yes	3.63×10^{9}	39	1.0×10^{-8}	0.38
	6	N	R	Yes	3.12×10^{9}	26	8.0×10^{-9}	0.31
	302	N	R	No	1.05×10^{10}	275	2.6×10^{-8}	0
CF	297	N	R	No	1.01×10^{10}	8835	8.8×10^{-7}	**33.65**
	333	M	R	No	2.25×10^{9}	13660	6.1×10^{-6}	**233.5**
	527	N	S	No	1.15×10^{10}	953	8.2×10^{-8}	3.15

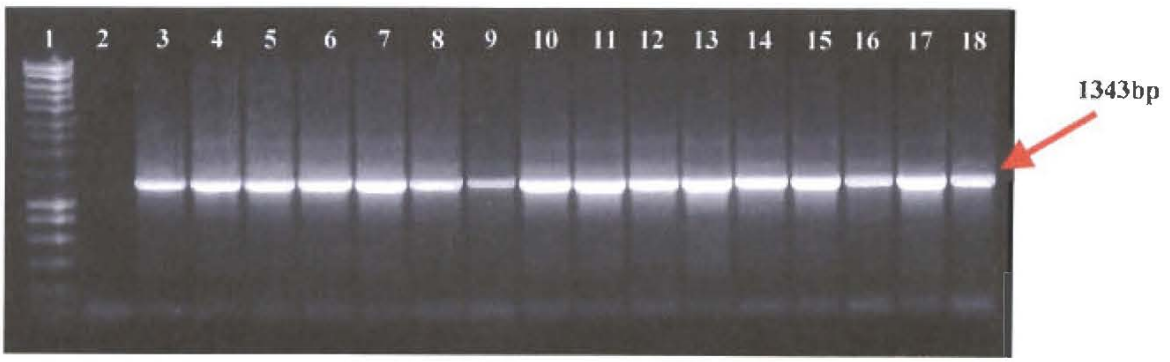
[a] Mean colony forming units calculated from 10^{-6}, 10^{-7} and 10^{-8}, or 10^{-7}, 10^{-8} and 10^{-9} dilutions of bacterial cells, all performed in triplicate.
[b] Mean colony forming units calculated from undiluted, 10^{-1} and 10^{-2} dilutions of bacterial cells, all performed in triplicate.
[c] Strains are considered hypermutable if their rate of mutation relative to PAO1 is greater than 20-fold (indicated in bold).
N – non-mucoid; M – mucoid; S – susceptible; R – resistant

3.3.4 Prevalence of IS elements in *oprD* of imipenem-resistant *P. aeruginosa*

It is well documented that resistance to imipenem in *P. aeruginosa* arises predominantly as a result of mutations in *oprD* (Segal *et al*, 2003; Köhler *et al*, 1999; Lambert, 2002; Huang & Hancock, 1993; Trias & Nikaido, 1990). Loss of this outer membrane porin, OprD, prevents the entry of imipenem into the bacterial cell, thereby resulting in resistance to imipenem (Köhler *et al*, 1999). Although loss of OprD is predominantly as a result of point mutations, frameshift mutations resulting in premature stop codons, or deletions within the *oprD* gene, the first instance of carbapenem resistance in clinical isolates as a result of insertional inactivation was recently described (Wolter *et al*, 2004). The *oprD* gene of five carbapenem resistant *P.aeruginosa* isolates contained an inserted IS element, IS*Pa1328*, and one strain contained an IS designated IS*Pa1635* (Wolter *et al*, 2004).

PCR assays were carried out on all (24) imipenem resistant strains included in this study to screen for the presence of IS elements that disrupt *oprD* and may thereby account for the imipenem resistance in these strains. Eight of the strains (6, 7, 8, 11, 286, 295, 311, 325) contained IS*6100*, and strains 5 and 12 were included as IS*6100*-containing, imipenem susceptible controls. Using primers OPRD3 (5`-ATG AAA GTG ATG AAG TGG AG-3`) and OPRD4 (5`- CTG TCG GTC GAT TAC AGG-3`) PCR assays were carried out (2.2.6) with primer elongation time increased to 90 seconds. Following AGE (2.2.4), amplification products corresponding to *oprD* (1343bp) were obtained for all but one isolate [Fig. 3.3.6].

A



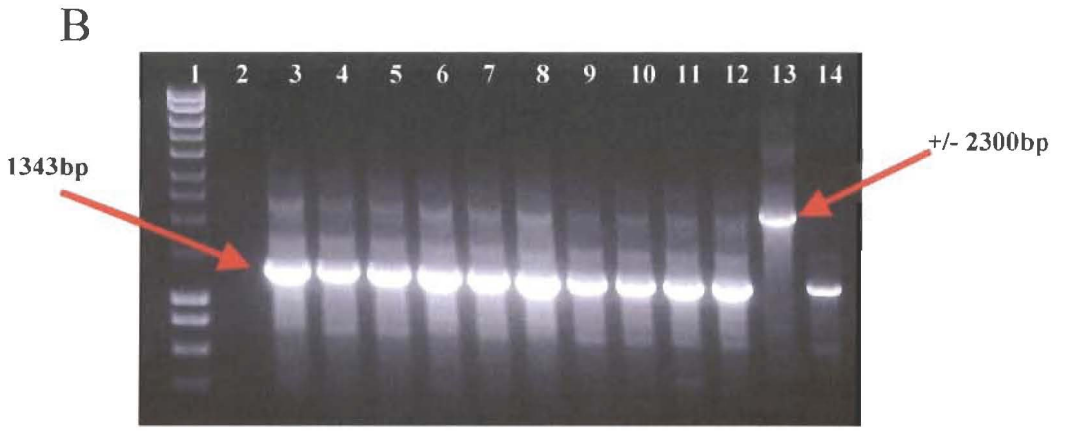


Figure 3.3.6: PCR amplification of *oprD* of imipenem-resistant *P. aeruginosa* isolates to determine the presence of insertion sequences.
A. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, +ve PAO1 control; lane 4, PACT5; lane 5, PACT7; lane 6, 280; lane 7, 286; lane 8, 289; lane 9, 295; lane 10, 297; lane 11, 299; lane 12, 300; lane 13, 302; lane 14, 306; lane 15, 311; lane 16, 325; lane 17, 333; lane 18, 335.
B. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, PAO1; lane 4, 506; lane 5, P19n; lane 6, 1; lane 7, 2; lane 8, 3; lane 9, 4; lane 10, 5; lane 11, 6; lane 12, 7; lane 13, 8; lane 14, 11.

The amplified product obtained from strain 8 was approximately 2300bp in size, suggesting an insertion of approximately 950bp in the *oprD* gene of this strain [Fig. 3.3.6 B]. The PCR product was purified (2.2.5.2) and sequenced on both strands (2.2.7). Analysis of the sequencing data obtained indicated that an IS element was inserted in *oprD* in strain 8. The nucleotide sequence of the *tnpA* gene of this element was most homologous to that of IS*Psy2* (Kamiunten *et al*, 2002), displaying 85% sequence similarity. The amino acid sequences of the transposases are 93.81% homologous [Fig. 3.3.8]. Both elements are flanked by 16bp imperfect inverted repeats, and both result in a 4bp duplication of the target upon transposition (Kamiunten *et al*, 2002) [Fig. 3.3.7]. The IS*Psy2*-like element transposed into *oprD* of strain 8 at nucleotide position 35 relative to the transcriptional start site of *oprD* [Fig. 3.3.7], in the opposite transcriptional orientation to *oprD*.

caatgaaagtgatgaagtggagcgccattgcactggGGAAGGTCTGAAGTAGCCGATGTTTTCCGGCT
ACTTCGCCATCGACACTTTCAAAAAACGGTAGCCGCTCAAATCCCGGTCAAACCTGCCGTCTATTTCA
GCCTTTTTTGCCGCTGCAAGCACCTCGCAGCAGTCACTTCACTCTTCACGGACGCACCTCTCCTGCAT
TGGAGAGCAAATGTCGACGCGCCATCCAAATGTTCGACAGGGCGAACAGCGTCACCATCTGAGCAGTG
TTTTTCACCAAGCCTCGGAAGCGCACTTTCGTATAACCAAACTGGCGCTTGATCACCCGAAATGGATG
CTCAACCTTGGAGCGAACCTGGGCCTTGGCTCTTTCGATCTTACGTATCGCTTTGTATAACGCGCTGC
GGTTACCGTGCTTTTTGTAAGTGCTGCGCCGGGCTGCAATCTGCCAGATGACTTGGCGCCCCTCATGC
TCTTCACGCTTCTCTACACCGGTATAGCCCGCATCAGCGCAGACCACGTTCTCACCACCGTGCAGCAG
TTTGTCGACTTGGGTTACGTCCGCGACGTTGGCCGCAGTGACCACCACGCTGTGCACAAGCCCGGACT
CATCGTCGACACCAATGTGAGCTTTGGCACCGAAGTAATACTGGTTGCCCTTCTTGGTCTGATGCATT
TCCGGGTCGCGTTTGCCGTCCTTGTTCTTGGTCGAACTGGGCGCATGAATCAGAGTGGCATCGACGAT
GGTGCCTTGTCGCAGTGACAAGCCACGGTCGCCCAAATAGCCATTGATCACGCCGAGAATACCGGTGG
CCAACTCATGCTTTTCCAGCAGGCGACGGAAGTTGAGGATGGTGGTTTCATCCGGGATTCGCTCCAGG
CTCAGACCGGAAAACTGGCGCAGGATCGTCGTCTCGTACAGCGCCTCTTCCATCGCCGGATCGCTGTA
ACCGAACCAGTTCTGCATTAGATGAATCCGCAGCATCGCCATCAGCGGATAGGCTGGACGGCCCCCTT
CGCCCTTTGGGTAATGAGGTTTGATCAGAGCAATCAAACCCTCCCAGGGTACGACCCGATCCATCTCT
ATCAGGAACAGCTCCTTGCGGGTCTGCTTGCGCTTACCGGCGTACTCGGCATCGGCGAAGGTCATTTG
TTTCATCATCGAAAAGCTCGGCGATTGGTGTCCGGGGATTTTGCCAAATCAGGAAGTCTTTTTCAGGA
TTTCCctggcggtttccgcaggtagcactcagttcgccgtggcc

Figure 3.3.7: Sequence of the IS*Psy2*-like element located within the *oprD* gene of *P. aeruginosa* strain 8. Lower case sequence represents *oprD*, upper case letters represent IS*Psy2*-like sequence. The start (ATG) codon of *oprD* is highlighted green; the start (ATG) and stop (TGA) codons of IS*Psy2*-like transposase are highlighted red. The 16bp inverted repeats of IS*Psy2*-like element are highlighted yellow, and the 4bp duplication of the *oprD* gene is highlighted blue.

Figure 3.3.8: Alignment of the amino acid sequences of the IS*Psy2* transposase and the transposase identified in *oprD* of *P. aeruginosa* strain 8. Amino acid residues that are conserved in both proteins are highlighted dark blue; non-homologous amino acid residues are indicated by light blue and white backgrounds.

3.4 Discussion

In non-CF *P. aeruginosa* isolates, IS*6100* has been identified in single copy within the resolvase gene of Tn*801*, which carries a bla_{TEM-21} gene and is adjacent to an *aac(3)-II* gene (Dubois *et al*, 2005; Dubois *et al*, 2002). IS*6100* was detected in 12 of the 48 non-CF isolates, and in none of the CF isolates, included in this study. Of these 12 IS*6100*-containing isolates, 7 were from GSH and 5 from RX, indicating that strains containing this element are prevalent in both hospitals.

The association of IS*6100* with Tn*801* in these 12 non-CF isolates was considered. In the absence of a control strain carrying Tn*801*, PCR assays to screen for the presence of bla_{TEM} and *aac(3)-II* were carried out. Interestingly, of all 12 IS*6100*-containing isolates that were screened, a PCR product corresponding to bla_{TEM}, as well as a PCR product corresponding to *aac(3)-II*, was obtained from strain 325 only. This suggests that the IS*6100* present on a single *Bam*HI fragment in strain 325, as determined by Southern hybridisation, may be associated with bla_{TEM} and *aac(3)-II*, and indeed with Tn*801*, in this strain. Additional PCR and sequence analysis will be necessary to confirm this hypothesis. TEM-21 mediates resistance to cephalosporins (Dubois *et* al, 2002; Arlet *et al*, 1999) and the acetyltransferase encoded by *aac(3)-II* mediates resistance to gentamicin and tobramycin (Dubois *et al*, 2002). The susceptibility of strain 325 to gentamicin, tobramycin, ceftazidime and cefepime is therefore unexpected. It may be that point mutations or insertional inactivation of the promoter regions of bla_{TEM} and *aac(3)-II* in strain 325 have resulted in reduced expression of these genes. Alternatively, mutation(s) within the structural genes themselves may have altered the substrate specificities of these enzymes. Of course, it is also possible that the bla_{TEM} gene identified in strain 325 does not encode the TEM-21 variant, but another TEM variant displaying a different substrate specificity. Again, sequence analysis of the PCR products obtained from strain 325 may provide further information with respect to identification and expression of these genes.

The association of IS*6100* with the mercury-resistant Tn*5041* (Yamane *et al*, 2004) in the non-CF, IS*6100*-containing strains in this study remains to be determined. The

presence of *rmtA* in this arrangement suggests that strains displaying resistance to the aminoglycosides, such as strain 286, may harbour IS*6100* in a similar arrangement.

The presence of IS*6100* flanking TNCP23 was not investigated at this stage, as this transposon has only been identified in CF *P. aeruginosa* strains (Klockgether *et al*, 2004). It is suggested that this mobile genetic element is responsible for conferring a survival advantage through the immobilisation of plasmid pKLC102 within the bacterial genome (Klockgether *et al*, 2004). It may be that selective pressures have led to similar transposon-mediated genetic alterations in non-CF isolates, in association with IS*6100*.

IS*6100* has been identified in CF *P. aeruginosa* isolates in genes associated with conferring a survival advantage (Kresse *et al*, 2003). It is not known whether this is due to specific integration of the element into these genes or due to the selection of these strains following random integration events (Kresse *et al*, 2003). What is apparent, however, is that the acquisition of IS*6100* seems to be beneficial to the bacterium. In order to determine the role of this element in non-CF *P. aeruginosa* strains from local hospitals, inverse PCR (Ochman *et al*, 1988) was carried out to investigate the genetic environment of IS*6100* in strain 6. The DNA sequences flanking IS*6100* in this strain could not, however, be elucidated using this method. Smears of DNA obtained following this procedure indicated that amplification of DNA had occurred, but that this amplification was mis-primed. The presence of a high concentration of digested genomic DNA in the ligation reaction would favour the formation of concatamers. Under such circumstances, the lengths of DNA between the primer binding sites would be variable, and amplification of these variable regions would lead to products varying in size, resulting in the smeared effect. IS*6100* was identified in strain 6 upon two DNA fragments of greater than 10kb in size and one DNA fragment of 2.2kb by Southern hybridisation [Fig. 2.3.4]. Thus, it is also possible that that the restriction sites resulting in the two large DNA fragments are located too far from IS*6100*, inhibiting the PCR amplification of such a large product. An alternative method for deducing the identity of the regions flanking IS*6100* is to create a genomic DNA library by shot-gun cloning. Sequence

analysis of insert DNA carrying IS*6100* would be carried out using primers specific for the vector molecule.

Hypermutable *P. aeruginosa* are being isolated with increasing frequency in both the CF and the non-CF setting, which has serious implications for the future with regard to antibiotic treatment of infections caused by these organisms as hypermutable strains are frequently pan-resistant (Oliver *et al*, 2004). Insertion of IS*6100* into *mutS*, which results in conversion to hypermutability, has been shown to be due to IS*6100*-mediated LCIs in CF *P. aeruginosa* strains (Kresse *et al*, 2003). No insertions were detected in *mutS* of the 12 strains containing IS*6100*. It could therefore be concluded that the *mutS* genes of all of these isolates did not contain IS*6100*. Additionally, no IS*6100*-mediated LCIs had occurred that would disrupt *mutS*, as a product of the correct size corresponding to an intact *mutS* gene was obtained.

Alteration of a number of genes other than *mutS* have been shown to result in conversion to hypermutability, including *mutL*, *mutT*, *mutY*, *mutM*, *mutD* and *uvrD* (Oliver *et al*, 2000). Mutation rates of 2.38-, 0.38- and 0.31-fold greater than that of PAO1 were obtained from IS*6100*-containing strains 311, 286 and 6, respectively. These IS*6100*-containing strains are therefore not hypermutable, suggesting that there is no association between IS*6100* and hypermutability in these strains. Moreover, this suggests that there is no association between IS*6100* and disruption of the genes that play a role in conversion to hypermutability in these strains. The MDR isolates, strain 6, strain 286 and strain 302, did not have significantly increased mutation rates relative to PAO1, suggesting no correlation between MDR and hypermutability in these strains. Though opinions differ as to the correlation between hypermutability and MDR, these results are in accordance with Gutiérrez *et al* (2004), who determined no relationship between MDR and hypermutability. Both strain 333 and strain 297 are CF hypermutable strains, displaying 233.5- and 33.65-fold increases in mutation rate relative to PAO1, respectively. The 7-fold increase in mutation rate of the mucoid CF strain 333 in comparison with the non-mucoid CF strain 297 suggests that there may be an association between hypermutability and conversion to mucoidy in these strains. As both of these characteristics are

commonly seen in CF *P. aeruginosa* isolates, however (Oliver *et al*, 2004; Govan & Deretic, 1996), it is equally likely that they may have arisen independently of each other.

Imipenem, a β-lactam antibiotic of the recently discovered carbapenem group, is frequently used to treat MDR pseudomonal infections (Lagatolla *et al*, 2004; Livermore, 2001). Resistance to imipenem is, however, emerging rapidly (Sasaki *et al*, 2004; Segal *et al*, 2003; Torres *et al*, 2000; Caldwell, *et al*, 1999), leaving few alternatives for treatment of *P. aeruginosa* infections. Imipenem resistance in *P. aeruginosa* has been shown to occur by the insertional inactivation of *oprD* (Wolter *et al*, 2005). Of the 25 imipenem resistant strains investigated in this study, only strain 8 had an insertion in *oprD*. Analysis of the sequencing data indicated that *oprD* was interrupted by an IS*Psy2*-like element in strain 8, displaying 85.09% nucleotide homology to IS*Psy2*. This insertional inactivation would account for lack of expression of *oprD* in this strain, and therefore resistance to imipenem. Little is known about IS*Psy2* other than it was identified adjacent to a virulence gene of *P. syringae* pv. eriobotryae NAE6, *psvA* (Kamiunten *et al*, 2002). This suggests that the IS*Psy2*-like element identified in strain 8 may have originally been acquired by this strain in association with a virulence factor(s), perhaps as part of a pathogenicity island. Following exposure to antibiotic selective pressure, *P. aeruginosa* strains in which the IS*Psy2*-like element had transposed into *oprD* were selected, as these strains were consequently resistant to imipenem. The resistance to imipenem of the remaining 24 strains may be as a result of point mutation(s) within *oprD* of these strains. Alternatively, carbapenem-hydrolysing β-lactamases of the IMP and VIM families, which result in high-level resistance to imipenem, may be present in these strains (Toleman *et al.*, 2004; Oh *et al.*, 2003; Sardelic *et* al., 2003; Lauretti *et al.*, 1999).

CHAPTER 4

SUMMARY AND GENERAL CONCLUSIONS

CF and non-CF *P. aeruginosa* isolates from local hospitals were screened for the presence of IS*6100*. PCR amplification and hybridization experiments detected this IS element in 12 of 48 non-CF *P. aeruginosa* isolates and in none of the 32 CF *P. aeruginosa* isolates included in this study. Furthermore, IS*6100* is present in non-CF *P. aeruginosa* isolates from both GSH and RX, indicating that the presence of this element is not confined to one hospital.

Using Southern hybridization experiments, the number of copies of IS*6100* present in the strains was determined. At least one copy of IS*6100* was detected in strain 325, with at least two copies in strain 286, strain 295, strain 303, strain 311, strain 7, strain 8, strain 11, PACT 7, strain 292 and strain 293. The presence of at least three copies of IS*6100* in strain 6 suggests that at least in this strain an LCI, which is associated with three copies of IS*6100*, may have occurred. Identification of the endpoints of the LCI in this strain may provide good insight into the role of IS*6100* in non-CF *P. aeruginosa* strains.

Strains 7, 8, 11, PACT 7 and 311 produced an identical hybridization pattern, suggesting that IS*6100* may be located within a similar genetic environment in these strains. This was also noted for strains 303, 292 and 293, suggesting that these groups of strains may be related. This was confirmed by genotyping using RAPD analysis and PFGE typing. It is interesting to note, however, that although strain 311 had a similar genetic arrangement for IS*6100* as strains 7, 8, 11 and PACT7, as seen by Southern hybridization, this strain was unique with respect to PFGE profiles. It may be that IS*6100* is located upon a mobile genetic element in these strains. This theory is also supported by the lack of a common genotypic profile among IS*6100*-containing *P. aeruginosa* isolates. Interestingly, however, strain 7, strain 8, strain 11, PACT 7 and strain 311 were isolated from GSH, while strain 303, strain 292 and strain 293 were isolated from RX. This suggests that IS*6100* is present on two different transmissible elements in these groups of strains, each of which is unique to the respective hospitals.

IS*6100* has previously been found in non-CF *P. aeruginosa* strains in association with transposons Tn*801* (Dubois *et al*, 2002; Dubois *et al*, 2004) and Tn*5041* (Yamane *et al*, 2003). That a PCR product corresponding to bla_{TEM} and one corresponding to *aac(3)-II* were detected in strain 325 suggests a possible association of IS*6100* with Tn*801* in this strain. It was previously suggested that these genes were acquired by *P. aeruginosa* from *Enterobacteriaceae*, in which Tn*801* was disrupted by IS*4321* as opposed to IS*6100* (Dubois *et al*, 2004). It would be interesting to determine the presence of such genetic arrangements in *Enterobacteriaceae* from GSH and RX, to establish whether a similar path of transmission has occurred.

No association was detected between IS*6100* and hypermutablity in the 12 non-CF *P. aeruginosa* strains harbouring the element. Similarly, an association between IS*6100* and imipenem resistance in the 8 imipenem resistant strains harbouring this element was detected. The resistance to imipenem displayed by strain 8, however, was found to be as a result of the insertional inactivation of *oprD* by an IS*Psy2*-like element. The prevalence of this IS element, as well as its association with imipenem resistance in other *P. aeruginosa* strains from local hospitals remains to be determined.

In conclusion, IS*6100* has been shown to be present in non-CF *P. aeruginosa* isolates from both GSH and RX. Surprisingly, IS*6100* was not present in *P. aeruginosa* isolates from CF patients. The similarity in IS*6100* hybridisation patterns observed between strains isolated from the same hospitals indicates that IS*6100* may be located extra-chromsomally, for example upon a plasmid, or, alternatively, it may be located chromosomally, within inserted foreign DNA sequences, such as transposons. Whichever form of mobile element IS*6100* is located within, the identically located *Bam*HI restriction sites in these acquired DNA sequences would result in the detection of IS*6100* upon the same size DNA fragments by Southern hybridisdation, as observed in two distinct groups in this study. Further investigation into the genetic environment of IS*6100* to confirm the location of this element is necessary, as transmission of IS*6100* to CF strains within hospitals could have a

negative impact on the care and outcome of treatment of *P. aeruginosa* infections in CF patients, should a strain carrying IS*6100* enter the CF population.

APPENDIX A

Media, Buffers and Solutions

A.1 Media

2X YT

Broth: Tryptone 16g
Yeast extract 10g
NaCl 5g

Made to 1l with dH_2O and autoclaved.

Agar: 2X YT broth 100ml
Agarose 1.5g

Autoclaved.

A.2 Buffers and Solutions

CTAB/NaCl solution (10% CTAB; 0.7M NaCl)

NaCl 4.1g
CTAB 10g

Dissolve the NaCl in approximately 80ml dH_2O and slowly add the CTAB.
Heat to 65°C to dissolve and adjust the volume to 100ml with dH_2O.

Denaturation solution

NaCl 43.83g
NaOH 10g

To 500ml with dH_2O.

ECL hybridization solution

ECL hybridization buffer 30ml
NaCl 0.88g
Blocking agent 1.5g

Mix at room temperature for 1 hour on a magnetic stirrer, then heat to 42°C for 1 hour with occasional stirring.

Gel tracking dye (6X)

Bromophenol blue	25mg
Sucrose	4g
0.5M EDTA	0.4ml

To 10ml with dH_2O.

0.25M HCl

Concentrated HCl	10.8ml

To 500ml with dH_2O.

Lysis buffer

30% lauroyl sarcosine	1.1.7ml
1M EDTA	17.5ml
100mg/ml Proteinase K	175µl

To 35ml with dH_2O.

Neutralisation solution

Tris	30.29g
NaCl	43.83g

Adjust pH to 7.5; make to 500ml with dH_2O.

Phenol

(in 10mM Tris, pH 7.6)

Commercial crystallized phenol	500g
8-hydroxyquinolone	0.6g
2M NaOH	7.5ml
dH_2O	130ml
1M Tris-HCl, pH 7.6	6ml

Liquefy at 40°C; aliquot and store at -20°C.

Primary Wash Buffer without urea

SDS	4g
20X SSC	25ml

To 1l with dH_2O; store at 4°C for up to 3 months.

SE buffer

5M NaCl	300µl
0.5M EDTA	1ml

To 20ml with dH_2O.

Solution 1

1M Tris-HCl (pH 8.0)	2.5ml
0.5M EDTA (pH 8.0)	10ml
20% (w/v) Glucose	5ml

To 100ml with dH_2O.

Solution 2

10N NaOH	2ml
20% (w/v) SDS	5ml

To 100ml with dH_2O.

Solution 3

5M Potassium acetate	60ml
Glacial acetic acid	11.5ml

To 100ml with dH_2O.

20X SSC

NaCl	175.32g
Tri-sodium citrate	88.23g

Adjust pH to 7.0; make to 1l with dH_2O; autoclave.

50X Tris-Acetate-EDTA (TAE) buffer

Tris	242g
Glacial acetic acid	57.1ml
0.5M EDTA	100ml

To 1l with dH_2O; autoclave.

10X Tris-Borate-EDTA (TBE) buffer

Tris	60.57g
Boric acid	27.82g
0.5M EDTA	10ml

To 500ml with dH_2O; autoclave.

Tris-EDTA (TE) buffer

1M Tris-HCl	5ml
1M EDTA	5ml

Adjust pH to 8.0; make to 500ml with dH_2O.

Tris-HCl

Tris	121.1g

Adjust pH to required level with concentrated HCl; make to 1l with dH_2O; autoclave.

X-gal (40mg/ml)

X-gal	0.4g

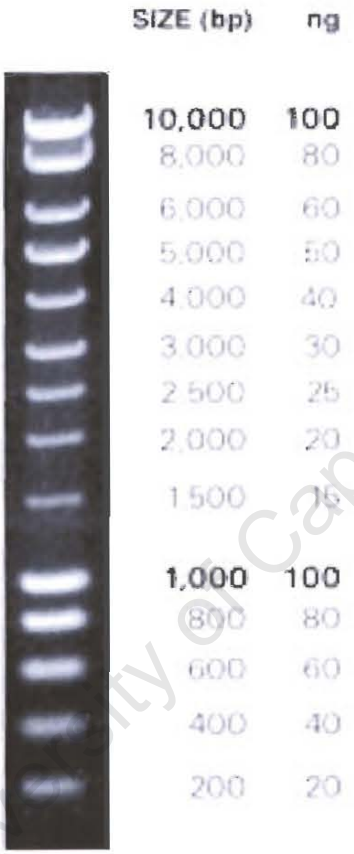
Dissolve in 10ml dimethyl sulfoxide (DMSO); add 1 volume dH_2O.

Store at -20°C.

APPENDIX B

Molecular Weight Markers

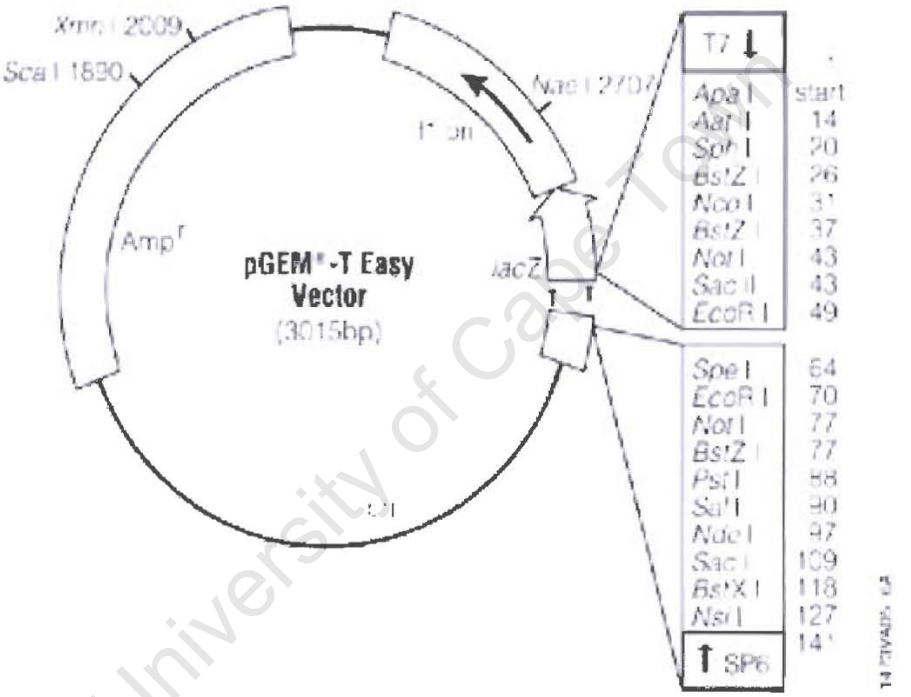
B.1 Hyperladder I [Bioline]



APPENDIX C

Maps of Plasmid Vectors

C.1 pGEM-T [Promega]



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