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**INVESTIGATION OF THE PREVALENCE AND
ROLE OF MOBILE GENETIC ELEMENTS
ASSOCIATED WITH AN AMINOGLYCOSIDE
RESISTANCE GENE, *aacC2a*, IN
*ACINETOBACTER BAUMANNII***

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Submitted in fulfilment of the requirements for the degree of Master of Science (Med)
in the Department of Clinical Laboratory Science,
Division of Medical Microbiology, Faculty of Health Sciences,
University of Cape Town

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UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

WITH LOVE, FOR MY PARENTS, STEPHEN AND CONSTANCE JONGWE

DECLARATION

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ABSTRACT

Mobile genetic elements contribute significantly to antibiotic resistance in *A. baumannii* isolates worldwide. In our local hospitals, the genetic arrangement IS1133_L/ISAb_a-1/IS1133_R-aacC2_a has been characterised in strains of *A. baumannii* spanning more than 23 years.

Investigation of sequences flanking this arrangement identified a 1 586 bp fragment from an *EcoRI*/*HindIII* genomic DNA library of *A. baumannii* strain MOS1. Sequence analysis of this fragment indicated that it contained a partial ISK_{pn12} element upstream of the left end of IS1133. PCR assays and sequence analysis using primers that are complementary to ISK_{pn12} indicated the presence of a full copy of ISK_{pn12} in *A. baumannii* MOS1. Southern hybridisation studies showed that MOS1 contains a single copy of ISK_{pn12} and reverse transcriptase PCR showed that the *tnpA* of this element is expressed during stationary and logarithmic growth phases. Other *A. baumannii* isolates that possess ISK_{pn12} also carry the IS1133_L/ISAb_a-1/IS1133_R-aacC2_a conserved genetic arrangement, whereas strains that do not carry this conserved region do not contain ISK_{pn12}. This finding suggests that ISK_{pn12} is part of the conserved genetic arrangement that has been identified in *A. baumannii* strains since 1983. The ISK_{pn12} element was not present in any other nosocomial isolates screened including representative isolates of *E. coli*, *P. aeruginosa*, and *K. pneumoniae*.

A carbapenem resistant *A. baumannii* strain MOS2 was isolated from the same patient as MOS1, following treatment with meropenem. Though MOS2 does not contain the conserved *aacC2a*-associated genetic arrangement, it carries at least ten copies of ISAb_a-1. The role of ISAb_a-1 was investigated in both isolates, *A. baumannii* MOS1 and MOS2.

ABBREVIATIONS

| | |
|-------------|--|
| % | – percentage |
| °C | – celcius |
| A. | – <i>Acinetobacter</i> |
| AME | – aminoglycoside-modifying enzyme |
| be | – base element |
| C. | – <i>Campylobacter</i> |
| CS | – conserved sequence |
| CTAB | – cetyltrimethylammonium bromide |
| DNA | – deoxyribonucleic acid |
| E. | – <i>Escherichia</i> |
| EDTA | – ethylenediaminetetra-acetic acid |
| ESBL | – extended spectrum β -lactamase |
| EtBr | – ethidium bromide |
| GSH | – Groote Schuur hospital |
| IPTG | – isopropyl- β -D-thio-galactosidase |
| IR | – inverted repeat |
| IS | – insertion sequence |
| K. | – <i>Klebsiella</i> |
| Kb | – kilo base |
| kDa | – kilo Dalton |

| | |
|--------------------------|------------------------------------|
| LB | – Luria Bertani |
| M | – molar |
| MBL | – metallo- β -lactamase |
| MDR | – multi-drug resistant |
| MGE | – mobile genetic element |
| μg | – micro gram |
| μl | – micro litre |
| mg | – milli gram |
| ml | – milli litre |
| mM | – milli molar |
| MIC | – minimum inhibitory concentration |
| ng | – nano gram |
| OMP | – outer membrane protein |
| ORF | – open reading frame |
| OXA | – oxacillinase |
| <i>P.</i> | – <i>Pseudomonas</i> |
| PAI | – pathogenicity island |
| PCR | – polymerase chain reaction |
| PBP | – penicillin binding protein |
| RNA | – ribonucleic acid |
| SDS | – sodium dodecyl sulphate |
| SHV | – sulfhydryl |

| | |
|--------------|--|
| SIM | – seoul-imipenemase |
| SOC | – super optimal broth with catabolite repression |
| TAE | – tris-acetate EDTA |
| Taq | – <i>Thermus aquaticus</i> |
| TEM | – temoniera |
| UK | – United Kingdom |
| USA | – United States of America |
| UV | – ultraviolet |
| VIM | – verona-integron-encoded MBL |
| v/v | – volume per volume |
| w/v | – weight per volume |
| YT | – yeast- tryptone |
| X-gal | – 5-bromo-4chloro-3indolyl- β -D-galactosidase |

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LITERATURE REVIEW

1.1 Microbiology and taxonomy of the *Acinetobacter* genus

Acinetobacter (*A.*) *baumannii* is an environmental bacterium that has become a major problem mainly in the hospital setting. It is a Gram-negative, non-fermentative, and non-motile opportunistic pathogen that does not form any spores. Although *Acinetobacter* species can appear Gram-positive on initial Gram stains, they are oxidase-negative, and this helps distinguish them from other Gram-negative organisms like *Pseudomonas* (*P.*), *Neisseria*, and *Moraxella*, (Allen & Hartman, 2005). *A. baumannii* grows on routine laboratory media as smooth, mucoid and non-pigmented colonies (Bergogne-Berezin & Towner, 1996). In liquid media, *A. baumannii* has a rod-shaped appearance during rapid growth and a coccobacillary appearance during the stationary phase. The morphologic characteristics of *A. baumannii* may also change depending on its exposure to antimicrobial agents (Bayuga *et al.*, 2002; Wilson *et al.*, 2004).

The genus *Acinetobacter* was not definitively established until 1971. Today more than 30 *Acinetobacter* species have been identified (Juni, 1972; Gerner-Smidt *et al.*, 1991; Vallenet *et al.*, 2008). Difficulties presented in identification of specific species in the laboratory using phenotypic tests have resulted in just 17 *Acinetobacter* species being named to date (Vallenet *et al.*, 2008). In most circumstances, species identification requires molecular techniques like DNA-DNA hybridisation (Bouvet & Grimont, 1986), amplified fragment length polymorphisms (Janssen *et al.*, 1997), amplified ribosomal DNA restriction analysis (Vanechoutte *et al.*, 1995), and biotyping (Bouvet & Grimont, 1987). Gerner-Smidt and colleagues termed four of their phenotypically similar strains *Acinetobacter calcoaceticus* - *A. baumannii* complex as standard tests could not distinguish them (Gerner-Smidt *et al.*, 1991).

1.2 Clinical significance of *Acinetobacter baumannii*

With regard to its habitat, *A. baumannii* is non-selective and is generally widely distributed in soil and water (Vallenet *et al.*, 2008). In humans, *A. baumannii* normally colonises wounds, the skin and mucous membranes. Up to 40 % of healthy adults can have skin colonisation, with higher rates among hospital personnel and patients (Glew *et al.*, 1977; Villegas & Hartstein, 2003; Allen & Hartman, 2005; Munoz-Price & Weinstein, 2008). The respiratory tract, blood, pleural fluid, peritoneum, urinary tract, surgical wounds, central nervous system, skin and eyes may be sites of infection or colonisation (Al-Khoja & Darrell, 1979; Bayuga *et al.*, 2002; Rodriguez-Bano *et al.*, 2004). *A. baumannii*-associated infections include pneumonia, meningitis, septicemia and urinary tract infections (Smith *et al.*, 2007). Less frequently, *A. baumannii* causes skin, soft tissue, abdominal, and central nervous system infections (Fournier & Richet, 2006). People mostly likely to get *A. baumannii* infections are those in intensive care units and those with serious underlying illnesses (Dijkshoorn *et al.*, 2007).

Although ubiquitous in the environment, *A. baumannii* has now become a key nosocomial pathogen worldwide. *A. baumannii*'s fitness in the hospital is mainly a result of its need for simple growth requirements, its ability to exploit various nutritional sources, and its ability to grow in a wide range of temperatures, pHs, salt concentrations and humidity levels (Bayuga *et al.*, 2002; Gusten *et al.*, 2002; Simor *et al.*, 2002; Iacono *et al.*, 2008). In this regard, *A. baumannii* shares its hardiness and survival abilities with other important Gram-negative nosocomial pathogens like *Escherichia (E.) coli*, *Klebsiella spp.*, *P. aeruginosa* and *Shigella spp.* (Kramer *et al.*, 2006).

A. baumannii was susceptible to most antibiotics in the 1970s. This nosocomial pathogen has now become a major cause of hospital-acquired infections, partly due to the widespread use of antibiotics in hospitals and *A. baumannii*'s ability to respond rapidly to antibiotic challenge, which selects for resistant strains (Thomas, 1998). As a result, multi-drug resistant (MDR) clones of *A. baumannii* are emerging and spreading throughout many geographic areas (Corbella *et al.*, 2000; Theaker *et al.*, 2003; Van Looveren & Goosens, 2004). The ability of *A. baumannii* to acquire foreign DNA has allowed it to develop antimicrobial resistance more rapidly than the

more traditional pathogenic bacteria, which require more time to acquire highly effective resistance mechanisms in response to antimicrobial exposure (Bergogne-Berezin & Towner, 1996).

Previously, reports of *A. baumannii* community acquired infections were rare (Dijkshoorn *et al.*, 2007), but this has increased recently (Gootz & Marra, 2008). An increased incidence of *A. baumannii* infections in the community is usually associated with a warm climate (Dijkshoorn *et al.*, 2007), cancer (Chen *et al.*, 2001; Anstey *et al.*, 2002; Leung *et al.*, 2006), diabetes mellitus (Falagas *et al.*, 2006; Alsultan *et al.*, 2009), chronic obstructive pulmonary disease and/or alcohol abuse (Falagas *et al.*, 2006). It is difficult to ascertain the source of these infecting *A. baumannii* strains within communities. *A. baumannii* isolates obtained from the hospital were thus compared with environmental isolates. The latter were entirely susceptible to antibiotics in clinical use, whereas the nosocomial isolates had MDR rates of up to 36.6 % (Zeana *et al.*, 2003). This and other similar studies revealed that nosocomial isolates were almost always related while the environmentally-derived isolates were unrelated, despite the fact that the environmental isolates were obtained from the same geographical area (Zeana *et al.*, 2003; Abbo *et al.*, 2005; Longo *et al.*, 2006). Poirel and his group discovered carbapenem resistant genes that had spread from *A. radioresistens* isolates to other *Acinetobacter* species within a hospital setup (Poirel *et al.*, 2008). These studies by Zeana and Poirel provide strong evidence of how the hospital has become the main reservoir of antibiotic resistant determinants.

1.3 Antibiotic resistance mechanisms in *Acinetobacter baumannii*

The emergence of antimicrobial resistance in many human pathogenic bacteria has become an international problem (Houghton, 2002; Romero *et al.*, 2006; Todd, 2006). Surveillance efforts show the development of many genera of bacteria that are resistant to all antibiotics (Gaynes & Edwards, 2005; Goossens, 2005). *A. baumannii* is one such pathogen and deserves close attention. Added to its intrinsic antibiotic resistance afforded by decreased membrane permeability and robust efflux systems (Gootz & Marra, 2008), this bacterium exhibits an

exceptional and highly impressive ability to rapidly acquire new antibiotic resistance genes from other bacterial genera through horizontal gene transfer (Mah *et al.*, 2001; Landman *et al.*, 2002). With vertical gene transfer, genes are inherited through cell division (Figure 1.1; Summers, 2006). Horizontal gene transfer on the other hand is driven by multiple systems, namely conjugation, transduction and transformation (Figure 1.2). However, not all bacteria can use all three processes. Horizontal gene transfer involves cell-to-cell transfer among closely and distantly related bacteria and gene transfer from one DNA molecule to another (Figure 1.2; Frost & Leplae, 2005; Summers, 2006; Dorman, 2009). The latter can take place regardless of similarity between the donating and recipient molecules (Hallet & Sherratt, 1997) and includes plasmids, transposons and integrons (Collis & Hall, 1995; Bennett, 2004).

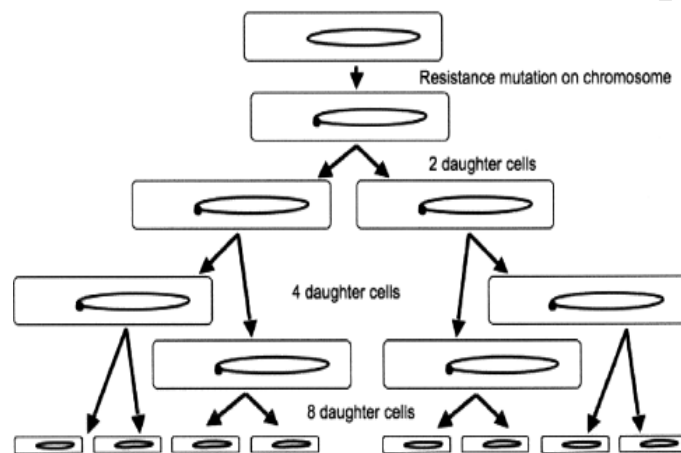


Figure 1.1: Vertical inheritance of resistance genes. Bacterial cells are represented by rectangles and their circular chromosomes by flattened circles. A spontaneous point mutation is represented by a small dot. Binary propagation through hundreds of cell divisions could yield trillions of cells with the same chromosomally borne resistance mutation (small dot). (Taken from Summers, 2006).

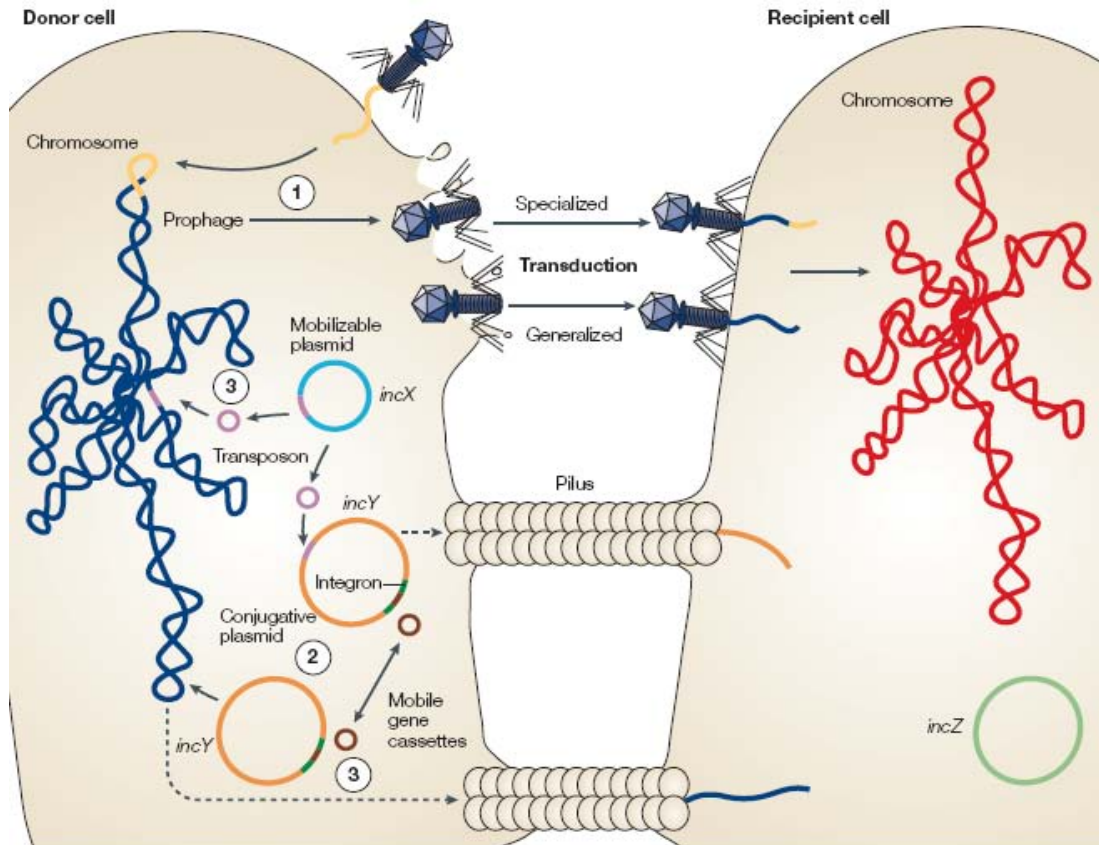


Figure 1.2: Transfer of DNA within and between bacterial cells. Transduction (1). The DNA genome (yellow) of a temperate phage inserts into the chromosome (dark blue) as a prophage; it later replicates, occasionally packaging host DNA alone (generalized transduction) or with its own DNA (specialized transduction), lyses the cell, and infects a naive recipient cell in which the novel DNA recombines into the recipient host cell chromosome (red). Conjugation (2). Conjugative plasmids (orange) use a protein structure (known as a pilus) to establish a connection with the recipient cell and to transfer themselves to the recipient cell. Alternatively, a copy of a small, multicopy plasmid or defective genomic island or a copy of the entire bacterial chromosome can be transferred to a naive cell, in which these genetic elements either insert into the chromosome or replicate independently if compatible with the resident plasmids (light green). Conjugative transposons and plasmids of Gram-positive bacteria (not shown) do not use pili. Transposition (3). Transposons (pink) integrate into new sites on the chromosome or plasmids by non-homologous recombination. Integrons (dark green) use similar mechanisms to exchange single gene cassettes (brown). (Taken from Frost & Leplae, 2005).

Phenotypic characteristics that can be acquired through horizontal gene transfer include those contributing towards pathogenesis, symbiosis and resistance to antimicrobial agents (Dorman, 2009). Undoubtedly, pathogens that can rapidly acquire antibiotic resistance have a selective advantage over those with more static genomes (Smith *et al.*, 2007). This has resulted in bacterial species resistant to an array of clinically useful antibiotics (Segal & Elisha, 1997; Hartzell *et al.*, 2007).

Antibiotic resistance in *A. baumannii* arises from various intrinsic and acquired mechanisms, which are expressed singly or in combination to enable survival of this pathogen in the presence of bacteriostatic and bactericidal agents. Such mechanisms include alterations of outer membrane and penicillin binding proteins, expression of efflux pumps that exude harmful substances, the expression of antibiotic inactivating enzymes, modifications of antibiotic resistance genes, and protection of the ribosome from antibiotics that target this macro molecular structure.

1.3.1. Alterations of outer membrane proteins and penicillin binding proteins

Porins are channel forming proteins that allow the transport of molecules across the hydrophobic lipid bilayer in bacteria (Garrett & Grisham, 2005). Compared to other Gram-negative bacteria, *A. baumannii* has a reduced number of porins (Sato & Nakae, 1991; Marti *et al.*, 2006). Smaller and fewer porins or outer membrane proteins (OMPs) in *A. baumannii* are associated with increased antibiotic resistance (Bou *et al.*, 2000, Fernández-Cuenca *et al.*, 2003, Mussi *et al.*, 2005). The most commonly described OMP in *A. baumannii* is the heat-modifiable protein HMP-AB (Gribun *et al.*, 2003) that belongs to the OmpA family, and is homologous to OprF in *P. aeruginosa* (Vila *et al.*, 2007). This family of channel proteins allow for the diffusion of larger solutes that cannot diffuse through smaller OMPs (Nikaido, 2003).

An investigation of the epidemic of MDR *A. baumannii* in New York City revealed carbapenem resistant isolates with reduced expression of 37-, 44-, and 47- kDa OMPs and increased class C cephalosporinase expression (Quale *et al.*, 2003). In the report, a small number of isolates were

studied and β -lactamase enzymes were not investigated. Similarly, in isolates from Madrid, the loss of 22-kDa and 33-kDa OMPs combined with the production of OXA-24 resulted in resistance to carbapenems (Bou *et al.*, 2000).

A 43-kDa protein in *A. baumannii* was identified as a homologue of OprD (a well-studied porin frequently associated with imipenem resistance in *P. aeruginosa*; Nikaido, 2003) and was involved in carbapenem diffusion (Dupont *et al.*, 2005). Another channel protein, CarO, a 29-kDa OMP which confers resistance to both imipenem and meropenem in *A. baumannii*, has been well characterised (Limansky *et al.*, 2002; Mussi *et al.*, 2005; Siroy *et al.*, 2005). CarO is a non-specific channel as it does not have any carbapenem-binding sites (Siroy *et al.*, 2005).

OmpW in *A. baumannii* is similar to its counterpart in *E. coli* and *P. aeruginosa*. The role of the OmpW OMP in *A. baumannii* is still unclear; however, its expression was decreased in an *in vitro* colistin-resistant *A. baumannii* mutant (Sara Martí, unpublished data in Vila *et al.*, 2007). Previously, OmpW expression was severely decreased in a ceftriaxone-resistant *Salmonella Typhimurium* strain (Hong *et al.*, 2006) and it may be that OmpW might also be involved in the uptake of this antibiotic in *A. baumannii* (Vila *et al.*, 2007). It will be necessary to carry out further studies to elucidate the role of OMPs in MDR *A. baumannii*. It would also be essential to characterise the structure of the outer membrane of *A. baumannii* that confers its special permeability features (Vila *et al.*, 2007).

Of the few studies undertaken, there is variability in the number of observed OMPs in *A. baumannii* (Cuenca *et al.*, 2003) and a similar pattern exists for penicillin binding proteins (PBPs) (Fernández-Cuenca *et al.*, 2003). Resistance to carbapenems can also be explained by the reduced expression of PBP-2 in *A. baumannii* (Fernandes-Cuenca *et al.*, 2003). Notably, these strains had no OMPs and produced β -lactamases, illustrating the interaction of different mechanisms of resistance against one antibiotic class.

1.3.2. Efflux pumps

Efflux pumps represent a single mechanism causing resistance against several compounds toxic to the bacterial cell, including different antibiotic classes (Perez *et al.*, 2007). Various classes of efflux pump families exist among the different bacterial species (Poole, 2005) and the AdeABC efflux pump in *A. baumannii* belongs to the resistance-nodulation-cell division family. This pump is responsible for the resistance of *A. baumannii* against aminoglycosides, cefotaxime, tetracyclines, erythromycin, chloramphenicol, trimethoprim, and fluoroquinolones (Magnet *et al.*, 2001). High-level carbapenem resistance can be achieved by the increased expression of the AdeABC efflux pump combined with class D β -lactamases expression (Marqué *et al.*, 2005). AdeABC overexpression is a result of point mutations in either the *adeR* or *adeS* genes that make up a two step regulator (*adeR*) and sensor (*adeS*) system (Marchand *et al.*, 2004).

Another multidrug efflux pump, AbeM, belonging to the multidrug and toxic compound extrusion family has been described in *A. baumannii* (Su *et al.*, 2005). Unlike the AdeABC efflux pump, the AbeM has substrate specificity and is responsible for the efflux of fluoroquinolones, trimethoprim, and aminoglycosides (Su *et al.*, 2005). This pump is similar to a PmpM pump in *P. aeruginosa*, VcmA in *Vibrio parahaemolyticus*, YdhE in *E. coli* and HmrH in *Haemophilus influenzae* (Su *et al.*, 2005).

1.3.3. Enzymatic inactivation of β -lactams

The β -lactam antibiotics are the antibiotics of choice in treatment of severe *A. baumannii* infections. This class of antibiotics includes penicillin derivatives, cephalosporins, monobactams, β -lactamase inhibitors and carbapenems (Holten & Onusko, 2000). Carbapenems are one of the last resort antibiotics left to treat *A. baumannii* infections, unfortunately, resistance to carbapenems is steadily increasing (Henwood *et al.*, 2002; Nordmann & Poirel, 2002; Segal *et al.*, 2004; Poirel & Nordmann, 2006a; Turton *et al.*, 2006; Le Hello *et al.*, 2008; Mugnier *et al.*, 2009) and this causes great concern as the rate of antibiotic resistance doesn't match the rate of antibiotic discovery (Fernandes, 2006).

Different mechanisms of β -lactam resistance have been reported and identified in *A. baumannii* and these include β -lactamase production (Danes *et al.*, 2002; Brown & Amyes, 2006; Dorsey *et al.*, 2006; Qi *et al.*, 2008; Kansal *et al.*, 2009), penicillin binding protein alterations (Fernández-Cuenca *et al.*, 2003), activity of efflux pumps (Perez *et al.*, 2007), and reduced penetration across the outer membrane porins (Bou *et al.*, 2000; Limansky *et al.*, 2002; Quale *et al.*, 2003). Nevertheless, β -lactamase production is the primary cause of resistance (Corvec *et al.*, 2003; Hall & Barlow, 2005; Ruiz *et al.*, 2007) or is often linked with other resistance mechanisms in the same antibiotic resistance strain (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003).

The most commonly used classification of β -lactamases is the Ambler classification (Ambler, 1980) that divides β -lactamases into four classes (Classes A, B, C and D; Ambler, 1980), based on their amino acid sequences. Class A, C, and D β -lactamases share a common β -lactam hydrolysis mechanism with the amino acid serine as their active site (Ambler, 1980; Jaurin & Grundstrom, 1981; Ouellette *et al.*, 1987). Class B β -lactamases on the other hand require a bivalent metal ion, mostly Zn^{2+} , for activity (Ambler, 1980, Hall & Barlow, 2005).

1.3.3.1. Class A β -lactamases

The class A β -lactamase variants temoniera (TEM), sulfhydryl (SHV), VEB, PER and CTX-M, have been detected mostly in *Enterobacter cloacae*, *Serratia marcescens*, and *Klebsiella* spp. but more recently in *Acinetobacter* spp. (Gootz & Marra, 2008; Walther-Rasmussen & Høiby, 2004; Vila *et al.*, 1993). Class A β -lactamases are present in *A. baumannii* isolates from all over the world. *A. baumannii* harboring the integron-borne VEB-1 has caused outbreaks in French and Belgian hospitals (Naas *et al.*, 2006a; Naas *et al.*, 2006b, Poirel *et al.*, 2003). SHV-12-producing *A. baumannii* strains have been reported in China (Huang *et al.*, 2004). In addition, an *A. baumannii* isolate harbouring both SHV-12 and TEM-116 was identified in Netherlands (Naiemi *et al.*, 2005). In Italy, TEM-92 was found in *A. baumannii* isolates (Endimiani *et al.*, 2007). CTX-M-2, an extended spectrum β -lactamase (ESBL) that hydrolyses cefotaxime and ceftriaxone, was found in epidemic strains of *A. baumannii* in a neurosurgical ward in Japan, as well as in *A. baumannii* strains isolated in Bolivia (Nagano *et al.*, 2004; Celenza *et al.*, 2006).

Interestingly, the dissemination of the *bla*_{CTX-M} gene is not as widespread in *Acinetobacter spp.* as among Enterobacteriaceae (Perez *et al.*, 2007; Gootz & Marra, 2008). *A. baumannii* strains with PER-1 demonstrate high-level resistance to penicillins and extended-spectrum cephalosporins but not to carbapenems (Perez *et al.*, 2007) and such strains are mostly prevalent in Turkey, Korea, France, Belgium, and Bolivia (Yong *et al.*, 2003; Kolayli *et al.*, 2005; Poirel *et al.*, 2005a; Celenza *et al.*, 2006; Naas *et al.*, 2006b). In 2006, a molecular and epidemiological analysis described PER-1 for the first time in the United States (Hujer *et al.*, 2006).

Another class A β -lactamase, SCO-1 was identified in *A. baumannii* (Poirel *et al.*, 2007; Papagiannitsis *et al.*, 2007). This enzyme has a 40 % homology to other known class A β -lactamases and hydrolyses penicillin at high levels. However, SCO-1 hydrolyses carbapenems and cephalosporins at very low levels. The *bla*_{SCO-1} gene has been identified on a 150 kilobase (kb) plasmid (Poirel *et al.*, 2007) where it was associated with an *umuDC*-type operon (Poirel *et al.*, 2007) that encodes *umuD* and *umuC* proteins involved in SOS responses (Smith & Walker, 1998).

Generally, detection of the level of resistance of ESBLs in *A. baumannii* is complicated by the presence of chromosomal cephalosporinases. Therefore, it is often uncertain to what extent class A ESBLs are distributed in *A. baumannii* (Perez *et al.*, 2007).

1.3.3.2. Class B β -lactamases

Metallo- β -lactamases (MBLs) can hydrolyze carbapenems and every other β -lactam antibiotic except aztreonam (Walsh, 2005; Walsh *et al.*, 2005). This class of β -lactamases was first described in a strain of *P. aeruginosa* found in Japan in 1988 (Watanabe *et al.*, 1991). MBLs have now been identified in most Gram-negative bacteria including *Acinetobacter* genomic species 13 TU (Poirel & Nordmann, 2006a; Lim *et al.*, 2007). However, only three types of metallo- β -lactamases, IMP, Verona-integron-encoded MBL (VIM), and Seoul-imipenemase (SIM), have been identified in *A. baumannii* (Da Silva *et al.*, 2002; Yum *et al.*, 2002; Lee *et al.*,

2004; Walsh *et al.*, 2005; Franklin *et al.*, 2006), all of which were initially identified in *Pseudomonas* species (Lauretti *et al.*, 1999; Lee *et al.*, 2005). As the MBLs are not intrinsic to *A. baumannii*, there is increasing evidence that they were acquired from a reservoir, *P. aeruginosa*, in the hospital setting (Walsh *et al.*, 2005; Lolans *et al.*, 2005; Toleman *et al.*, 2005; Hanson *et al.*, 2006), highlighting the important continuous flow of genetic information within the hospital environment.

IMP MBLs in *A. baumannii* are usually detected as part of a class 1 integron (Huang *et al.*, 2008). Several IMP MBLs have been described so far: IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, and IMP-11 (Walsh, 2005; Walsh *et al.*, 2005). Most of the IMP β -lactamases in *A. baumannii* have been reported in Japan (Nishio *et al.*, 2004), but they seem to have first appeared in Hong Kong (Chu *et al.*, 2001). An MBL in *A. baumannii* in the Americas has been reported from an IMP-producing isolate from Brazil (Gales *et al.*, 2003; Tognim *et al.*, 2006).

The broad-spectrum SIM-1 MBL possesses 69 % identity with IMP-12 MBL and 64 % identity with IMP-9 MBL. There is intriguing genetic evidence to suggest that the *bla*_{SIM-1} cassette may have originated from the *P. alcaligenes* In55044 superintegron (Lee *et al.*, 2005). Thus far, SIM-1 has only been reported in *A. baumannii* isolates from South Korea (Lee *et al.*, 2005), where this determinant might also be prevalent (Park *et al.*, 2005).

VIM-1 MBL was first identified in 1997 in Italy in a *P. aeruginosa* isolate (Lauretti *et al.*, 1999); *A. baumannii* harboring VIM-2 has been reported in Korea (Weldhagen, 2004). Both variants are often associated with class 1 integrons in *A. baumannii* (Poirel & Nordman, 2002; Yum *et al.*, 2002; Weldhagen, 2004).

IMP and VIM β -lactamases have stronger hydrolytic activity against carbapenems than do carbapenem-hydrolyzing oxacillinases while the SIM variant confers a moderate level of carbapenem resistance (Yum *et al.*, 2002; Lee *et al.*, 2004; Lee *et al.*, 2005; Walsh *et al.*, 2005).

1.3.3.3. Class C β -lactamases

All *A. baumannii* isolates, like other Gram-negative organisms, have a chromosomally encoded *ampC* cephalosporinase gene much like the species specific *ampC* gene in *E. coli* (Gootz & Marra, 2008). The class C cephalosporinases hydrolyze penicillins and narrow-spectrum and extended-spectrum cephalosporins, but not cefepime or carbapenems (Morohoshi & Saito, 1977). The *ampC* gene is not inducible with β -lactams, therefore it is normally expressed at low levels (Joly-Guillou *et al.*, 1988; Blechschmidt *et al.*, 1992; Perilli *et al.*, 1996; López-Hernández *et al.*, 2001; Poirel & Nordmann, 2006b). Thus, many clinical isolates of *A. baumannii* are resistant to ceftazidime (Joly-Guillou *et al.*, 1987; López-Hernández *et al.*, 2001). Sequence analysis of *ampC* flanking regions has failed to identify the presence of any of the regulatory regions, *ampR* or *ampD* (Perilli *et al.*, 1996). Nevertheless, the presence of an insertion sequence, IS*Aba-1*, upstream of the *ampC* gene has been associated with increased levels of resistance to β -lactams in *A. baumannii* isolates by providing promoter sequences within this IS element (Corvec *et al.*, 2003; Segal *et al.*, 2004).

1.3.3.4. Class D β -lactamases

The most common carbapenemases detected in *A. baumannii* are carbapenem-hydrolyzing oxacillinases (Poirel & Nordmann, 2006a). There exists nine clusters of carbapenem-hydrolyzing oxacillinases (Walther-Rasmussen & Høiby, 2006; Queenan & Bush, 2007) of which four, including, OXA-23-like, OXA-40-like, OXA-51/69-like and OXA-58, have been identified in *A. baumannii*. The first description of such an OXA carbapenemase in *A. baumannii* was OXA-23, which was obtained from a clinical isolate found in Scotland in 1985 before the introduction of carbapenems. Since then, this plasmid-encoded enzyme, initially named ARI-1 (acinetobacter resistant to imipenem) has been discovered internationally (Dalla-Costa *et al.*, 2003; Jeon *et al.*, 2005; Brown & Amyes, 2006; Segal *et al.*, 2007). The OXA-23-like group consists of OXA-23, OXA-27, and OXA-49. OXA-24, OXA-25, OXA-26, and OXA-40 constitute the second group which shares 60 % identity with the OXA-23-like group (Dorsey *et al.*, 2006). OXA-51/69-like

carbapenemases include OXA-64, OXA-65, OXA-66, OXA-68, OXA-70, OXA-71, OXA-78, OXA-79, OXA-80, OXA-82 (Perez *et al.*, 2007), OXA-90, OXA-130, OXA-131, and OXA-132 (Alsultan *et al.*, 2009). The OXA-51/69-like β -lactamases are naturally occurring chromosomal enzymes in *A. baumannii*; they have been identified in isolates from all over the world, and their expression varies according to the presence of the insertion sequence, IS*Aba-1* (Poirel & Nordmann, 2006a; Alsultan *et al.*, 2009). OXA-58, a plasmid-borne carbapenemase, (Poirel *et al.*, 2005b; Qi *et al.*, 2008) has been identified in strains from France, England, Argentina, Spain, Turkey, Romania, Austria, Greece, Scotland, and Kuwait (Marque *et al.*, 2005; Coelho *et al.*, 2006; Pournaras *et al.*, 2006). All these variants of carbapenem-hydrolyzing oxacillinases were initially identified in carbapenem resistant clinical isolates of *A. baumannii* (Brown & Amyes, 2006; Dorsey *et al.*, 2006; Qi *et al.*, 2008).

The significant contribution of class D β -lactamases to carbapenem resistance in *A. baumannii* has been emphasised, particularly when they are accompanied by IS*Aba-1* and IS*Aba-3* (Marque *et al.*, 2005). The widespread presence of Class D β -lactamases may indicate that oxacillinases are also an essential component of the genetic make-up of *Acinetobacter* spp. (Walther-Rasmussen & Høiby, 2006).

1.3.4. Resistance to aminoglycosides

The aminoglycoside antibiotics function by specifically binding to an aminoacyl site on the 16S rRNA of the 30S ribosomal subunit where they interfere with bacterial protein synthesis (Magnet & Blanchard, 2005). While nucleotide substitution of the aminoacyl site, decreased membrane permeability, and the previously described AdeABC multidrug efflux pump play a role in aminoglycoside resistance, it is the aminoglycoside-modifying enzymes (AMEs) that mainly mediate resistance to aminoglycosides in *A. baumannii*. Aminoglycoside resistance by AMEs is a major unwelcome feature in the multidrug-resistant phenotype of *A. baumannii* (Bergogne-Berezin & Towner, 1996) and is mediated by the alteration of the amino- or hydroxyl groups of the antibiotic (Dever & Dermody, 1991; Van Looveren *et al.*, 2004). The AMEs include

phosphotransferases, acetyltransferases, and nucleotidyltransferases, most of which are encoded on integrons (Table 1.1). Resistance to aminoglycosides as a result of the activity of all three types of AMEs has been identified in *A. baumannii* (Bergogne-Berezin & Towner, 1996; Seward *et al.*, 1998; Nemeč *et al.*, 2004).

Table 1.1: Aminoglycoside modifying enzymes reported in *A. baumannii*

| Enzyme | Substrate | Reference |
|-------------------------|---------------|---|
| Acetylating | | |
| AAC(2")I | Gm, Neo, Tob | Dowding, 1979; Elisha & Steyn, 1991 |
| AAC(3)I | Gm | Vila <i>et al.</i> , 1993 |
| AAC(3)II | Gm, Tob, Net | Murray & Moellering, 1980; Elisha & Steyn, 1994 |
| AAC(3)Va | Gm, Tob | Elisha & Steyn, 1991; Shaw <i>et al.</i> , 1993 |
| AAC(3)IV | Gm, Tob | Shaw <i>et al.</i> , 1993 |
| AAC(6') | Amik, Km, Tob | Lambert <i>et al.</i> , 1990; Lambert <i>et al.</i> , 1993; Murray & Moellering, 1979; Shannon <i>et al.</i> , 1978 |
| Adenylating | | |
| AAD(2") | Gm, Km, Tob | Shaw <i>et al.</i> , 1993; Elisha & Steyn, 1994; Segal & Elisha, 1997 |
| AAD(3")(9) ^a | | Devaud <i>et al.</i> , 1982; Goldstein <i>et al.</i> , 1983; Murray & Moellering, 1980; Vila <i>et al.</i> , 1993 |
| ANT(2")I | Gm, Km, Tob | Murray & Moellering, 1979 |
| ANT(3")I | Sm | Shannon <i>et al.</i> , 1978 |
| Phosphorylating | | |
| APH(3')I | Km, Neo | Devaud <i>et al.</i> , 1982; Goldstein <i>et al.</i> , 1983; Shaw <i>et al.</i> , 1993 |
| APH(3")I | Sm | Elisha & Steyn, 1989 |
| APH(3')II | Km, Neo | Murray & Moellering, 1979 |
| APH(3')III | Amik, Km, Neo | Joly-Guillou <i>et al.</i> , 1987; Murray & Moellering, 1979; |
| APH(3')IV | Km, Neo | Lambert <i>et al.</i> , 1988; Martin <i>et al.</i> , 1988; Lambert <i>et al.</i> , 1990; Shaw <i>et al.</i> , 1993; Vila <i>et al.</i> , 1993 |
| APH(3")VI | Amik, Km, Neo | Bergogne-Bérézin & Towner, 1980 |

Modification of a table from Bergogne-Bérézin & Towner, 1996.

^a Enzymatic activity detectable only *in vitro*.

Amik, amikacin; Km, Kanamycin; Tob, Tobramycin; Gm, Gentamycin; Neo, Neomycin; Net, Netilmicin; Sm, Streptomycin

Similar AMEs can be found in unrelated isolates of *Acinetobacter* spp. and particular genes are not necessarily restricted to specific regions of the world. Hence, similar integrons have been found in genotypically distinct isolates from different locations worldwide (Seward *et al.*, 1998;

Seward, 1999). This finding has been confirmed throughout Europe (Gallego & Towner, 2001; Nemeč *et al.*, 2004; Turton *et al.*, 2005), where a remarkably stable class 1 integron with the same variable region was retrieved from isolates that are genotypically unrelated, indicating their importance in the dissemination of antibiotic resistance genes. Some strains harbour more than one aminoglycoside resistance gene, with as many as six different resistance genes being identified in some isolates (Bergogne-Bérézin & Towner, 1996).

A novel gene *aac(6')-Ig*, was identified in *A. haemolyticus*, where it confers resistance to amikacin (Lambert *et al.*, 1993). This gene has not been identified in any other species and may be used to facilitate the identification of this *Acinetobacter* spp. (Lambert *et al.*, 1993).

Aminoglycoside resistance in *A. baumannii* may be due to methylation of the 16S ribosomal RNA (Lee *et al.*, 2006), a common mechanism used by other bacterial species (Doi & Arakawa, 2007; Doi *et al.*, 2007). Naturally occurring aminoglycosides are synthesised by some *Actinomycete* spp., which in turn are intrinsically resistant to this group of antibiotics (Cundliffe, 1989). Intrinsic aminoglycoside resistance in *Actinomycete* spp. is mostly a result of ribosomal protection due to the methylation of specific nucleotides within the aminoacyl site on the 16S rRNA in the 30S ribosomal subunit by methylase enzymes. This way, the aminoglycosides will not bind to the 30S ribosomal subunit, and so serves as a self defense mechanism (Cundliffe, 1989). Unfortunately, this resistance mechanism has been identified in pathogenic bacteria (Doi & Arakawa, 2007). The 16S rRNA methylases are often located on transposons within conjugative plasmids, hence their widespread identification in nosocomial pathogens (Benveniste & Davies, 1973; Galimand *et al.*, 2003; Galimand *et al.*, 2005; González-Zorn *et al.*, 2005; Wachino *et al.*, 2006a; Wachino *et al.*, 2006b; Doi *et al.*, 2007).

1.3.5. Resistance to fluoroquinolones

Fluoroquinolones inhibit bacterial DNA synthesis by targeting the DNA gyrase (GyrA-GyrB) and topoisomerase IV (ParC-ParE) proteins, which have essential functions in DNA replication. Fluoroquinolone resistance in *A. baumannii* is mediated by modifications in the structure of DNA

gyrase mediated by mutations in the quinolone resistance *gyrA* and *parC* genes (Vila *et al.*, 1995; Vila *et al.*, 1997; Seward & Towner, 1998; Eliopoulos, 2004). These changes result in a lower affinity for the binding of the quinolone to the DNA-enzyme complex.

The first plasmid-mediated quinolone resistance gene, *qnrA*, was reported in 2002 from a *Shigella dysenteriae* isolate (Tran & Jacoby., 2002). It encodes a protein QnrA, which binds to the gyrase and prevents its inactivation by fluoroquinolones. Other gyrase-protecting proteins, QnrB in *Klebsiella (K.) pneumoniae* (Jacoby *et al.*, 2006), QnrC in *Proteus mirabilis* (Wang *et al.*, 2009), and QnrS, have been identified on plasmids. Genes encoding QnrA and QnrB occur as integron cassettes and are seen increasingly in bacterial isolates obtained from infected patients worldwide (Tran & Jacoby, 2002; Jacoby *et al.*, 2006; Summers, 2006). The plasmid-mediated quinolone resistance genes, *qnrA* and *qnrB*, and the gene encoding the AME with the capacity to modify ciprofloxacin (*aac(6')-Ib-cr*) have not been detected in *A. baumannii* (Cattoir & Nordmann., 2009), but have been identified in other Gram-negative bacteria, including *Enterobacter* and *Klebsiella* species (Robicsek *et al.*, 2005). It will be interesting to determine whether the spread of integron-based *qnr* genes diminishes the occurrence of the gyrase mutants (Summers, 2006).

In *A. baumannii*, fluoroquinolone resistance also results from reduced outer membrane permeability, or over expression of the non-specific AdeABC efflux pump (Magnet *et al.*, 2001). Fluoroquinolone resistance in *A. baumannii* could also be the result of the expression of the substrate specific AbeM efflux pump, as revealed by expression studies of an *A. baumannii* *abeM* gene cloned into *E. coli* (Su *et al.*, 2005).

1.3.6. Resistance to tetracyclines

In bacteria, tetracycline inhibits protein synthesis by binding to the 30 S ribosomal subunit (Roberts, 1996). Mechanisms contributing to tetracycline resistance include the effect of efflux pumps, and ribosomal protection. TetA and TetB are specific transposon-mediated efflux pumps; TetB effluxes both tetracycline and minocycline, whereas TetA drives the efflux of tetracycline

only (Guardabassi *et al.*, 2000; Huys *et al.*, 2005). Ribosomal protection is mediated by a protein, TetM, which has 100 % homology to the *S. aureus* counterpart (Ribera *et al.*, 2003). TetM shields the ribosome from the action of tetracycline, doxycycline, and minocycline (Bertrand *et al.*, 2005).

Tigecycline, a representative of a new class of antibiotics, glycylicyclines, is related to tetracyclines and is often used to treat complicated skin and intra-abdominal infections (Institute of Medicine, 1998). Bloodstream infections caused by tigecycline-resistant *A. baumannii* isolates have been reported (Peleg *et al.*, 2007; Ruzin *et al.*, 2007). Resistance to tigecycline in *A. baumannii* isolates was a result of the over expression of AdeABC efflux pumps (Ruzin *et al.*, 2007). The tigecycline-resistant strains had the AdeRS two-component system, which regulates AdeABC efflux pump expression, disrupted by the insertion sequence ISAb_a-1. The insertion of ISAb_a-1 resulted in an over expression of *adeABC* by providing promoter sequences for this operon (Ruzin *et al.*, 2007). TetX, a plasmid-borne flavin-dependent mono-oxygenase degrades tigecycline; however, this enzyme has not been identified in *A. baumannii* clinical isolates (Moore *et al.*, 2005).

1.3.7. Resistance to polymyxins

Polymyxin B and polymyxin E (colistin) are peptide antibiotics isolated from *Bacillus colistinus* (Montefour *et al.*, 2008). Colistin became commercially available in 1959; but due to its nephrotoxicity it was rarely used. However, in the absence of less toxic antibiotics with activity against MDR *A. baumannii*, colistin is being used more frequently. Colistin acts as a cationic detergent, resulting in modifications in the lipopolysaccharide of *A. baumannii* by acidification, acylation, or presence of antigens that interfere with binding of the antibiotic to the cell membrane (Peterson *et al.*, 1987; Montefour *et al.*, 2008). As a result, intercellular substances leak through the bacterial membrane, resulting in cell death (Montefour *et al.*, 2008). Reports of resistance to colistin in *A. baumannii* raise concern about the long term use of this antibiotic (Gales *et al.*, 2001; Tysall *et al.*, 2002; Reis *et al.*, 2003).

At Groote Schuur hospital (GSH) in South Africa, carbapenem resistant *A. baumannii* isolates are on the increase and colistin is administered as the last treatment option (Gounden *et al.*, 2009). Fortunately, there have not been any reported cases of resistance or adverse side effects to colistin in this hospital (Gounden *et al.*, 2009).

1.4. The role of mobile genetic elements in antibiotic resistance in *Acinetobacter baumannii*

Bacterial DNA is highly dynamic and the genetic content of bacterial species is in permanent flux (Schmidt & Hensel, 2004). Microbial genomes can host variable and frequently significant amounts of foreign DNA, with *E. coli* and *Salmonella enterica* genomes comprising up to 17 % of foreign DNA (Ochman *et al.*, 2000). Horizontal gene transfer is an important mechanism which seems to be the primary force by which bacteria genetically adapt to novel environments and by which bacterial populations diverge and form separate, evolutionary distinct species (Ochman *et al.*, 2000; Schmidt & Hensel, 2004).

Mobile genetic elements (MGEs) carry genes that encode proteins responsible for the process of horizontal gene transfer (Frost & Leplae, 2005). These elements also harbour clusters of resistance genes, the proteins of which confer resistance to several unrelated families of antibiotics (Milkman, 1999; Hacker & Carniel, 2001). There are generally two groups of MGEs; those that are transferable between bacterial cells and those transferable within the same bacterial cell from one genetic location to another (Sobecky & Hazen, 2009; Frost & Leplae, 2005). Plasmids and conjugative resistance transposons make up the group of MGEs transferable between cells. Those MGEs with the ability to move between genetic locations within a bacterial cell include resistance transposons, gene cassettes and insertion sequence elements. This second group of MGEs is transferable between bacterial cells when located on mobile plasmids (Hacker & Carniel, 2001; Sobecky & Hazen, 2009). MGEs therefore contribute significantly to the spread of resistance determinants in bacteria, including *A. baumannii*.

1.4.1. Plasmids

Plasmids are small, circular, secondary, dispensable chromosomes. Plasmids are found in essentially all types of bacteria and so constitute a large pool of mobile genetic information (Mims *et al.*, 2004). These DNA molecules have the ability to promote their own transfer between bacterial cells and so they play a significant role in bacterial adaptation and evolution (Snyder & Champness, 2002). Plasmids range in size from 2 kb, comprising 2 or 3 genes, to 10% or more of the host cell chromosome, accommodating at least 400 genes (Snyder & Champness, 2002). This sub-group of MGEs does not accommodate the core genes needed by the cell for basic growth and reproduction, but rather carry genes that may be periodically useful to enable the cell to exploit particular environmental conditions, like the ability to survive and thrive in the presence of potentially lethal antibiotics (Snyder & Champness, 2002).

Plasmids replicate independently of the main bacterial chromosome, although most replication functions are provided by the host cell. Conjugative plasmids allow for the transfer of plasmids between bacterial cells and can exhibit broad or narrow host range (Mims *et al.*, 2004; Bennett, 2008). For the latter, transfer is restricted generally to and between a small number of similar bacterial species. Broad host range denotes an element able to transfer between widely different bacterial species. The determinant of host range has not been widely investigated, but one possibility is that it reflects the nature of the surface receptor on the potential recipient cell needed by the particular conjugation machinery of the plasmid (Snyder & Champness, 2002; Bennett, 2008). If the potential recipient cell lacks this structure, then plasmid transfer to it will not occur. If distribution of the receptor is limited, then the plasmid will exhibit a narrow host range. Another possibility is that although transfer of the plasmid is successful, the recipient cell is unable to support its replication (Snyder & Champness, 2002; Bennett, 2008). Although multiple plasmid carriage is very common (Snyder & Champness, 2002) a plasmid belonging to a different compatibility group from those already in the host cell may act as a host range determinant.

Plasmid-encoded antibiotic resistance encompasses most, if not all classes of antibiotics currently in clinical use and includes resistance to many that are at the forefront of antibiotic therapy (Montefour *et al.*, 2008). More than 25 years ago it was demonstrated that *Acinetobacter* spp. can acquire antimicrobial resistance factors through plasmid conjugation (Goldstein *et al.*, 1983) and that plasmids can readily differ among *A. baumannii* strains (Murray & Moellering, 1980). In the early 1990s, plasmids had been identified in nearly 80 % of multi-resistant isolates of *Acinetobacter* (Gerner-Smidt, 1989; Seifert *et al.*, 1994). However, there had been a few examples in which plasmid-mediated transfer of resistance genes in *Acinetobacter* was demonstrated and this could have been a reflection of the absence of suitable test systems for detecting such transfer at that time (Seifert *et al.*, 1994). By the turn of the century, there has been an increased recovery of plasmid-encoded resistance genes from *A. baumannii* (Héritier *et al.*, 2005; Poirel *et al.*, 2005; Bertini *et al.*, 2007; Segal *et al.*, 2007).

Carbapenem-hydrolyzing oxacillinases represent the main mechanism of resistance to carbapenems in *Acinetobacter* spp. (Bonomo & Szabo, 2006). The oxacillinase-encoding genes are mostly located on transferable plasmids (Héritier *et al.*, 2005; Poirel *et al.*, 2005; Bertini *et al.*, 2007). Attempts to transfer the oxacillinase genes from *A. baumannii* to *E. coli* as the recipient strain failed either because the plasmids possess restricted host ranges (Bertini *et al.*, 2007; Héritier *et al.*, 2005; Poirel *et al.*, 2005) or because the transcriptional regulatory sequences in *A. baumannii* and *E. coli* differ (Segal & Elisha, 1999).

The carbapenem resistance conferring gene *bla*_{OXA-23} is almost always associated with the insertion sequence *ISAba-1* in *A. baumannii* isolates (Corvec *et al.*, 2007). A 60 kb plasmid obtained from various *A. baumannii* isolates in France had such an arrangement (Le Hello *et al.*, 2008). It may be that *A. radioresistens* acts as a source of the *bla*_{OXA-23}-like gene family currently emerging as the source of carbapenem resistance in *A. baumannii* worldwide (Corvec *et al.*, 2007; Poirel *et al.*, 2008). A model proposed is that a plasmid-mediated *ISAba-1* element originating from *A. baumannii* transposed into *A. radioresistens* targeting flanking regions of a chromosomal *bla*_{OXA-23}-like gene. This would have formed a transposon-like structure and also enhanced the expression of the *bla*_{OXA-23}-like gene (Poirel *et al.*, 2008). This structure may have

then transposed and targeted a plasmid within the *A. radioresistens* genome. In turn, this resultant plasmid may have conjugated into *A. baumannii*, thus spreading the resistance determinant in the latter species. It is possible that the genetic exchange between these two species may have occurred in humans, since both *A. baumannii* and *A. radioresistens* are identified on the human skin, especially in hospitalised patients (Seifert *et al.*, 1997; Berlau *et al.*, 1999; Poirel *et al.*, 2008). The identification of the same plasmid types in *A. radioresistens* and *A. baumannii* provides further evidence for gene exchange between those two species (Poirel *et al.*, 2008).

An epidemic, multidrug-resistant *A. baumannii* strain ACICU from Italy harbours a single chromosome and two plasmids, pACICU1 and pACICU2. Carbapenem resistance in *A. baumannii* ACICU is conferred by the OXA-58 oxacillinase, whose gene was detected in the plasmid pACICU1 (Iacono *et al.*, 2008). Plasmid pACICU1 carried two copies of the *bla*_{OXA-58} gene, and these likely originated from direct repetition of the IS*Aba-2*-*bla*_{OXA-58}-IS*Aba-3* mobile element (Iacono *et al.*, 2008). The duplication of the *bla*_{OXA-58} gene has also been described in other unrelated *A. baumannii* strains and is generally associated with increased resistance to imipenem and meropenem (Bertini *et al.*, 2007). The duplication of this genetic determinant was probably facilitated by the presence of multiple IS26 elements dispersed within the plasmid region (Bertini *et al.*, 2007; Iacono *et al.*, 2008).

Another plasmid-located *bla*_{OXA-58} conferring carbapenem resistance to *A. baumannii* was described (Chen *et al.*, 2008). The plasmid pTVICU53, recovered from an *A. baumannii* clinical isolate in Taiwan, contained open reading frames (ORFs) from multiple origins including plasmid pMAC (from *A. baumannii* ATCC 19606T), plasmid pAB2 (from *A. baumannii* ATCC 17978; GenBank accession number CP000523), plasmid pKLH201 (from *A. calcoaceticus* KHW14; GenBank accession number AJ251307), and the *A. baumannii* genome (of ATCC 17978; NC_009085). Hybrid IS elements, IS1008 and IS*Aba-3*, were also observed upstream of the *bla*_{OXA-58} on pTVICU53. Electro-transformation of the original or recombinant plasmid into different *A. baumannii* strains conferred a 64- to 256-fold increase in their carbapenem minimum inhibitory concentrations (MICs). Deletion of promoters provided by IS1008 resulted in reduced

carbapenem resistance (Chen *et al.*, 2008). Transformation of shuttle vector pOXA-58-3, bearing IS1008-ISAb_a-3-*bla*_{OXA-58}, into *E. coli* provided only a mild increase in carbapenem MICs (<2 µg/ml) observed. Similar results, where mild resistance to carbapenems was observed following transformation of resistance plasmids into *E. coli*, had previously been demonstrated by Héritier and colleagues (2005). Although carbapenem resistance was insignificant in the transformed *E. coli*, Southern hybridisation experiments revealed the presence of the plasmid-borne *bla*_{OXA-58} in this host (Chen *et al.*, 2008). This observation could mean that the plasmids in *A. baumannii* have promoter sequences different from those recognised in *E. coli* (Segal & Elisha, 1999) or that other co-factors from *A. baumannii* were required for the full function of carbapenem resistance (Chen *et al.*, 2008).

A site-specific recombination mechanism was observed on an 8.963 kb plasmid, pABVA01, from an OXA-24-producing *A. baumannii* strain VA-566/00 from Italy (D'Andrea *et al.*, 2009). Comparative analysis with other sequenced *A. baumannii* plasmids revealed that the genetic organisation of pABVA01 was very similar to that of p2ABAYE (Vallenet *et al.*, 2008) and of pAB0057 (Adams *et al.*, 2008), two small *Acinetobacter* plasmids previously detected in French and American clinical isolates, respectively. One of the major differences between pABVA01 and those plasmids was in the region containing *bla*_{OXA-24}. In p2ABAYE and pAB0057, *bla*_{OXA-24} was replaced by unique regions of different size and composition (Figure 1.3a and 1.3b). A similar arrangement of intervening DNA modules inserted at the same position suggested the occurrence of a conserved recombination site where different DNA modules could be inserted by a site-specific recombination mechanism (D'Andrea *et al.*, 2009). Analysis of the sequences at the recombination junctions revealed, on both sides, the presence of conserved inverted repeats (IRs) separated by a 6 base pair (bp) variable region. These structures share high homology with *dif*-like binding sites which act as targets of the XerC and XerD recombinases that normally convert plasmid and chromosome dimers to monomers during cell division (Summers & Sherratt, 1988). XerC and XerD proteins and similar recombination sites are also involved in other site-specific recombination mechanisms such as the integration of phage CTX-Φ at the *dif1* site of the *Vibrio cholerae* larger chromosome (Val *et al.*, 2005). They have also been exploited for artificial gene excision by site-specific recombination in *E. coli* and *Bacillus subtilis* (Bloor &

Cranenburgh, 2006). These findings suggest that the XerC/XerD-like sites could act as site-specific recombination targets responsible for mobilisation of discrete DNA modules within *Acinetobacter* plasmids and chromosomes, and that *bla*_{OXA-24} could be mobilised by a similar mechanism.

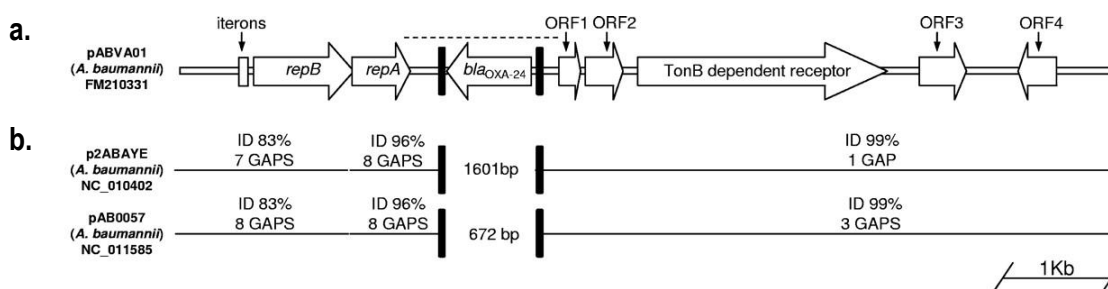


Figure 1.3: (a) Linear map of plasmid pABVA01. The dashed line indicates the sequenced region of plasmid from isolate VA-566/00 (b) Comparison of plasmid pABVA01 with plasmids p2ABAYE and pAB0057. For each segment of homology the percentage of nucleotide identity and gaps inserted into alignments are shown. Filled boxes represent the putative recombination sites represented by the IRs homologous to the XerC/XerD binding sites. Sizes of the different DNA modules inserted between the IRs in place of the *bla*_{OXA-24} module are also shown (Taken from D'Andrea *et al.*, 2009).

Analysis of the genetic context of *bla*_{OXA-24} did not reveal structures typically involved in DNA mobilisation (insertion sequences, transposons, integrons or other genes encoding known recombinases; D'Andrea *et al* 2009). However, *bla*_{OXA-24} was carried on a DNA module inserted between conserved inverted repeats homologous to XerC/XerD binding sites which, in other plasmids, flank DNA modules of different sizes and compositions. This suggests the occurrence of a unique site-specific recombination method that may play a role in the plasticity of *Acinetobacter* plasmids and in the mobilisation of resistance genes (D'Andrea *et al.*, 2009).

1.4.2. Transposons

Transposons are non-self-replicating genetic elements. They can move independently between DNA molecules within bacterial cells, but require plasmids or bacteriophages to move between cells (Figure 1.2) (McClintock, 1950; Frost & Leplae, 2005; Summers, 2006). These genetic structures contain inverted repeats, a transposase gene and at least one antibiotic resistance gene, for example tetracycline resistance in Tn10 or ampicillin resistance in Tn3. Thus, transposons differ from IS elements in that they encode at least one functional gene that changes the phenotype of the cell in a predictable fashion (Summers, 2006). Transposons are differentiated by their structure, genetic relatedness and transposition mechanism, which does not generally require DNA homology between the transposon and insertion site (Bonafede *et al.*, 1997; Frost & Leplae, 2005; Summers, 2006).

Transposons jump randomly within bacterial cells and occasionally integrate into each other resulting in mosaic structures in which highly recombinogenic regions are included (Bonafede *et al.*, 1997; Culebras & Martinez, 1999; Summers, 2006;). One such example is the transposon Tn21 in which the transposable integron In2 integrated into a mercury resistant transposon related to Tn501 (Summers, 2006). Thus, the acquisition of novel traits is a common occurrence in transposon evolution (Bonafede *et al.*, 1997). Transposons could therefore either be composite or complex systems. In composite transposons, a non-transposable DNA sequence whose expression changes the bacterial hosts' phenotype is sandwiched between two insertion sequences arranged as direct or inverted repeats. The direct repeat arrangement is less genetically stable as compared to the inverted repeat, however, the direct repeats allow the transposon to migrate to other sites where the same IS element is found in a given genome (Bennett, 2008). Complex transposons on the other hand do not have transposing and non-transposing functions assembled in an obvious arrangement (Bennett, 2008).

In one surveillance study in Italy, 6.6 % of clonally related *A. baumannii* isolates harboured a TEM-92 extended spectrum β -lactam gene (Endimiani *et al.*, 2007). Tn3-like transposons are often responsible for resistance to a number of β -lactam antibiotics and the *bla*_{TEM-92} gene was

linked to one such transposon (Endimiani *et al.*, 2007). The isolates in this study were resistant to all β -lactams tested, except the carbapenems.

Transposon-related carbapenem resistance has been reported elsewhere (Poirel *et al.*, 2008). The *bla*_{OXA-23} gene, linked to IS*Aba-1* in Tn2006 and to IS*Aba-4* in Tn2007 was identified in carbapenem-resistant *A. baumannii* isolates (Corvec *et al.*, 2007). The Tn2006 structure had two inverted copies of IS*Aba-1* flanking the *bla*_{OXA-23} gene, together with a truncated ATPase gene. The transposon Tn2007 only had a single copy of IS*Aba-4*. Interestingly, Tn2007 was associated with a partial ATPase-encoding gene downstream of the *bla*_{OXA-23} gene which was similar to the one flanking Tn2006. Further studies identified a truncated ATPase gene associated with *bla*_{OXA-23} in carbapenem resistant *A. baumannii* isolates (Corvec *et al.*, 2007). The ATPase gene may act as an insertion site for transposons (Corvec *et al.*, 2007).

Transposons and insertion sequences clearly play an important role, in conjunction with integrons, in ensuring that particular novel genes become established in a new gene pool. Should transposons be located on unstable plasmids in bacteria, the transposon could integrate and stabilise within the chromosome. Transposons are thus relevant for the dissemination of antibiotic resistance genes in *Acinetobacter spp.* (Devaud *et al.*, 1982; Palmen & Hellingwerf, 1997), either by integration in transferable plasmids or by direct integration within the bacterial chromosome.

1.4.3. Insertion sequence elements

Insertion sequence (IS) elements are small transposable genetic elements that range in size from 800 to 2500 bp and encode only those functions required for its transposition (Depardieu *et al.*, 2007). Currently, over 1 000 IS elements have been identified in approximately 200 Gram-negative and Gram-positive bacterial species and in archaea (Depardieu *et al.*, 2007). IS elements are assigned to 17 families based on their structural and functional characteristics (Table 1.2; Mahillon & Chandler, 1998; Chandler & Mahillon, 2002; Depardieu *et al.*, 2007). The

classification of IS elements into various families is mostly a guideline as variations in characteristics of IS elements within the families are tolerable and uncertainties even exist in some of the families (Mahillon & Chandler, 1998).

Table 1.2: Major features of prokaryote IS families

| Family | Group(s) | Size range (bp) ^a | DR (bp) ^b | ENDS ^c | IR ^d | No. of ORFs ^e | Comments ^f |
|-------------|----------|------------------------------|----------------------|-------------------|-----------------|--------------------------|----------------------------|
| IS1 | | 770 | 9 (8–11) | GGT | Y | 2 | Phage λ integrase? |
| IS3 | IS2 | 1,300–1,350 | 5 | TGA | Y | 2 | DD(35)E |
| | IS3 | 1,200–1,300 | 3 (4) | | | 2 | |
| | IS51 | 1,300–1,400 | 3 (4) | | | 2 | |
| | IS150 | 1,400–1,550 | 3–5 | | | 2 | |
| | IS407 | 1,200–1,250 | 4 | | | 2 | |
| IS4 | | 1,300–1,950 | 9–12 | C(A) | Y | 1 | DDE |
| IS5 | IS5 | 1,100–1,350 | 4 | GG | Y | 1 | DDE |
| | IS427 | 800–1,000 | 2–3 | Ga/g | | 2 | |
| | IS903 | 1,000–1,100 | 9 | GGC | | 1 | |
| | IS1031 | 850–950 | 3 | GAG | | 1 | |
| | ISH1 | 900–1,150 | 8 | | | 1 | |
| | ISL2 | 800–1,100 | 2–3 | | | 1 | |
| IS6 | | 750–900 | 8 | GG | Y | 1 | DD(34)E |
| IS21 | | 1,950–2,500 | 4 (5, 8) | TG | Y | 2 | DDE |
| IS30 | | 1,000–1,250 | 2–3 | | Y | 1 | DD(33)E |
| IS66 | | 2,500–2,700 | 8 | GTA | Y | >3 | |
| IS91 | | 1,500–1,850 | 0 | | N | 1 | ssDNA Rep |
| IS110 | | 1,200–1,550 | 0 | | N | 1 | Site-specific recombinase |
| IS200/IS605 | | 700–2,000 | 0 | | N | 1 (2) | Complex organization |
| IS256 | | 1,300–1,500 | 8–9 | Gg/a | Y | 1 | DDE, eukaryotic relatives |
| IS630 | | 1,100–1,200 | 2 | | Y | 1 | DDE, eukaryotic relatives |
| IS982 | | 1,000 | ND ^g | AC | Y | 1 | DDE |
| IS1380 | | 1,650 | 4 | Cc/g | Y | 1 | |
| ISAs1 | | 1,200–1,350 | 8 | C | Y | 1 | |
| ISL3 | | 1,300–1,550 | 8 | GG | Y | 1 | |

^a Size range represents the typical range of each group.

^b Length of direct target repeats. Less frequently observed lengths are included in parentheses.

^c Conserved terminal base pairs. Capital letters (and capital letters within parentheses) refer to mostly (and often) conserved bases. Lowercase letters separated by slashes indicate alternative conservation at that position.

^d Presence (Y) or absence (N) of terminal inverted repeats.

^e ORF, open reading frame. Number in parentheses indicates the possible involvement of a second ORF in the transposition process.

^f DDE represents the common acidic triad presumed to be part of the active site of the transposase. ssDNA, single-stranded DNA.

^g ND, not determined. (Taken from Mahillon & Chandler, 1998).

IS elements are typically bound by short repeat sequences of up to 40 bp in an indirect orientation. These IRs are specific for each element, and their presence and integrity are required for transposition, which may or may not be site specific (Grindley & Reed, 1985; Mahillon & Chandler, 1998; Depardieu *et al.*, 2007). Repeat sequences of 2 to 14 bp, characteristic for each element, are generated in a direct orientation upon insertion of an IS element into the target DNA (Figure 1.4; Depardieu *et al.*, 2007). Many IS elements carry a single ORF encoding a transposase. Some IS elements carry several ORFs on a single strand or on both strands, the products of which may also play a role in the regulation of the transposition process (Mahillon & Chandler, 1998; Chandler & Mahillon, 2002; Depardieu *et al.*, 2007). Of particular interest, IS elements may contain partial or complete promoter sequences, often located at their extremities and in an outward orientation and capable of activating the expression of flanking genes (Figure 1.4; Mahillon & Chandler, 1998; Chandler & Mahillon, 2002).

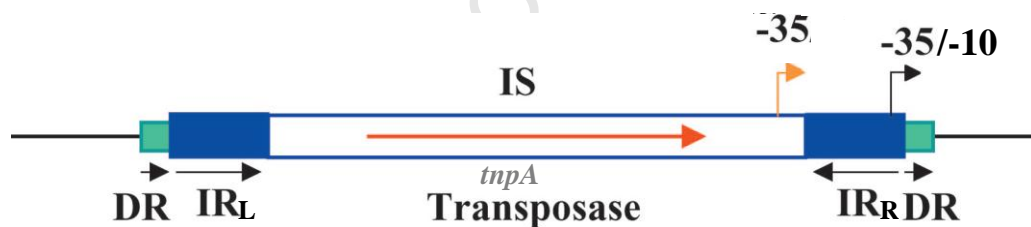


Figure 1.4: Schematic representation of an IS element. IR_L and IR_R , inverted repeats; -35 and -35/-10, approximate locations of promoter consensus sequences. The direct repeats, DR, are not parts of insertion sequences but are generated within the host DNA upon the integration of an IS (Adapted from Grindley & Reed, 1985; Depardieu *et al.*, 2007).

There is extraordinary variability in the distribution of the IS elements of the different families among bacterial species. For some IS families, e.g. IS21, the host range includes both Gram-positive and Gram-negative genera (Yeo *et al.*, 1998). For the IS3 family, the hosts are widely spread amongst different genera (Labigne-Roussel & Corvalin, 1983; Steibl & Lewecke, 1995;

Mahillon & Chandler, 1998). In some instances, IS elements are confined to a geographical area (Huh *et al.*, 2004); IS1542, IS1251 and IS1476 are confined to UK/Ireland (Darini *et al.*, 1999), United States of America (USA) and, Canada (Mackinnon *et al.*, 1997) respectively. Some IS elements are restricted to few hosts, such as IS6110, which has been found only in mycobacteria of the tuberculosis complex (Thierry *et al.*, 1990; Mahillon & Chandler, 1999). IS1 is unique to *E. coli* (Nymann *et al.*, 1983) while IS1222 is found only in strains closely related to *Enterobacter agglomerans* (Steibl & Lewecke, 1995). IS1491 is common amongst pseudomonads (Yeo, *et al.*, 1998), IS2000 is unique to *K. pneumoniae* (Aubert *et al.*, 2003), and ISAba-1 is thought to be unique to *A. baumannii* (Segal *et al.*, 2005).

IS elements may be present on the chromosome, plasmids, or both as single or multiple copies. Intercellular transfer is possible if the IS elements reside on conjugative plasmids (Depardieu *et al.*, 2007). A number of factors account for the varied IS copy numbers in different bacterial genera; these include the transposition frequency of the IS element into the same strain (Nymann *et al.*, 1983), and the genomic plasticity of the strains (Mahillon & Chandler, 1998). Most IS elements transpose readily, whilst others, such as IS200, rarely do so (Beuzón *et al.*, 2004).

The involvement of IS elements in altered expression of resistance-conferring or resistance modulating genes, consisting in some cases of the activation of silent genes, has been suggested (Poirel & Nordmann, 2006a, 2006b; Depardieu *et al.*, 2007; Munoz-Price & Weinstein, 2008; Chen *et al.*, 2008). The promoter regions in IS elements could be used by pathogens to over express antibiotic resistance genes. Such events have been described in the *Enterobacteriaceae*, strict aerobic and anaerobic Gram-negative bacteria, staphylococci, and enterococci (Depardieu *et al.*, 2007), resulting in resistance to many classes of antibiotics. Thus, IS elements constitute a group of genetic elements that may effect multiple antibiotic resistance in clinical bacterial isolates.

The expression of antibiotic resistance genes is a result of DNA polymerase binding to promoter sequences upstream of the gene to be expressed (Garret & Grisham, 2005). Insertion of an IS element upstream of a resistance gene may result in a hybrid promoter with an alternative, a new

IS-borne -35 region, or a completely new IS-borne promoter containing both the -35 and the -10 regions (Figure 1.4). The canonical consensus sequence for the -35 and the -10 regions are TTGACA and TATAAT, respectively, with a 17 bp spacing distance for optimal promoter activity as determined for *E. coli* (Lisser & Margalit, 1993).

The chromosomally encoded AmpC and OXA-51-like β -lactamases are intrinsic to *A. baumannii* (Munoz-Price & Weinstein, 2008). Usually, such β -lactamases have low expression levels that do not contribute to clinically significant resistance; however, the integration of IS*Aba-1* containing promoter sequences, upstream of *ampC* and of *bla*_{OXA-51} in *A. baumannii* increases β -lactamase production, resulting in limited treatment options for patients infected with these strains (Corvec *et al.*, 2003; Segal *et al.*, 2003; Poirel & Nordmann, 2006b).

Hybrid promoters that result in increased expression of associated resistance genes have been reported (Poirel & Nordmann, 2006b; Chen *et al.*, 2008). An IS*Aba-3*-like element interrupted by a portion of IS1008 was integrated upstream of *bla*_{OXA-58} gene in an *A. baumannii* clinical isolate in Taiwan (Chen *et al.*, 2008). The -10 promoter was located in the IS*Aba-3*-like element while the -35 promoter, which conferred higher levels of expression, was in the IS1008 domain. In the presence of hybrid promoters; if nosocomial pathogens have the option to use those promoters conferring the strongest expression of resistance genes towards antibiotics, then the end of the antibiotic era may actually be reached sooner than expected.

1.4.4. Integrons and gene cassettes

Antibiotic resistance genes can be located within integron-borne cassettes, which provide a remarkably efficient means for mobilising various resistance genes (Stokes & Hall, 1989). Integrons are phylogenetically diverse and ancient, complex genetic elements, which have been implicated in dissemination of antibiotic resistant genes (Stokes & Hall, 1989; Gootz & Marra, 2008; Labbate *et al.*, 2009). They are capable of capturing genes by a site-specific recombination mechanism that often carries gene cassettes containing antibiotic resistance genes (Stokes & Hall, 1989). Integrons are divided into different classes based on the sequence of their integrase

gene (Hall *et al.*, 1999; Rowe-Magnus *et al.*, 2001). Class 1 integrons have a gene encoding an IntI1 integrase (Figure 1.5; Recchia & Hall, 1995). Class 2 integrons are related to Tn7-like transposons and have an *intI2* gene that encodes an integrase with partial homology to IntI1 (Young *et al.*, 1994; Recchia & Hall, 1995). Class 3 integrons are associated with the integrase Int I3 that has 60.9 % homology with the IntI1 integrase (Arakawa *et al.*, 1995). Class 4 integrons have a gene encoding an IntI9 integrase (Hochhut *et al.*, 2001) that has 53 % homology to IntI2. Class 1 integrons are the most common in clinical isolates of Gram-negative bacteria (Levesque *et al.*, 1995), including acinetobacters (Koeleman *et al.*, 2001; Gombac *et al.*, 2002; Severino & Magalhaes, 2004). Class 2 integrons have been identified in Acinetobacter species (Sallen *et al.*, 1995; Jones *et al.*, 1997; Ploy *et al.*, 2000; Koeleman *et al.*, 2001) but are rare, and class 3 integrons have emerged in enterobacteriaceae (Correia *et al.*, 2003).

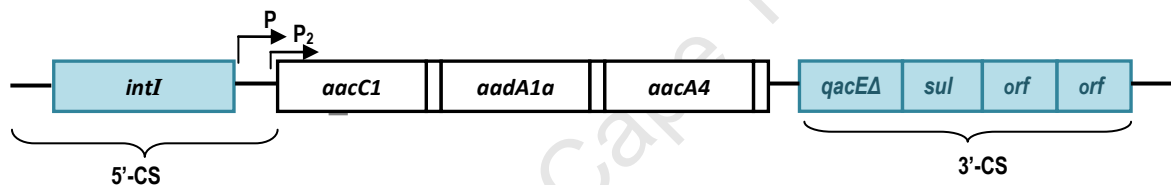


Figure 1.5: Structure of an integron. Integrons are site-specific recombination elements that mediate the acquisition and spread of genes among bacterial populations. The 5'-CS contains an integrase gene (*intI*) followed by one primary *attI* recombination site (gray box). The 3'-CS is characterized by *qacEΔ1*, *sul1*, and two uncharacterised genes, *orf5* and 6. Several cassettes, in this case *aacC1*, *aadA1*, and *aacA4*, can be inserted in no specific order between the two conserved regions. Each of the cassettes code for one gene and have one 59-bp recombination site (white box). The transcription of the system is controlled by a strong promoter (P) located upstream of the gene cassettes and associated with a weaker promoter (P₂). This structure favours the arrangement of genes in tandem, which are transferred as single elements among bacterial populations (Adapted from Recchia & Hall, 1995; Martínez & Baquero, 2002, and Bonomo & Szabo, 2006).

Class 1 integrons are marked by the presence of two conserved regions between which gene cassettes are integrated at a recombination site known as *attI* (Figure 1.5; Stokes & Hall, 1989; Recchia & Hall, 1995). The identities and number of the gene cassettes within a given integron varies (Stokes & Hall, 1989). The 5'-conserved sequence (CS) of an integron contains the gene

intI that encodes a site-specific recombination enzyme, integrase (Stokes & Hall, 1989; Hall & Collis, 1995).

The 5'-CS also contains a promoter, P_1 , and a second promoter, P_2 , which drive the expression of any integrated gene cassettes. Integrons have a truncated quaternary ammonium compound resistance gene *qacE Δ 1*, a *sul1* sulfonamide resistance gene, and two open reading frames (ORFs), ORF5 and ORF6, of unknown function (Hall & Collis, 1995).

Gene cassettes are small, non-replicating, double stranded circular DNA molecules (Recchia & Hall, 1995). They encode antibiotic resistance genes and vary considerably in total length from 262 to 1549 bp depending on the size of the gene, which covers most of the cassette length (Recchia & Hall, 1995; Figure 1.5). Their circular state is more of an intermediary stage as they get transferred between integrons or as they get reshuffled within a particular integron (Recchia & Hall, 1995). Gene cassettes lack promoters and are often found within integrons between the 5'- and 3'- CSs (Recchia & Hall, 1995). Cassette integration occurs at the cassette integration site (*attI*) of the integron (Hall & Collis, 1995) or at non-specific sites within plasmids and chromosomes (Recchia & Hall, 1995; Segal & Elisha, 1997). Gene cassettes have a recombination site known as a 59-base element (59-be) that varies in size from 57 to 141 bp and is located at the 3'- end (Recchia & Hall, 1995; Toleman *et al.*, 2006). The 59-be is a family of recombination sites that act as substrates to integrase-mediated recombination and is reformed whenever the gene cassette is excised from the integron (Recchia & Hall, 1995; Toleman *et al.*, 2006).

The origin of the antibiotic resistance genes on cassettes is subject to speculation. The discovery of resistance gene families, like the dihydrofolate reductase B family, which were found only in combination with a 59-be, suggested a pool of antibiotic resistance genes different from the pool of resistance genes that are not linked to gene cassettes (Fluit & Schmitz, 1999). The ability of gene cassettes to integrate at secondary sites further complicates the tracing of the origin of the resistance genes (Segal & Elisha, 1997; Fluit & Schmitz, 1999).

Gene cassettes carry antibiotic resistance genes that encode activity against a wide range of antibiotics and antibiotic classes. Resistance to chloramphenicol is encoded either by one of seven gene cassettes encoding a chloramphenicol acetyltransferase or by one of the three known efflux pumps (Gootz & Marra, 2008). Resistance to rifampin and erythromycin encoded on gene cassettes has also been described (Houang, 2000). Most gene cassettes encode resistance to antibiotics that have been in use for a relatively long period of time. Notably, gene cassettes encoding resistance to β -lactams, including *bla*_{IMP}, *bla*_{VIM}, *bla*_{VEB-1}, *bla*_{oxa15}, *bla*_{oxa19}, *bla*_{oxa20}, and *bla*_{oxa21}, have been described (Mugnier *et al.*, 1998; Poirel *et al.*, 1999; Laraki *et al.*, 1999; Lauretti *et al.*, 1999; Poirel *et al.*, 2000; Nordmann & Poirel, 2002). This indicates the important role played by cassettes in antibiotic resistance in *A. baumannii* and so causing great concern.

Gene cassettes can be inserted one after the other, in different combinations (Hall & Stokes, 1990; Stokes & Hall, 1991; Parent & Roy, 1992; Stokes *et al.*, 1993; Naas *et al.*, 1998), into the *attI* of integrons to produce impressive resistance gene arrays, such as the two copies of *oxa2* in In1 (Stokes & Hall, 1992). It is because each cassette is inserted at the same point that the gene cassettes from the 5'-CS indicate the order of addition, the one nearest the 5'-CS being the latest addition (Bennett, 2008). As many as five (Stokes & Hall, 1991; Stokes *et al.*, 1993) to 30 (Vaisvila *et al.*, 2001) gene cassettes can be integrated into one integron. Various combinations of gene cassette in integrons play a vital role in the creation of new gene arrays, operons, or pathogenicity islands responsible for MDR *A. baumannii* strains.

Integron-based antibiotic resistance has been frequently described in *Enterobacteriaceae* (Fluit & Schmitz, 1999) and clinical isolates of *Pseudomonas* spp. (Bunny *et al.*, 1995; Kazama *et al.*, 1995). Until the late 1990s, descriptions of integrons in *Acinetobacter* were rare (Gonzalez *et al.*, 1998; Seward & Towner, 1998; Petersen *et al.*, 2000; Ploy *et al.*, 2000; Gallego & Towner, 2001). The first report on the presence of integrons in isolates of *A. baumannii* was in Chile (Gonzalez *et al.*, 1998). Various studies have described antibiotic resistance genes located on integrons in *Acinetobacter* species (Seward *et al.*, 1998; Ploy *et al.*, 2000; Gallego & Towner, 2001; Gombac *et al.*, 2002; Navia *et al.*, 2002; Nemec *et al.*, 2004; Zarrilli *et al.*, 2004).

Although class 1 integrons are the most prevalent in clinical isolates of antibiotic resistant Gram-negative bacteria (Rowe-Magnus *et al.*, 2001). Surprisingly, class 2 integrons were more frequent in *A. baumannii* biotype 9 isolates (Sallen *et al.*, 1995; Jones *et al.*, 1997). These isolates were previously known to exhibit higher levels of resistance as well as broader resistance patterns to β -lactams, sulfonamides, trimethoprim, tetracycline, chloramphenicol, and aminoglycosides (Sallen *et al.*, 1995; K.E. Sossa, personal communication in Gonzalez *et al.*, 1998).

Although many variants of acquired *bla* genes are detectable in *A. baumannii*, some metallo- β -lactamase variants are not commonly found in this bacterium (Gootz & Marra, 2008). Three imipenem-resistant *A. baumannii* clones with *bla*_{VIM} alleles on class 1 integrons were identified in a study conducted in Greece (Tsakris *et al.*, 2008). One of the clones harbored a *bla*_{VIM-4} allele; a point mutant of the *bla*_{VIM-1} variant which was first identified in a carbapenem-resistant *P. aeruginosa* clinical isolate (Pournaras *et al.*, 2002). This variant has now been detected in numerous *P. aeruginosa* clones in Greece (Pournaras *et al.*, 2003), and in pseudomonads and enterobacteria in several other European countries, Australia and Tunisia (Luzzaro *et al.*, 2004; Ktari *et al.*, 2006; Libisch *et al.*, 2006).

Extended spectrum β -lactamases have rarely been identified in *A. baumannii* (Perez *et al.*, 2007; Gootz & Marra, 2008). In 2002, a *bla*_{CTX-M-2} gene was identified in an *A. baumannii* nosocomial isolate (Arduino *et al.*, 2002) on an integron, In35. The integron, In35, also had *bla*_{OXA-23} and *aac(6')-Ib* gene cassettes, and partial gene sequences of *sul1* and *qacEL*, a metal efflux pump gene. Identification of In35 in *Proteus mirabilis*, *K. pneumoniae*, *Enterobacter cloacae*, *S. marcescens* and *Salmonella* spp. recovered from the same hospital as the *A. baumannii* isolate suggested interspecies transfer of this genetic element (Arduino *et al.*, 2002).

In one study, strains of *A. baumannii* that had infected multiple patients in one hospital contained significantly more integrons in their genomes than those that had only infected single patients (Koeleman *et al.*, 2001). The presence of integrons in these strains suggested that the more

antibiotic-resistant isolates containing more integrons were more fit for hospital infection than non-integron-containing isolates (Koeleman *et al.*, 2001).

Although integrons are immobile, they are sometimes found as part of transposons or plasmids, through which they can be transferred between different pathogens. Class 1 integrons can be found in Tn21 and Tn21-related transposons (Schmidt *et al.*, 1989; Parent & Roy, 1992; Sundstrom *et al.*, 1993). Plasmids and chromosomal DNA thus, represent platforms on which gene arrays are assembled and reassorted (Schmidt *et al.*, 1989) in which the latter may result in the formation of pathogenicity islands.

1.4.5. Pathogenicity islands

In some pathogens, foreign DNA, in the form of IS elements, gene cassettes, transposons and integrons, is assembled in one area of the organisms' chromosome or plasmids to form pathogenicity islands (PAIs). These islands represent distinct clusters of virulence genes, which are outcomes of horizontal gene transfer, (Smith *et al.*, 2007) and they extend the range of habitats that can be colonised by bacterial species. Genes located within PAIs have been used as diagnostic markers for pathogen identification in clinical specimens and for the differentiation of pathogenic strains from closely related non-pathogenic strains (Schmidt & Hensel, 2004). Toxins encoded by genes on PAIs can be used as tools in cell biology (Schiavo & van der Goot, 2001). Protein secretion systems, often found in PAIs, can be used to deliver heterologous antigens for vaccination strategies with live carrier strains (Rüssmann *et al.* 1998; Gentshev *et al.* 2002). PAI-encoded virulence determinants, such as secretion systems, may be targets for novel forms of therapeutic intervention of bacterial infections.

PAIs are a sub-group of genomic islands sharing a set of unifying features (Hentschel and Hacker, 2001). These features include **i)** the carriage of one or more virulence genes; **ii)** their presence in the genomes of pathogenic bacteria but not in the genomes of a non-pathogenic representative of the same species or a closely related species; **iii)** the relatively large genomic

size ranging from 10 to 200 kb; **iv**) a different G + C content to that of the core genome, as well as different codon usage; **v**) an association with tRNA genes or, as in *A. baumannii*, within ATPase genes (Fournier *et al.*, 2006; Shaikh *et al.*, 2009; Post & Hall, 2009); **vi**) the association with mobile genetic elements and mobility genes, often flanked by direct repeats; and **vii**) a more mosaic-like structure rather than homogeneous segments of horizontally acquired DNA (Schmidt & Hensel, 2004).

PAIs have been identified in various pathogenic bacteria. The 40 kb *cag* PAI in *Helicobacter pylori* contributes to the pathogen's virulence by encoding type IV secretion system genes. Type IV secretion systems play a role in host cell invasion, inactivation of phagocytic cells, apoptosis, and interference with intracellular transport processes (Censini *et al.*, 1996). *P. aeruginosa* strain PAO1 has a 50 kb PAI, PAG1, consisting of 51 ORFs, most of which encode dehydrogenases and paraquat-inducible proteins involved in redox recycling and detoxification (Stover *et al.*, 2000; Larbig *et al.*, 2003). PAG1 thus plays an important role in protecting strain PAO1 against oxidative damage (Liang *et al.*, 2001; Schmidt & Hensel, 2004; Yoon *et al.*, 2005).

Recently, PAIs were described in strains of *A. baumannii* (Fournier *et al.*, 2006; Smith *et al.*, 2007; Iacono *et al.*, 2008). An 86 kb resistance island (*AbaR1*) carrying 45 resistance genes acquired by horizontal gene transfer from various Gram-negative species was identified in *A. baumannii* strain AYE (Fournier *et al.*, 2006). Strain AYE is resistant to several β -lactams, aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, and rifampin (Smith *et al.*, 2007). *AbaR1* was also inserted into an ATPase gene that did not have any resistance genes in the antibiotic-susceptible strain *A. baumannii* SDF. This highlights the exceptional ability of *A. baumannii* to gather and assemble foreign genes (Fournier *et al.*, 2006). A further PAI, *AbaR2*, was identified in a multi-drug resistant *A. baumannii* strain ACICU (Iacono *et al.*, 2008). *AbaR2* is smaller and only carries seven resistance genes. The gene cassette array identified in a class 1 integron located on both *AbaR1* and *AbaR2* is completely different despite identical genes flanking the 5' and 3' conserved regions. The presence of these related resistance islands in AYE and ACICU suggests that *AbaR1* and *AbaR2* may originate from an island in a common *A. baumannii* ancestor before diverging into the two different clonal groups (Iacono *et al.*, 2008).

In another study, antibiotic resistance genes were located on seven of 28 putative alien islands identified in *A. baumannii* ATCC 17978 as determined by high density pyrosequencing and transposon mutagenesis (Smith *et al.*, 2007). Strain ATCC 17978 is resistant to β -lactams however, it shows weak resistance to tetracycline and it is aminoglycoside (kanamycin) sensitive (M.G. Smith & M. Snyder, unpubl. in Smith *et al.*, 2007). The presence of seven antibiotic-resistance islands is surprising, especially since this strain was isolated in or around 1951 (Baumann, 1968), prior to the development of the macrolides (erythromycin), glycopeptides (vancomycin), and cephalosporins and other latter generations of β -lactams.

There are examples of highly mobile PAIs that can be converted into phages or plasmids and these include VSP-2, VSP-I and VSP-II of *Vibrio cholera* (Jermyn & Boyd, 2002; O'Shea & Boyd, 2002; Rajanna *et al.*, 2003; Jermyn & Boyd, 2005; Murphy & Boyd, 2008). Between highly mobile PAIs and those stably integrated within the host genome, various intermediate forms of PAIs can be found (Murphy & Boyd, 2008). Some gene clusters can be found on both PAIs and plasmids, indicating that the corresponding PAI carries an integrated plasmid. It would be interesting to determine any such mobile PAIs in *A. baumannii*.

With so much genetic exchange within *A. baumannii* and between *A. baumannii* and other genera, it would be interesting to determine the transcriptional regulation mechanisms of antibiotic resistance genes.

1.5 Transcriptional regulation of antibiotic resistance genes in *Acinetobacter baumannii*

A complex multimeric RNA polymerase regulates transcription in *E. coli*. The subunits of the RNA polymerase, α , β , and β' all have their specific roles in transcription. A sigma factor, δ , allows the core polymerase ($\alpha_2\beta\beta'$) to recognise different promoter sequences (Garret & Grisham, 2005). In *E. coli*, the crucial step of initiation of mRNA synthesis is strongly influenced by the sequence of two hexamers, the -35 and -10 boxes (Harley & Reynolds, 1987) with consensus sequences

TTGACA and TATAAT respectively, optimally separated by 17 bp for *E. coli*. The closer sequences are to the consensus sequences, the stronger the promoter (Garret & Grisham, 2005). The first two bases of the hexamers are known to be the best conserved ones among the various promoters sequenced (Harley & Reynolds, 1987).

In 1997, an *aadB* cassette from *A. baumannii* strain SUN was identified within a secondary site on plasmid pRAY (Segal & Elisha, 1997). The cassette was regulated by promoter sequences different from those used by *E. coli* (Segal & Elisha 1999). In 2003, putative -35 and -10 boxes (TTGTTC and TATGAT) separated by 17 bp were detected in a mobile element about 100 bp upstream of the start codon in the *A. baumannii ampC* gene (Corvec *et al.*, 2003). These promoter sequences also differed from the *E. coli* consensus sequences by three bases and one base, respectively, the first bases being conserved. *A. baumannii* thus, recognises similar and not necessarily identical promoter sequences from *E. coli* (Segal & Elisha, 1997; Segal & Elisha, 1999). The aminoglycoside resistance genes *aac(6')-Ig* and *aac(6')-Ij* in *A. haemolyticus* and *Acinetobacter* sp. 13 have promoter sequences similar to $E\delta^{70}$ in *E. coli*, however, they are separated by 21 and 15 bp respectively (Lambert *et al.*, 1993). This unusual spacing has also been recognised in the promoter sequences of the genes involved in tryptophan synthesis in *A. calcoaceticus* (Haspel *et al.*, 1990), esterase biosynthesis in *A. calcoaceticus* (Kok *et al.*, 1995), and in *aac(6')-Ig* (Lambert *et al.*, 1993), *bla_{OXA-27}* (Segal *et al.*, 2003), and *ampC* (Segal *et al.*, 2004, Corvec *et al.*, 2003) expression in *A. baumannii*. Interestingly, a promoter located in IS*Aba-1*, upstream of a *bla_{OXA-23}* gene, expressed the *bla_{OXA-23}* gene in *E. coli* more readily than it did in *A. baumannii* (Segal *et al.*, 2007), even though IS*Aba-1* may be specific to *Acinetobacter* spp. (Segal *et al.*, 2005).

1.6 Aims of this study

Since the treatment options for MDR *A. baumannii* infections are generally limited to carbapenems, the emergence of imipenem and meropenem resistance is a growing cause for concern (Rice, 2006). *A. baumannii* acquires most resistance genes on mobile genetic elements through horizontal gene transfer from other pathogens (Mah *et al.*, 2001; Landman *et al.*, 2002).

Some mobile genetic elements, like *ISAbal-1*, enable increased expression of resistance genes by providing preferred promoter sequences (Corvec *et al.*, 2003; Segal *et al.*, 2005), and may be responsible for the plasticity observed in the *A. baumannii* genome (Segal *et al.*, 2005). The role of these mobile genetic elements in the spread, maintenance and expression of antibiotic resistance genes in *A. baumannii* is of great interest.

In 2005, a carbapenem susceptible *Acinetobacter* isolate, MOS1, was obtained from a patient at GSH, Cape Town. The patient was treated with meropenem. Two weeks later, another *A. baumannii* isolate, MOS2, was obtained from the same patient. MOS2 was carbapenem resistant. It may be that MOS2 arose due to MOS1 acquiring a carbapenem resistance gene following antibiotic selective pressure, or that the patient harboured both MOS1 and MOS2, and following carbapenem treatment, the resistant MOS2 was selected for.

Strain MOS1 has a genetic arrangement in which an aminoglycoside resistance gene, *aacC2a*, is linked to *IS1133* interrupted by a full copy of *ISAbal-1* (Figure 1.6; Segal *et al.*, 2003; Jacobson, 2007). This genetic arrangement has been conserved for more than 20 years in *A. baumannii* clinical isolates in Cape Town, South Africa (Elisha & Steyn, 1991; Jacobson, 2007). The T to A transversion within the *IS1133* right inverted repeat is believed to have stabilized this genetic arrangement in *A. baumannii* at least since 1983.

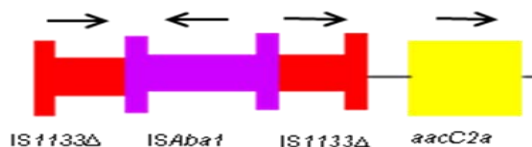


Figure 1.6: Genetic arrangement of the aminoglycoside resistance gene, *aacC2a* (yellow), downstream of a portion of *IS1133* (red). *IS1133* is interrupted by a full copy of *ISAbal-1* (purple) in strain MOS1. This genetic arrangement has been conserved for over 20 years in *A. baumannii* isolates obtained from patients at GSH and Red Cross War Memorial children's hospital. The arrows indicate direction of transcription. The shaded regions represent inverted repeats of the respective insertion sequence elements. (Adapted from Segal *et al.*, 2003; Jacobson, 2007).

Following PFGE genotyping of *A. baumannii* isolates collected over a 23 year period, it is interesting to note that all isolates that harbour the *aacC2a* gene have the same PFGE profile (Jacobson, 2007). Included in that group of isolates was MOS1. MOS2, however has a different PFGE profile, is resistant to carbapenems, and carries an *ISAb_a-1*-linked *bla_{OXA-23}* gene, suggesting that these two strains are genetically distinct. This also suggests that the patient from which MOS1 and MOS2 were isolated, was co-infected with these two strains, and that following antibiotic treatment, MOS2 was selected for.

It may be that when MOS1 and MOS2 originally diverged, the *aacC2a*-associated conserved region in MOS1-like strains, as part of a larger mobile genetic element, was replaced via horizontal gene transfer by a genetic arrangement containing the *ISAb_a-1*-linked *bla_{OXA-23}* gene to give rise to MOS2-related strains. Sequences flanking the *IS1133_L/ISAb_a-1/IS1133_R-aacC2a* genetic arrangement may give further insight into the origin and role of these genetic arrangements in *A. baumannii* MOS1 and MOS2.

The aims of this study are thus:

- i. To screen for the presence of large mobile genetic elements
- ii. To characterize further sequences associated with the *IS1133_L/ISAb_a-1/IS1133_R-aacC2a* conserved genetic arrangement in MOS1
- iii. To determine the role of the mobile genetic elements associated with this conserved region in MOS1
- iv. To investigate the role of *ISAb_a-1* in MOS1 and MOS2

MATERIALS AND METHODS

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2.1 Bacterial strains and antibiotic susceptibility testing

A. baumannii strains MOS1 was obtained from a patient admitted to GSH, Cape Town, South Africa, in 2005. Following two weeks of meropenem treatment, strain MOS2 was obtained from the same patient. Selected carbapenem resistant and susceptible isolates obtained from 1983 to 2008 from patients at GSH and Red Cross War Memorial Children's Hospital, Cape Town, were also included in this study.

The antibiotic susceptibility profiles are indicated in Table 2.1. Susceptibility testing with selected antibiotics was carried out using the disc diffusion method in the National Health Laboratory Service laboratory, GSH, according to the guidelines of the National Committee for Clinical Laboratory Standards.

Escherichia coli JM109 (Promega, USA) was used as the recipient in transformation studies (2.5).

A selection of isolates including *A. lwoffii*, *A. calcoaceticus*, *P. aeruginosa*, *Campylobacter (C.) jejuni*, *K. pneumoniae* and *E. coli* were included for screening of mobile genetic elements (Table 2.2).

Table 2.1. Antibiotic susceptibility profiles of *A. baumannii* isolates included in this study

| Bacterial isolate | Antibiotic resistance profile | Year obtained |
|--------------------------|--------------------------------------|----------------------|
| MOS1 | Gm, Amik, Cipro, SXT | 2005 |
| MOS2 | Gm, Amik, Cipro, SXT, IMP, MPM | 2005 |
| 794 | Gm, Amik, Cipro, SXT, IMP, MPM | 2008 |
| A2 | Gm, Cipro, SXT, IMP, MPM | 2006 |
| A3 | Gm, Amik, IMP, MPM, Tob | 2006 |
| A4 | Gm, Amik, Cipro, SXT, IMP, MPM, Tob | 2006 |
| A5 | Gm, Amik, Cipro, SXT, IMP, MPM, Tob | 2006 |
| A7 | Gm, Amik, IMP, MPM, Tob | 2006 |
| A9 | Gm, Amik, Cipro, SXT, IMP, MPM | 2006 |
| A10 | Gm, Cipro, SXT, IMP, MPM | 2006 |
| A19 | Gm, Amik, Cipro, SXT, IMP, MPM, Tob | 2006 |
| A21 | Amik | 2006 |
| A40 | Gm, Cipro, SXT, IMP, MPM | 2006 |
| A91 | Gm, Amik, Cipro, SXT, IMP, MPM | 2006 |

Gm, Gentamicin; Amik, Amikacin; Cipro, Ciprofloxacin; SXT, Cotrimoxazole;
Tob, Tobramycin; IMP, Imipinem; MPM, Meropenem

Table 2.2. Bacterial isolates used to screen for mobile genetic elements

| Organism | Isolates included |
|-------------------------|---|
| <i>E. coli</i> | JE1, JE2, LK111, MTH |
| <i>P. aeruginosa</i> | P5, P26, P28, PACT1, PACT2, PACT3, PACT4, PACT7 |
| <i>K. pneumoniae</i> | 795, JK1, JK2, K1, KAT |
| <i>C. jejuni</i> | 390 |
| <i>A. lwoffii</i> | 2, 3 |
| <i>A. calcoaceticus</i> | BD413 C91 |

2.2 DNA extraction

2.2.1 Genomic DNA extraction

Genomic DNA was obtained using the phenol/ chloroform method (Ausubel *et al.*, 1987). Frozen cultures of MOS1 and MOS2 bacterial strains were streaked onto two times Yeast-Tryptone (2 x YT) agar (Appendix A) plates. A single colony of each strain was inoculated into 5 ml 2 x YT broth (Appendix A), without antibiotic selection, and incubated to saturation at 37°C overnight with shaking. A 2 ml aliquot of the culture was centrifuged in an Eppendorf centrifuge 5417C (Germany) and the pellet resuspended in TE buffer (Appendix A). Bacterial cell lysis was enhanced by using 0.5 % (v/v) of the detergent sodium dodecyl sulphate (SDS). Cellular proteins were removed by digestion with 100 µg/ml of the non-specific protease, proteinase K (Merck, Germany), for an hour at 37°C.

To remove cell wall debris, denatured protein, and polysaccharides, selective precipitation with cetyltrimethylammonium bromide (CTAB) was used. A chloroform/isoamyl alcohol (24:1) mixture was added and centrifuged to extract CTAB-protein/polysaccharide complexes, leaving nucleic acids in solution. Genomic DNA was precipitated using 0.6 volumes isopropanol and the pellet was washed with 70 % ethanol before drying in a fume hood for 5-10 minutes and resuspending in 100 µl TE buffer. The quality of the DNA was confirmed by electrophoresis on a 1 % (w/v) agarose gel (2.3.2) and by using it as a template in a polymerase chain reaction (PCR) (2.6). DNA was quantified by using a Nanodrop spectrophotometer, ND-1000 UV/Vis (USA). DNA was stored at 4°C until required.

When screening recombinant clones, ten recombinant colonies were resuspended in 20 µl distilled water. After boiling for ten minutes and rapidly cooling on ice to prevent any nucleases degrading the released DNA, the sample was centrifuged briefly. A 2 µl aliquot of the supernatant was used as template in PCR (2.6).

2.2.2 Small-scale plasmid DNA extraction

The vector pUC19 (Appendix B) was used for cloning in this study. Plasmid DNA was extracted according to the manufacturer's instructions, using a Miniprep spin kit (Qiagen, Germany). *E. coli* JM109 containing pUC19 was streaked onto LB agar (Appendix A) plates containing 100 mg/ml ampicillin (Bodene, South Africa), 40 mg/ml 5-bromo-4chloro-3indolyl- β -D-galactosidase (X-gal, Roche, Germany), and 4 mg/ml isopropyl- β -D-thio-galactosidase (IPTG, Roche, Germany). A single blue colony was selected and inoculated into 5 ml LB broth (Appendix A) containing 100 mg/ml ampicillin and incubated at 37°C overnight with shaking. The cell mass was precipitated in a 1.5 ml eppendorf tube by centrifugation and the pellet resuspended in Buffer P1 containing 100 μ g/ml RNaseA, 10 mM EDTA, and 50 mM Tris.Cl. The cells were lysed using SDS (1 %; w/v)-containing Buffer P2. The NaOH (200 mM) in Buffer P2 causes cellular proteins, chromosomal, and plasmid DNA to denature. The lysate was finally neutralised with Buffer N3 to recover the plasmid. The high salt concentration in Buffer N3 (3 M potassium acetate) causes the denatured macromolecular structures to precipitate. Following centrifugation, the supernatants were transferred to a QIAprep spin column and cleaned by successive washes with Buffer PB and Buffer PE. Plasmid DNA was eluted by adding 50 μ l Buffer EB, allowing this to stand at room temperature for a minute and then centrifuging at 13 000 rpm for one minute.

Plasmid DNA was quantified by comparison to known concentrations of the molecular weight marker, HyperLadder I™ (Bioline, UK, Appendix C) and by using a Nanodrop spectrophotometer, ND-1000 UV/Vis (USA). The quality of plasmid DNA was confirmed by restriction enzyme digestion (2.3.1) followed by electrophoresis on a 1 % (w/v) agarose gel (2.3.2). DNA was stored at 4°C until required.

Where necessary, plasmids were extracted using the large scale plasmid DNA extraction protocol (2.2.3).

2.2.3 Large-scale plasmid DNA extraction

Plasmids were extracted using a Midiprep spin kit (Qiagen, Germany) where necessary according to manufacturer's instructions. A single blue colony was picked from a freshly streaked selective plate containing 100 mg/ml ampicillin, 40 mg/ml X-gal and 4 mg/ml IPTG. The colony was inoculated into a starter culture of 5 ml LB broth containing 100 mg/ml ampicillin and incubated at 37°C for 8 hours with shaking. The starter culture was diluted 1/500 - 1/1000 in a total volume of 50 ml and incubated for 12-16 hours at 37°C with shaking. Bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C and resuspended in 6 ml Buffer P1 with RNase A (100 µg/ml; Roche, Germany) added to it. Subsequently, 60 ml of P2 lysis buffer and 6 ml chilled Buffer P3 were added to the mixture. The lysate was immediately transferred to the barrel of a QIAfilter cartridge, incubated at room temperature for 10 minutes for optimal performance and filtered into a HiSpeed Midi Tip previously equilibrated with Buffer QBT. The HiSpeed Midi Tip was washed with 20 ml Buffer QC. Plasmid DNA was eluted with 5 ml Buffer QF in a clean receiver tube and precipitated with 3.5 ml isopropanol. This was mixed and allowed to stand for 5 minutes. The plasmid DNA was then bound to a QIAprecipitator using a syringe, washed with 70 % ethanol, and eluted with 0.5 ml Buffer TE into a clean microcentrifuge tube.

Plasmid DNA was quantified by comparison to known concentrations of the molecular weight marker, HyperLadder I™ (Bioline, UK, Appendix C) and by using a Nanodrop spectrophotometer, ND-1000 UV/Vis (USA). The quality of plasmid DNA was confirmed by restriction enzyme digestion (2.3.1) followed by electrophoresis on a 1 % (w/v) agarose gel (2.3.2). DNA was stored at 4°C until required.

2.3 DNA analysis and purification

2.3.1 Restriction enzyme digestion

Genomic DNA (5 µg) and pUC19 (2 µg) were digested with 10 U of *EcoRI* (Roche, Germany) and 10 U of *HindIII* (Roche, Germany) or 10 U of *BamHI* (Roche, Germany) in a total volume of 20 µl in 1 X Buffer B. Digestion of genomic DNA proceeded at 37°C for 6 hours and plasmid DNA

at 37°C for 3 hours. Double, instead of single digests, were performed to reduce the chances of the cloning vector, pUC19, recombining to itself during ligation (2.4). Religated pUC19 can be transformed into *E. coli* JM109 competent cells and this reduces the chance of obtaining recombinant clones. The restriction enzyme *EcoRI* recognises the sequence G↓AATTC, *HindIII* recognises A↓AGCTT, and *BamHI* recognises G↓GATCC. All the enzymes therefore produce overhangs following restriction digestion. Using two enzymes to digest pUC19 results in a linear plasmid with a different restriction site on either end, thus preventing pUC19 from religating. Double digests ensured genomic DNA fragments of smaller molecular size, rather than larger fragments produced using only one enzyme. This was done to increase the religation of fragments into pUC19, which is known to not readily take up larger DNA fragments (Brown, 2003). The efficiency of the digestion was confirmed on a 1 % agarose gel (2.3.2).

2.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis resolves DNA fragments on the basis of their differing rates of migration, which in turn are dependent on their size. Since DNA carries a negative electric charge due to the phosphate groups on its sugar-phosphate backbone (Brown, 2003; Garrett & Grisham, 2005), it will migrate towards the positive electrode in the presence of an electric current. Smaller fragments migrate faster than larger ones as they pass through the agarose gel pores with more ease. In this study, DNA was resolved on horizontal gels of 1 or 2 % (w/v) agarose (Lonza, USA) dissolved in 1 X TAE buffer (Appendix A). The agarose was dissolved by heating the solution in a microwave oven and then allowed to cool. Ethidium bromide (EtBr, Fluka, Switzerland) was added at a final concentration of 10 ng/μl, and the gel poured into a mould of the appropriate size with a comb at one end. Once the gel had set, the comb was removed and the gel submerged in 1 X TAE buffer in an electrophoresis tank. EtBr is a fluorescent dye that intercalates between the adjacent base pairs of DNA. The fluorescent yield of EtBr-DNA complexes is greater than that of unbound dye. EtBr absorbs ultraviolet (UV) light at 302 nm (Sambrook *et al.*, 1989). DNA was thus visualized at 302 nm by UV transillumination using an UVITEC UV light box (UK) and the results captured by an UVITEC camera (Japan).

DNA samples were loaded with 5 X loading buffer (Bioline, UK) containing sucrose, bromophenol blue and ethylenediaminetetra-acetic acid (EDTA). Sucrose increases the samples density, ensuring that the DNA remains in the well and migrates evenly. Bromophenol blue allows one to monitor the rate of migration of the DNA samples, and EDTA chelates Mg^{2+} ions thereby inhibiting enzymatic activity that may degrade the DNA.

The molecular weight marker HyperLadder I™ or IV™ (Bioline, UK, Appendix C) was loaded alongside DNA samples in 1 or 2 % agarose gels, respectively. The markers are both molecular weight and quantitative markers. Each band corresponds to a specific size and known DNA concentration. The size and the approximate concentration of the DNA samples could thus be determined.

Electrophoresis was performed at 90 volts for 1-2 hours depending on the size of the gel.

2.3.3 Agarose gel extraction of DNA

DNA was electrophoresed and excised from agarose gels using a MiniElute gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Three volumes of QG buffer were added to one volume of the excised gel fragment. Following 10 minutes of incubation at 50°C, one gel volume of isopropanol was added and mixed by inverting several times to help precipitate the DNA. The solution was transferred to a MiniElute column and centrifuged for 1 minute, allowing the plasmid to bind to the matrix. The flow through was discarded, 500 µl QG buffer was added to the column and centrifuged for another minute. The column was washed with 750 µl buffer PE and residual ethanol was removed by an additional centrifugation step for a minute. The DNA was eluted with 10 µl purified distilled water dropped onto the centre of the column membrane and allowing it to stand at room temperature for a minute. Following centrifugation, the eluant was collected and stored at -20°C to prevent it from degradation in the absence of buffering and chelating agents.

2.3.4 Genomic DNA precipitation

Digested genomic DNA was recovered using the ethanol precipitation method by Ausubel *and* colleagues (1987). The digested sample was made up to 100 μ l with purified distilled water. Sodium acetate at 3 M and 300 μ l 100 % ethanol were added to the solution and incubated at -70°C for an hour. DNA was precipitated, washed with 1 ml 70 % ethanol and air dried for 5-10 minutes. The DNA was resuspended in 10 μ l distilled water at room temperature for an hour. DNA was stored at -20°C to prevent it from degradation in the absence of buffering and chelating agents.

2.4 DNA ligation

Digested *A. baumannii* MOS1 genomic DNA (2-5 μ g; 2.3.1) was ligated to 50-100 ng similarly digested pUC19. The final reaction volume of 10 μ l also contained 1 U T4 DNA ligase (Roche, Germany) in 1 X buffer. The ligation reaction proceeded overnight at 4°C.

2.5 Transformation of *E. coli* JM109 cells

Transformation was performed using *E. coli* JM109 competent cells (Promega, USA) following the manufacturer's instructions. The competent cells were placed on ice until just thawed after which an aliquot of the ligation reaction was added. The mixture was incubated for 30 minutes on ice to decrease the membrane potential of the cells (Panja *et al.*, 2006) in preparation for the next step. To allow the uptake of recombinant plasmids, the mixture was heat shocked for 45 seconds in a 42°C water bath after. The tubes were immediately placed on ice for two minutes to maintain the transformed plasmid within the competent cells. This was achieved by increasing the membrane potential of the competent cells (Panja *et al.*, 2006). The transformation reaction was made up to 1 ml with SOC medium (Appendix A) and incubated at 37°C for one hour with shaking to allow for the expression of the *amp* gene. A 100 μ l aliquot was diluted 1:10 with SOC medium, and 100 μ l aliquots of both the diluted and undiluted cultures plated on LB agar plates containing 100 mg/ml ampicillin, 100 mg/ml IPTG, and 200 mg/ml X-gal.

The cloning vector pUC19 harbours an ampicillin resistance gene, hence the selection of transformants on ampicillin-containing agar. X-gal was included as a colour indicator of β -galactosidase activity, which is induced by IPTG. pUC19 carries a *lacZ'* gene that encodes the amino terminal (α -peptide) of the enzyme β -galactosidase (Brown, 2003). When pUC19 is introduced into bacteria containing the carboxyl terminal (ω -peptide) of β -galactosidase, e.g. *E. coli* JM109, the bacterial and plasmid components of the protein complement each other (α -complementation) to produce a functional enzyme. X-gal is hydrolysed by β -galactosidase to give a non-diffusible blue dye, resulting in blue colonies on media with X-gal. If the α -peptide sequence in pUC19 is interrupted by cloning in the multiple cloning site (Appendix B), α -complementation cannot take place and colonies will appear white. Thus, putative recombinants will appear as white colonies on agar plates with ampicillin, X-gal and IPTG, and these were subcultured onto 2 x YT agar plates with 100 mg/ml ampicillin. The plates were incubated over night at 37°C.

2.6 Polymerase chain reaction

The polymerase chain reaction (PCR) is a primer-directed enzymatic reaction that results in the selective amplification of a chosen region of a DNA molecule (Figure 2.1). Any region of DNA can be amplified, given that sequences at the borders of the required region are known. Two short oligonucleotides, known as primers, must hybridize to the DNA molecule, one on each strand of the double helix. The primers, thus, restrict the region that will be amplified. Amplification is carried out by a thermo-stable DNA polymerase I enzyme from the bacterium *Thermus aquaticus* (*Taq* polymerase; Saiki *et al.*, 1988). The reaction conditions (Table 2.3) involve an initial denaturation step at 95°C for 5 minutes, followed by multiple cycles of DNA denaturation at 95°C for 45 seconds to five minutes, annealing of primers at 42 to 65°C for 30 seconds to a minute, and DNA extension by the polymerase at 72°C for one minute per kb of target DNA. A final extension step at 72°C for 5 minutes completes the reaction. Thermostable polymerase is used as it can withstand the high temperatures required to denature the DNA template.

The reaction components consisted of 25 mM MgCl₂, unless specified, 2.5 mM each dNTP, 20 pmoles forward and reverse primer each and 2.5 U GoTaq Flexi DNA Polymerase (Promega, USA) in the appropriate buffer, made up to 50 µl with distilled water. Typically, 10-100 ng of DNA (2.2) was added as template. A negative control with no DNA was included to detect possible contamination of any of the reagents. PCR was carried out in a GeneAmp® PCR system 2 700 (Applied Biosystems, Singapore) and amplification products were analysed by agarose gel electrophoresis (2.3.2).

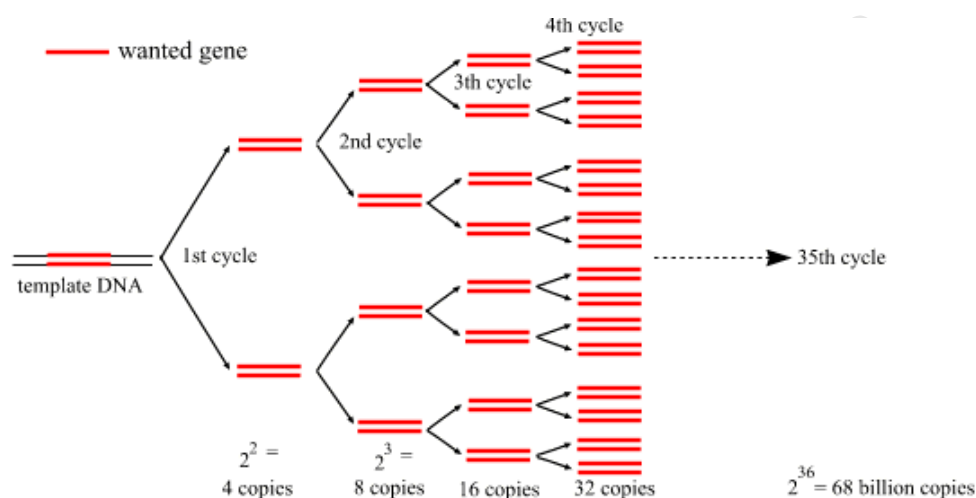


Figure 2.1: Diagrammatic representation of the polymerase chain reaction (PCR). (Taken from <http://users.ugent.be/~avierstr/principles/pcrcopies.gif>)

Primers were designed using DNAMAN software (Version 4.0, Lynnon Biosoft) and synthesised in the Molecular and Cell Biology Department, University of Cape Town. The oligomers were synthesized using a Polygen DNA synthesizer (Germany) with the standard procedure and purification. They were stored at 50 mM in Milli-Q water at -20°C. Primers used in this study are listed in Table 2.3.

Table 2.3. Primers and PCR assays used for amplification of target gene(s) or regions

| Target | Primers | Primer sequence (5'→3') | *PCR cycle (X 35) | product size (bp) |
|---------------------------------|--|--|-----------------------------------|-------------------|
| ATPase gene | ^a 42-156F ^a 46-358R | ATT GAT GTA CCG CCA TTA GTG AAC CAT CTT TGG GCA | 95°C (1') 58°C (45'') 72°C (45'') | 253 |
| ATPase gene linked to IS26 | ^a 42-156F ^b IS26F2 | ATT GAT GTA CCG CCA TTA TCA CTC CAC GAT TTA CC | 95°C (1') 58°C (45'') 72°C (4') | ≥ 2 175 |
| | ^b IS26F2 ^a 46-358R | TCA CTC CAC GAT TTA CC GTG AAC CAT CTT TGG GCA | 95°C (1') 58°C (45'') 72°C (4') | ≥ 2 175 |
| ATPase gene linked to IS1133 | ^a 42-156F ^c IS1133F5 | ATT GAT GTA CCG CCA TTA GAT GTT TGA GCA GTA GC | 95°C (1') 58°C (45'') 72°C (4') | ≥ 967 |
| | ^c IS1133F5 ^a 46-358R | GAT GTT TGA GCA GTA GC GTG AAC CAT CTT TGG GCA | 95°C (1') 58°C (45'') 72°C (4') | ≥ 967 |
| <i>aacC2a</i> | ^c <i>aacC2a</i> F ^c <i>aacC2a</i> R | CGC GGA AGG CAA TAA C GCT TCT CAA GAT AGG TG | 95°C (1') 51°C (45'') 72°C (1') | 786 |
| Sequences upstream of IS1133 | ^d M13R ^c IS1133F5 | CAG GAA ACA GCT ATG AC GAT GTT TGA GCA GTA GC | 95°C (1') 58°C (45'') 72°C (4') | ≥ 1 014 |
| IS1133 | ^c IS1133F2 ^c IS1133F5 | GTT CCG GTC TTT TTG AG GAT GTT TGA GCA GTA GC | 95°C (45'') 52°C (45'') 72°C (1') | 949 |
| ISK _{pn12} | ^b ISK _{pn12} F ^b ISK _{pn12} R3 | CTT CAG CCC TAT TTC CC GGT CAG GAC TCA TTG CT | 95°C (1') 60°C (45'') 72°C (45'') | 735 |
| | ^b ISK _{pn12} F ^b ISK _{pn12} R2 | CTT CAG CCC TAT TTC CC AAC GGT CGC AGC GAG AG | 95°C (1') 56°C (45'') 72°C (45'') | 704 |
| | ^b ISK _{pn12} F2 ^b ISK _{pn12} R2 | TTC ATA ACG GCA GGC CA AAC GGT CGC AGC GAG AG | 95°C (1') 52°C (45'') 72°C (45'') | 321 |
| ISK _{pn12} <i>tnpA</i> | ^b ISK _{pn12} F ^b ISK _{pn12} R | CTT CAG CCC TAT TTC CC GCA GAG CAC AAG ACC AT | 95°C (1') 52°C (45'') 72°C (45'') | 230 |
| 16 S rRNA | ^c 16SF ^c 16SR | ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA | 95°C (45'') 50°C (45'') 72°C (1') | 449 |
| IS <i>Aba-1</i> <i>tnpA</i> | ^e HRF ^e HRR | CAC GAA TGC AGA AGT TG CGA CGA ATA CTA TGA CAC | 95°C(1') 50°C (45'') 72°C (45'') | 550 |

* - initial denaturation was 95°C for 5 minutes and final elongation was 72°C for 5 minutes for all assays. Conditions were cycled 35 times. ^a Fournier *et al.*, 2006 ; ^b This study; ^c Jacobson, 2007; ^d Yanisch-Perron *et al.*, 1985; ^e Segal *et al.*, 2005

2.7 Sequencing and sequence analysis

Purified PCR products and recombinant plasmids were sequenced using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. Additional primers were designed and used for sequencing reactions to complete the entire sequence. The dideoxy sequence reaction protocol was followed. The dideoxy method utilises a DNA polymerase to catalyse the synthesis of DNA in the presence of modified deoxy and dideoxynucleotides (Garrett & Grisham, 2005). The polymerase is genetically engineered and lacks all traces of exonuclease activity that might otherwise degrade the DNA. Chain elongation occurs from the 3' end of a DNA primer that is annealed to the target template DNA until a dideoxynucleotide is incorporated. Elongation is terminated as the dideoxynucleotide is modified and lacks a 3' hydroxyl group. The products are thus a population of oligonucleotide fragments whose lengths are determined by the distance between the 5' termini of the primer used to initiate DNA synthesis and the sites of the dideoxynucleotide termination. Four reactions are run in parallel, each with a dNTP labeled with one of four different fluorescent dyes to provide a tracer for the products of the polymerization process. The sequencing products are visualized by autoradiography following their separation on a polyacrylamide gel based on their size. Smallest fragments migrate the fastest upon electrophoresis and fragments differing by a single nucleotide are readily resolved. As the differently sized fragments pass down the gel, a laser beam excites the dye and the emitted energy is detected by a fluorometer. The emitted light identifies the final base in the fragment. This way, a whole sequence is determined and will be displayed as a graph using computer based programs.

Automated sequencing was performed at the Core Sequencing Facility at the University of Stellenbosch, Cape Town. Sequences were edited in Chromas (Version 2.31, Techelysium Pty Ltd) and analysed using DNAMAN software (Version 4.0, Lynnon Biosoft). A standard nucleotide-nucleotide BLAST analysis (blastn; www.ncbi.nlm.nih.gov/BLAST) of the GenBank database was performed for all sequences.

2.8 Southern hybridisation

2.8.1 Preparation of labelled probe

Gel extracted (2.3.3) PCR products (2.6) were used as probes in Southern hybridisation experiments. The probe was diluted to 10 ng/ μ l and denatured by boiling for five minutes. Rapid cooling on ice for five minutes kept the denatured probe from reannealing before 10 μ l of the labeling reagent (horseradish peroxidase; Amersham, UK) was added. The peroxidase is complexed with a positively charged polymer that forms ionic interactions with the negatively charged single-stranded DNA probe. Addition of an equal volume of glutaraldehyde causes covalent bond formation between the probe and peroxidase as the mixture is incubated at 37°C for 10 minutes.

2.8.2 Hybridisation, signal generation and detection

Genomic DNA (5 μ g) was digested (2.3.1) for 6 hours and electrophoresed on a 0.8 % agarose gel (2.3.2) at 30 volts overnight. The gel was washed for 15 minutes in 0.25 M depurinating buffer (Appendix A), twice for 20 minutes in denaturing buffer (1.5 M NaCl and 0.5 M NaOH; Appendix A), and twice for 20 minutes in neutralising buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5; Appendix A). The gel was rinsed with distilled water between each of the washes. The single stranded genomic DNA was immobilized overnight onto a Hybond-N+ membrane (Amersham, UK) by capillary transfer. Immobilizing allows for downstream manipulation of the DNA to detect required sequences using a specific probe.

The transferred DNA was fixed onto the Hybond-N+ membrane by cross-linking with a UV fixer (Hoefer Scientific Instruments, USA) for 1200 counts at wavelength of 245 nm. Cross-linking is achieved by the formation of bonds between the positively charged amino groups on the membrane and the thymine residues in DNA (Khandjian, 1986). The membrane was incubated at 42°C in a hybridization oven (Techne Hybridisation HB 1D, England) with gentle shaking in prehybridization buffer (Appendix A) for at least 15 minutes.

The probe was added to the prehybridization buffer and left to hybridize over night at 42°C. Two 20 minute washes with primary wash buffer (Appendix A) at 42°C were sufficient to remove unbound probe from the membrane. Following two five-minute washes with secondary wash buffer (Appendix A), the membrane was incubated for a minute in a mixture of equal volumes of detection reagent 1 and 2 (Amersham, UK). Detection reagent 1 decays to hydrogen peroxide. The hydrogen peroxide is reduced to water and oxygen by the peroxidase bound to the probe (2.8.1). The oxygen produced oxidizes luminol in detection reagent 2 to produce light.

The presence of an enhancer in the detection reagents prolongs and increases the light output, allowing it to be detected on a blue-light sensitive film. After excess detection reagent was drained off, the blot was wrapped in cling film, placed in a film cassette and exposed to an X-ray film (AGFA, CP-BU, 100NIF) for a minute, or longer if necessary. The film was then placed in developer (G128, AGFA, Belgium) for 3 minutes, briefly rinsed in tap water, and placed in Rapid fixer (G333C, AGFA, Belgium) for 3 minutes. Following a brief rinse in tap water, the film was hung to dry and scanned using a Canon scanner, CanoScan 5600F (Japan).

2.9 RNA extraction and purification

RNA was extracted from bacterial cultures at time points corresponding to logarithmic and stationary phases. Using the hot acid phenol procedure (Aiba *et al*, 1981), harvested cells were resuspended in 0.02 M sodium acetate, 0.5 % SDS and 1 mM EDTA (acetate/SDS buffer). Samples were incubated with gentle shaking at 60°C in the presence of redistilled phenol. The aqueous phase was re-extracted with phenol and the RNA chilled at -70°C in 70 % ethanol. Following resuspension of the precipitate in acetate/SDS buffer, the RNA was ethanol precipitated two more times and finally resuspended in 50 µl RNase-free distilled water. The RNA was quantified using a Nanodrop spectrophotometer, ND-1000 UV/Vis (USA) and the quality determined by agarose gel electrophoresis (2.3.2). RNA was stored as 5 µl aliquots at -70°C until required.

To remove contaminating DNA from RNA samples prior to reverse-transcriptase PCR (2.10), the RNA was treated with RQ1 DNase (Promega, USA), an endonuclease that cleaves double- and single-stranded DNA molecules. The DNase was added at a concentration of 1 unit per microgram of RNA together with the appropriate buffer. The reaction was made up to 10 μ l with RNase-free distilled water and incubated for 30 minutes at 37°C. To inactivate the DNase which may interfere with downstream reactions, a microlitre of RQ1 DNase stop solution was added and incubated at 65°C for 10 minutes.

2.10 Reverse transcriptase PCR

Reverse transcriptase PCR is a technique used to determine gene expression. An RNA-dependant DNA polymerase is used to synthesise cDNA from RNA transcripts in the presence of a reverse primer. The cDNA obtained is then used as template in a standard PCR (2.6).

A 15 μ l reaction with DNase treated RNA (2 μ l; 2.9) and reverse primer (0.5 μ g) complementary to the gene of interest was incubated for five minutes at 70°C to denature the RNA secondary structure. Following rapid cooling on ice, the reverse transcriptase moloney murine leukaemia virus (M-MLV, 200 units; Promega, USA), 5 μ l 5 X M-MLV reaction buffer, 10 μ l nuclease-free dNTPs, and 25 units of Recombinant RNasin® Ribonuclease Inhibitor (Roche, Germany) were added. The reaction was made up to 25 μ l with RNase-free distilled water and incubated at 42°C for an hour to synthesize cDNA.

PCR amplification (2.6) was carried out using appropriate primers and 2 μ l of the cDNA as template.

RESULTS AND DISCUSSION

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3.1 Investigation of the presence of an interrupted ATPase gene in *A. baumannii* strains MOS1 and MOS2

An *A. baumannii* isolate, MOS1, was obtained from a patient admitted to GSH in 2005. Susceptibility tests indicated that MOS1 was resistant to the aminoglycosides gentamicin and amikacin, and susceptible to meropenem and imipinem (Table 2.1). PCR assays indicated that MOS1 contains a copy of *aacC2a* downstream of an *ISAbal-1*-interrupted copy of *IS1133* (Figure 3.1; Jacobson, 2007).

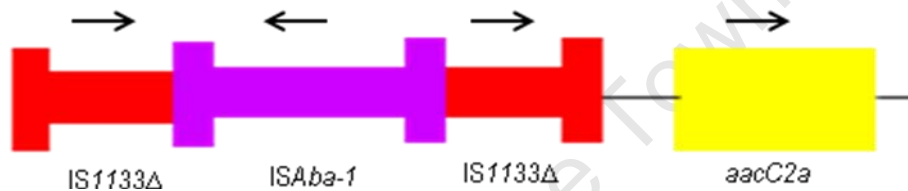


Figure 3.1: Genetic arrangement of an aminoglycoside resistance gene, *aacC2a* (yellow), downstream of an *IS1133* portion (red). *IS1133* is interrupted by a full copy of *ISAbal-1* (purple) in strain MOS1. This genetic arrangement has been conserved for almost 26 years in *A. baumannii* clinical isolates from Cape Town. The arrows indicate direction of transcription (Adapted from Jacobson, 2007).

MOS2, isolated two weeks later from the same patient following treatment with meropenem, was resistant to gentamicin, amikacin, imipinem and meropenem. MOS2 did not carry a copy of *aacC2a* (Jacobson, 2007). Resistance to gentamicin and amikacin in this strain may be due to the expression of other aminoglycoside resistance genes (Table 1.1; Bergogne-Berezin & Towner, 1996), overexpression of the AdeABC multidrug efflux pump (1.3.2; Magnet *et al.*, 2001), methylation of the 16S rRNA (1.3.4; Lee *et al.*, 2006), or a combination of these mechanisms.

Notably, MOS2 had a copy of *bla_{OXA-23}* (Segal *et al.*, 2007), which would account for the carbapenem resistance in this isolate. MOS1 did not carry a copy of *bla_{OXA-23}* (Segal *et al.*, 2007). It may be that when these strains originally diverged, a mobile genetic element carrying the

*bla*_{OXA-23} gene replaced the conserved genetic arrangement, possibly present on a large mobile genetic element in MOS1, following horizontal gene transfer. Integrons are associated with gene cassettes that have a 59 base on their 3'- end (Figure 1.5; Recchia & Hall, 1995). Sequence analysis of the conserved genetic arrangement (Figure 3.1) did not reveal the presence of a 59 base element, indicating that this arrangement does not contain features of an integron. Therefore, should MOS1 harbour a large IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a*-associated mobile genetic element, it may be part of a transposon or a PAI located on either a plasmid or within the chromosomal DNA of this strain.

Transposons, PAIs, and other large genomic regions are located within the ATPase gene of *A. baumannii* (Fournier *et al.*, 2006; Corvec *et al.*, 2007; Iacono *et al.*, 2008; Shaikh *et al.*, 2009). To determine whether the ATPase gene was interrupted in MOS1 and MOS2, a PCR assay (2.6) with primers 42-156F and 46-358R (Table 2.3; Figure 3.2; Fournier *et al.*, 2006) was carried out. The primers 42-156F and 46-358R amplify a 253 bp region within the ATPase structural gene and flank the insertion site in which PAIs were identified (Fournier *et al.*, 2006).

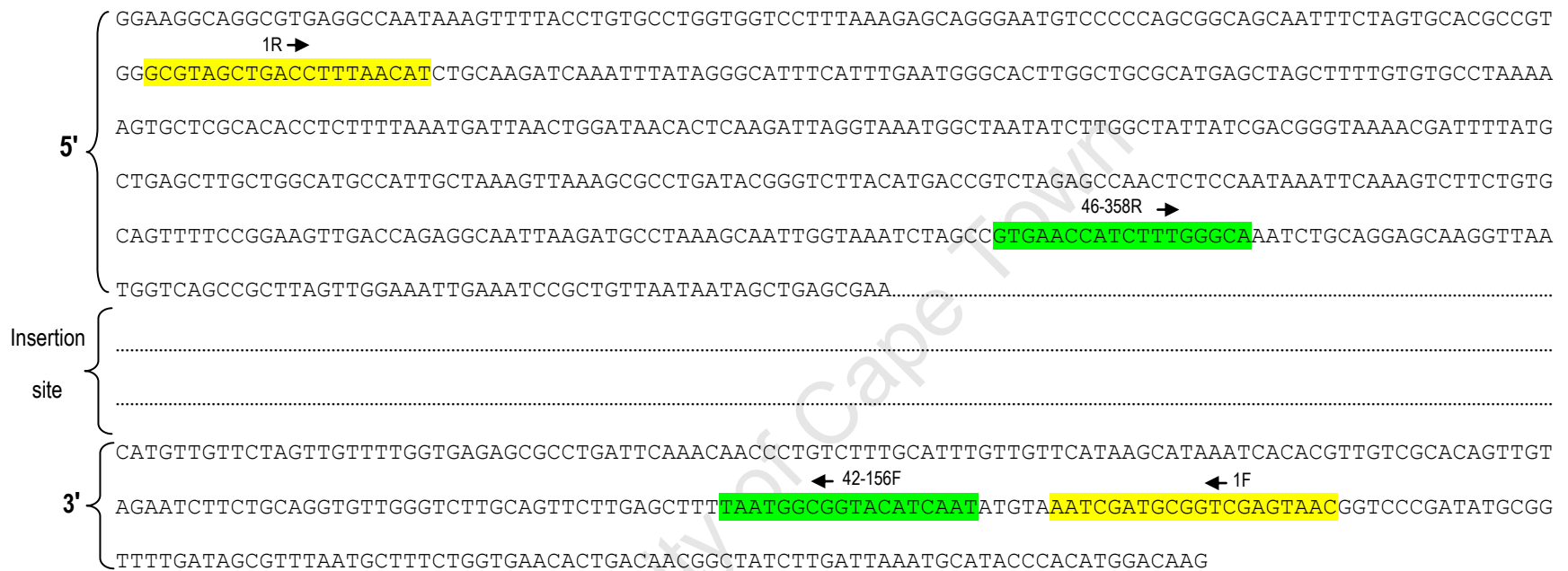


Figure 3.2: Nucleotide sequence of a partial ATPase gene. The primers used in the study by Fournier and colleagues (2006) to detect for the presence of an interrupted ATPase gene are highlighted in green. The primers used by Shaikh and colleagues are highlighted in yellow. The direction of the primers is indicated by arrows. The 5' and 3' – ends of the ATPase gene are indicated on the left. The region between the 5' and 3' where large genomic regions can insert is indicated by dots (.....) and is labelled on the left. In the absence of an interrupting genomic DNA region, the primers 42-156F and 46-358R will yield a 253 bp fragment in a PCR assay.

A. baumannii strain A21 is susceptible to most of the clinically available antibiotics except amikacin. This isolate was therefore included as a positive control in the absence of a strain with a known intact ATPase gene. A no DNA control was also included to detect the presence of contamination in the PCR reagents. Following PCR amplification, the PCR amplicons were analysed by agarose gel electrophoresis (2.3.2).

No product was obtained from the no DNA control, indicating no contamination of the reagents used. A product of the expected size (253 bp) was not obtained from the susceptible strain A21. Following a number of optimisations including increasing the annealing time to four minutes to allow for amplification of larger products, no product was obtained from A21. A 20 kb AbaG1 genomic island interrupted an ATPase gene in the *A. baumannii* susceptible isolate SDF (Fournier *et al.*, 2006). Another isolate, *A. baumannii* ATCC 17978 (Bauman *et al.*, 1968), had a 13 kb genomic island inserted within an ATPase gene (Iacono *et al.*, 2008). The presence of such a large insertion in the ATPase gene of A21 would prevent amplification of the 253 bp region in this PCR assay and could account for the lack of product observed.

Following amplification, no 253 bp PCR product was obtained from MOS1, suggesting the presence of a disrupted ATPase gene in this isolate. The IS1133_L-aacC2a genomic region in MOS1 is 3 397 bp (Figure 3.1). Should this arrangement lie on a large mobile element inserted within the ATPase gene in MOS1, the mobile element would be greater than 3 397 bp.

Sequences downstream of the IS1133_L-aacC2a conserved genetic arrangement have been characterised in a previous study (Figure 3.3; Segal *et al.*, 2003). Primers on either side of this region, IS26F2 and IS1133F5 (Table 2.3; Figure 3.3), were used in combination with the ATPase-specific primers, 42-156F and 46-358R (Table 2.3; Fournier *et al.*, 2006), to identify further sequences that may be associated with putative transposons or PAIs in MOS1. IS26F2 and IS1133F5 are complementary to sequences within the IS26 and IS1133_L portion, respectively. A21 and MOS2 were included as negative controls as they do not harbour the IS1133_L-aacC2a conserved genetic arrangement (Jacobson, 2007). Following amplification, the PCR products were analysed on a 1 % agarose gel (2.3.2).

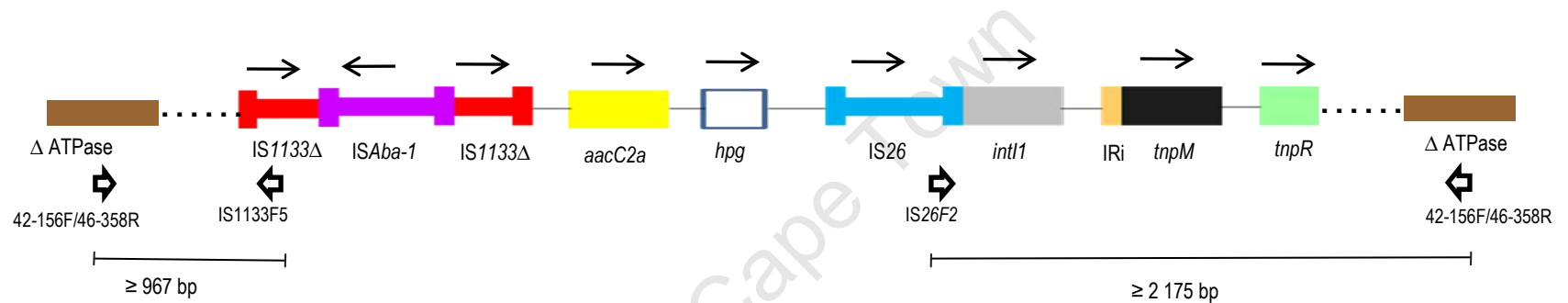


Figure 3.3: Schematic representation of sequences associated with *aacC2a* (yellow; Segal *et al.*, 2003); *hpg*, hypothetical protein; insertion sequence element, *IS26*; *intl1*, a portion of an integrase gene; *IRi*, left inverted repeat flanking integrons; *tnpR* and *tnpM* are Tn21-related sequences. The bracket shows the region that has been conserved for over 25 years in *A. baumannii* isolates obtained from patients at Groote Schuur and Red Cross children's hospitals. The closed arrows indicate direction of transcription. The open arrows indicate primers used to identify sequences on either side of the insert (Jacobson, 2007). A putative interrupted ATPase gene is indicated in brown. The dots represent unknown sequence that may form part of a putative genomic island. The expected product sizes used in combination with ATPase specific primers are indicated.

As expected, no product was obtained from the no DNA control or from A21 or MOS2 as these isolates do not contain a copy of IS1133. The absence of a PCR product from MOS1 suggests the location of the IS1133_L/ISAb_a-1/IS1133_R-aacC2_a conserved region on a large genomic region in MOS1. However, in the absence of control strains with an intact ATPase gene and one with an interrupted ATPase gene, it is difficult to interpret the results conclusively.

A. baumannii has an impressive ability to acquire resistance genes from other pathogens through horizontal gene transfer (Mah *et al.*, 2001; Landman *et al.*, 2002). The acquired resistance genes are often inserted in large genomic regions like transposons and PAIs. PAIs range from 10 – 200 kb (Schmidt & Hensel, 2004) in pathogenic bacteria, with the largest PAI identified in *A. baumannii* being 86 kb (Fournier *et al.*, 2006). PCR amplification using *Taq* polymerase proceeds at 1 minute/kb of genomic DNA (Promega, USA). Identification of large genomic regions in *A. baumannii* therefore requires elongation times greater than that which can be achieved using *Taq* polymerase.

In a study by Fournier and colleagues (2006), 5 of 22 *A. baumannii* isolates gave no PCR products with the ATPase-specific primers, 42-156F and 46-358R (Table 2.3). The presence of a large mobile genetic element inserted within an ATPase gene was suggested (Fournier *et al.*, 2006) and confirmed using whole genome sequencing. Thus, the identification and characterisation of such large regions of the genome would more readily be achieved through whole genome sequencing. In the study by Shaikh and colleagues (2009), a total of 10 *A. baumannii* isolates yielded no product in PCR assays using primers that flanked the ATPase insertional region (Figure 3.2). However, further investigation of the 10 isolates indicated that eight isolates carried ATPase genes interrupted by large genomic islands. Thus, even though the PCR fails to yield any amplicons, there is still a possibility that the ATPase gene will be interrupted by large genomic regions.

3.2. Characterisation of sequences associated with *aacC2a* in *A. baumannii* MOS1

The sequences downstream of the IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* conserved region have been characterised (Figure 3.3; Segal *et al.*, 2003). In the absence of whole genome sequencing, a standard cloning procedure (Sambrook *et al.*, 1989) was used to detect sequences upstream of the IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* genetic arrangement.

To obtain sequences upstream of IS1133, an *EcoRI*/*Bam*HI genomic DNA library of MOS1 was constructed (2.3.1; 2.4; 2.5) since the IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* genetic arrangement does not have *EcoRI* or *Bam*HI restriction sites. Recombinant isolates were selected on agar containing gentamicin since the aminoglycoside resistance gene, *aacC2a*, confers resistance to gentamicin and tobramycin (Seward *et al.*, 1998; Nemeč *et al.*, 2004). Recombinant *E. coli* JM109 growing in the presence of gentamicin selection would therefore contain a copy of *aacC2a* gene, along with the associated genetic arrangement (Figure 3.1), including sequences upstream of IS1133 *tnpA*.

To eliminate the digestion buffer and restriction enzymes that may interfere with downstream manipulation of the DNA, the genomic DNA was purified by ethanol precipitation (2.3.4) and the plasmid by the agarose gel extraction protocol (2.3.3). The quality of the DNA was analysed by agarose gel electrophoresis (2.3.2; Figure 3.4).

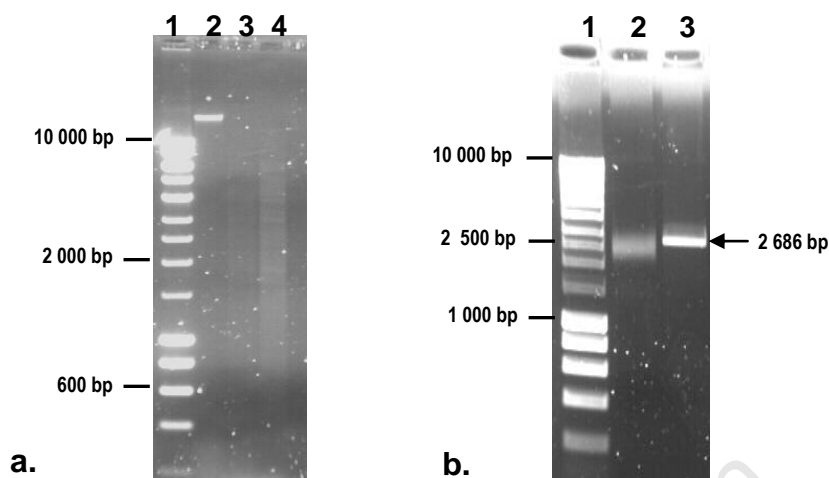


Figure 3.4: Agarose gel electrophoresis following digestion of genomic DNA and pUC19 used to construct a MOS1 *EcoRI/BamHI* genomic library. **a)** MOS1 genomic DNA used for library construction **Lane 1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** undigested MOS1 genomic DNA; **Lane 3:** *EcoRI/BamHI* digested MOS1 genomic DNA before purification; **Lane 4:** *EcoRI/BamHI* digested MOS1 genomic DNA following purification by ethanol precipitation (2.3.4). **b)** pUC19 used for library construction **Lane 1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** undigested pUC19; **Lane 3:** gel purified *EcoRI/BamHI* digested pUC19

Undigested MOS1 genomic DNA (Figure 3.4a; Lane 2) was included to check the efficiency of digestion. A product of the expected size, 2 686 bp, was obtained for linear pUC19 (Figure; 3.4b; Lane 3). Undigested pUC19 (Figure 3.4b; Lane 2) was included to check the efficiency of digestion. The undigested pUC19 (Figure 3.4b; Lane 2) is circular and supercoiled and therefore migrates faster than the linear pUC19 on an agarose gel.

Following ligation (2.4), competent cells were transformed in the absence of any plasmid to ensure that they were not contaminated with pUC19. The transformation efficiency of the competent cells, calculated as (number of colonies/ concentration of transformed pUC19) x $(10^3\text{ng}/\mu\text{g})$ x dilution factor, was determined by transforming the cells with undigested pUC19. The efficiency of the ligase enzyme was determined by transforming competent cells with religated pUC19 previously digested with *EcoRI*. The transformation efficiency of the ligase

control was then compared to that obtained using undigested pUC19. To ensure the digestion of pUC19, apart from analysis on an agarose gel (Figure 3.4b; Lane 3), competent cells were transformed with linear pUC19. Competent cells should not take up linear DNA. Following transformation of the ligation mix into *E. coli* JM109 competent cells (2.5), recombinants were selected for on agar containing gentamicin, X-gal and IPTG (2.5; Table 3.1).

Table 3.1. *E. coli* JM109 transformants obtained using pUC19 and MOS1

| Sample transformed | Colonies |
|---|----------|
| No DNA control | 0 |
| Undigested pUC19 | 1 083 |
| Religated pUC19 | 97 |
| Linear pUC19 | 0 |
| pUC19 (<i>EcoRI/BamHI</i>) + MOS1 (<i>EcoRI/BamHI</i>) | 95 |

No recombinants were obtained from the no DNA control following transformation of competent cells in the absence of pUC19 (Table 3.1) indicating that the competent cells were not contaminated. The competent cells used in this study had a transformation efficiency of 1.083×10^9 cfu/ μ g (Table 3.1) and therefore appropriate for use in this cloning procedure. A transformation efficiency of 9.7×10^7 cfu/ μ g was obtained from the ligase efficiency control (Table 3.1), indicating the efficiency of the ligase enzyme to religate digested DNA. There were no colonies obtained following transformation of the *E. coli* JM109 competent cells with linear pUC19 (Table 3.1), indicating complete digestion of the cloning vector.

A total of 95 recombinants were obtained from *E. coli* JM109 cells transformed with pUC19 ligated to *EcoRI/BamHI* digested MOS1 genomic DNA following selection on gentamicin (Table 3.1). Gentamicin resistance in *A. baumannii* may result from expression of other aminoglycoside

resistance genes apart from *aacC2a* (Bergogne-Berezin & Towner, 1996). To ensure that gentamicin resistance in these recombinants was due to the expression of *aacC2a* and to screen for sequences upstream of the IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* genetic arrangement, the library was screened for *aacC2a*. The *aacC2a*-specific primers *aacC2aF* and *aacC2aR* (Table 2.3; Figure 3.5) were used in a PCR assay (2.6) on DNA obtained from pools of 10 recombinants (2.2).



Figure 3.5: Schematic representation of *aacC2aF* and *aacC2aR* primers (arrows) in relation to the conserved genetic region in *A. baumannii* strain MOS1. A 786 bp product is obtained in a PCR assay using the indicated primers. IS1133 (red) is interrupted by a full copy of IS*Aba-1* (purple). (Adapted from Jacobson, 2007).

A no DNA control was included to detect contamination. *E. coli* JM109, *E. coli* JM109 (pUC19), and pUC19 were included to ensure that *aacC2aF* and *aacC2aR* primers were not complementary to sequences within the *E. coli* JM109 genome or pUC19. MOS1 genomic DNA obtained from the boiling method and from the phenol/chloroform method (2.2.1) was included as positive controls. Following PCR amplification, the amplicons were analysed by agarose gel electrophoresis (2.3.2; Figure 3.6).

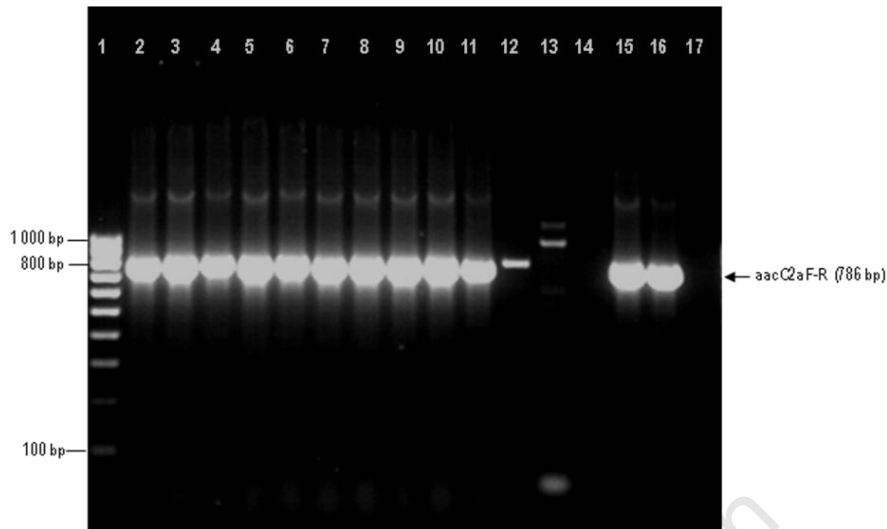


Figure 3.6: PCR amplicons of the *aacC2a* gene from MOS1 *EcoRI/BamHI* recombinant clones. **Lane 1:** Hyperladder IV (Biolone; Appendix C); Genomic DNA extracted from transformants in pools 1-10 (**Lanes 2-11**) were included; **Lane 12:** *E. coli* JM109; **Lane 13:** *E. coli* JM109 (pUC19); **Lane 14:** pUC19; **Lane 15:** MOS1 (genomic DNA obtained from the boiling method); **Lane 16:** MOS1 (genomic DNA obtained from phenol/chloroform extraction); **Lane 17:** no DNA control. The arrow indicates the size of the product corresponding to the *aacC2a* gene (786 bp) and relevant marker sizes are indicated.

No product was obtained from the no DNA control (Figure 3.6; Lane 17), indicating that the PCR reagents used in this assay were not contaminated. Non-specific amplification was observed with the negative controls *E. coli* JM109 and *E. coli* JM109 (pUC19) indicating regions complementary to *aacC2aF* and *aacC2aR* in *E. coli* JM109 and pUC19. Optimisations of the assay did not inhibit this non-specific amplification. A product of the expected size (786 bp) corresponding to the *aacC2a* structural gene, was obtained from the two MOS1 positive controls (Figure 3.6; Lanes 15 and 16). All 10 pools gave a product of the expected size following amplification with *aacC2aF* and *aacC2aR* primers, (Figure 3.6; Lanes 2-11), suggesting the presence of an *EcoRI/BamHI* insert harbouring the IS1133_L/ISAb_a-1/IS1133_R-*aacC2a* genetic arrangement in all the colonies.

To determine which of the recombinant colonies from all pools resulted in the PCR products obtained above, PCR assays using primers *aacC2aF* and *aacC2aR* (Table 2.3; Figure 3.5) were

carried out on individual colonies from each of the 10 pools. A product of 786 bp, corresponding to *aacC2a*, was obtained from all 95 colonies (data not shown). Thirty-five recombinant clones were randomly selected for small-scale plasmid extraction (2.2.2). The plasmid pUC19 was extracted from *E. coli* JM109 (pUC19) as a positive control. Following plasmid extraction, the plasmids were electrophoresed on a 1 % agarose gel (Figure 3.7).

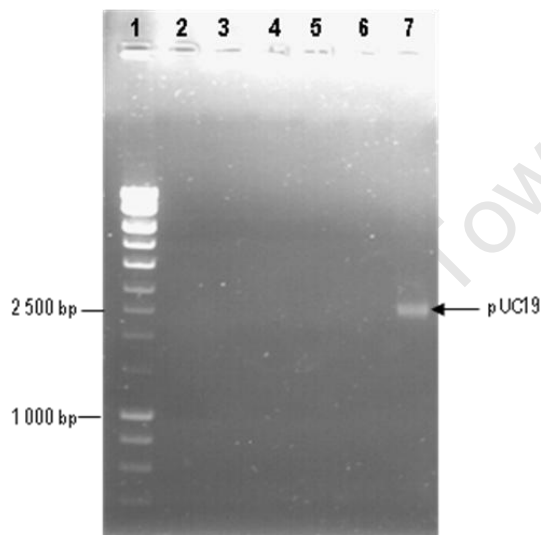


Figure 3.7: Agarose gel electrophoresis of DNA extracted from selection of recombinant clones of a MOS1 *EcoRI/BamHI* library. **Lane 1:** Hyperladder I (Bioline); **Lane 2 – 6:** clones 1-5; **Lane 7:** pUC19.

The plasmid pUC19 was extracted from *E. coli* JM109 (Figure 3.7; Lane 7). No plasmids were obtained from any of the thirty-five transformants (data not shown). It may be that the recombinant plasmids were unstable and integrated into the *E.coli* JM109 genome. Should this be the case, a recombination site may lie directly or many nucleotides upstream of the IS1133_L/ISAb_a-1/IS1133_R-*aacC2a* genetic arrangement in MOS1.

Although all of the recombinant clones were positive for the *aacC2a* PCR (Figure 3.6), no recombinant plasmids were obtained from the MOS1 *EcoRI/BamHI* recombinant clones (Figure 3.7). It was therefore necessary to determine if the recombinant plasmids had integrated into the

E. coli JM109 genome. A PCR assay (2.6) using the primers IS1133F5 and M13R (Table 2.3; Figure 3.8) was carried out on five recombinants. IS1133F5 is complementary to sequences in the left portion of IS1133 (Figure 3.8). M13R is a pUC19-specific primer that lies just outside of the multiple cloning site (Appendix B).

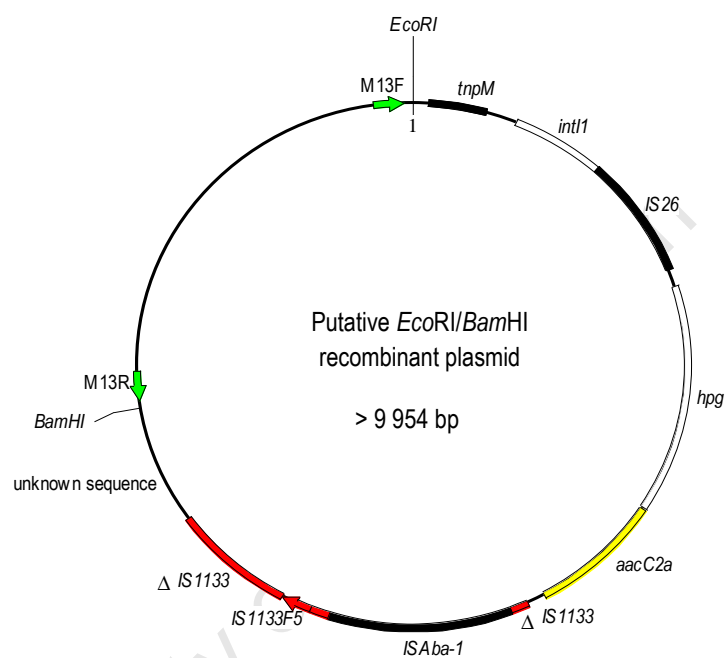


Figure 3.8: Schematic representation of a putative *EcoRI/BamHI* recombinant plasmid from *A. baumannii* strain MOS1. The restriction sites are indicated. **IS1133** interrupted by a full copy of *ISAbA-1* is indicated in red. Unknown sequences upstream of Δ **IS1133** are indicated in blue. **M13 primers** are indicated in green and the **IS1133F5 primer** as a red arrow. *aacC2a*, aminoglycoside resistance gene; *hpg*, hypothetical protein; insertion sequence element, *IS26*; *int1*, a portion of an integrase gene; *tnpM* is a Tn21-related sequences.

A no DNA control was included to detect contamination. The plasmid pRK002 (Appendix B; Jacobson, 2007) contains the conserved genetic arrangement (Figure 3.1), and was included as a positive control. Following amplification, the PCR products were analysed by agarose gel electrophoresis (2.3.2; Figure 3.9).

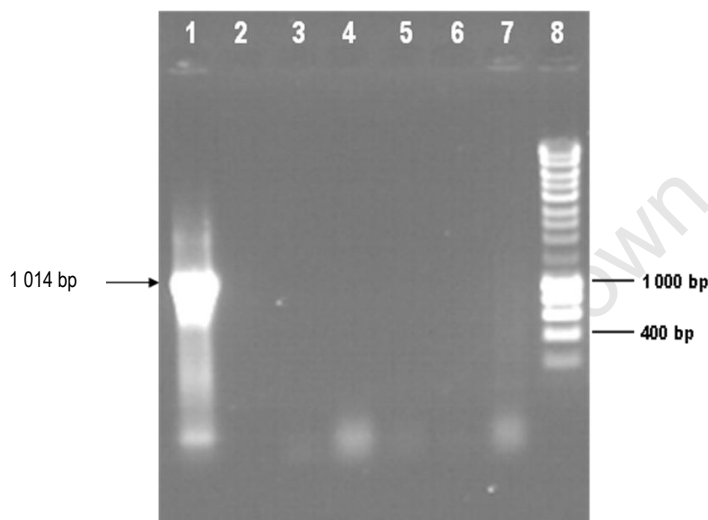


Figure 3.9: Agarose gel electrophoresis following PCR amplification with the primers IS1133F5 and M13R on MOS1 *EcoRI/Bam*HI recombinant clones. **Lane 1:** pRK002 (Appendix B); Genomic DNA extracted from five randomly selected transformants (**Lanes 2-6**) was included; **Lane 7:** no DNA control. **Lane 8:** Hyperladder I (Bioline; Appendix C). Relevant marker sizes are indicated and an expected product size (1 014) indicated by an arrow.

No product was obtained from the no DNA control (Figure 3.9; Lane 7), indicating that the PCR reagents used in this assay were not contaminated. A product of the expected size (1 014 bp) corresponding to the region between IS1133F5 and M13R (Table 2.3), was obtained from the positive control, pRK002 (Appendix B; Figure 3.9; Lane 1). There was no product of the expected size (> 1 014 bp) obtained from any of the clones used to test for recombination (Figure 3.9; Lanes 2-6), suggesting that the recombinant plasmid may have been very large and unstable and that it integrated into the *E. coli* JM109 genome, or that there may be a recombination site upstream of the left portion of IS1133.

As sequences upstream of the IS1133_L-*aacC2a*-associated genetic arrangement could not be obtained from the MOS1 *EcoRI/BamHI* library, another genomic library, using different restriction enzymes, was constructed.

MOS1 genomic DNA and pUC19 were therefore digested with *EcoRI* and *HindIII* (2.3.1 Figure 3.10a). Importantly, there is a *HindIII* site in the right hand portion of IS1133. Recombinants obtained would therefore contain more sequence upstream of the right hand portion of IS1133. *EcoRI* digested pUC19 was included to investigate the efficiency of the ligase enzyme (Figure 3.10b; Table 3.2). Following purification (2.3.3; 2.3.4) of *EcoRI* and *BamHI* MOS1 genomic DNA and pUC19, the quality of the DNA was analysed by agarose gel electrophoresis (2.3.2; Figure 3.10).

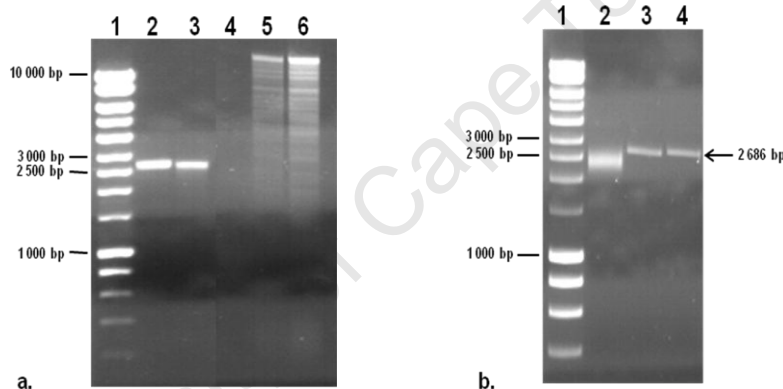


Figure 3.10: Agarose gel electrophoresis following MOS1 genomic DNA and pUC19 digestion. **a) Lane 1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** *EcoRI/HindIII* digested pUC19; **Lane 3:** *EcoRI/HindIII* digested pUC19 following agarose gel purification (2.3.3); **Lane 4:** BLANK; **Lane 5:** *EcoRI/HindIII* digested MOS1 genomic DNA; **Lane 6:** *EcoRI/HindIII* digested MOS1 genomic DNA following purification by ethanol precipitation (2.3.4). **b) *EcoRI* digested pUC19 used as a ligation control** **Lane 1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** undigested pUC19 **Lane 3:** *EcoRI* digested pUC19; **Lane 4:** *EcoRI* digested pUC19 following agarose gel purification (2.3.3). Marker sizes are indicated and the arrow indicates the 2 686 bp product of linear pUC19.

A product of the expected size, 2 686 bp, was obtained for linear pUC19 (Figure; 3.10a; Lane 3 and Figure 3.10b; Lane 4). Undigested pUC19 (Figure 3.10b; Lane 2) was included to check the

efficiency of digestion. The undigested pUC19 (Figure 3.10b; Lane 2) is circular and supercoiled and therefore migrates faster than the linear pUC19 on an agarose gel.

Following ligation (2.4), competent cells were transformed in the absence of any plasmid to ensure that they were not contaminated with pUC19. The efficiency of the ligase enzyme was determined by transforming competent cells with religated pUC19 previously digested with *EcoRI* (Figure 3.10b). To ensure the digestion of pUC19, apart from analysis on an agarose gel (Figure 3.10a; Lanes 3 and 4), competent cells were transformed with linear pUC19. Following transformation of the ligation mix into *E. coli* JM109 competent cells (2.5), recombinants were selected for on agar containing ampicillin, X-gal and IPTG (2.5; Table 3.2).

Table 3.2. *E. coli* JM109 transformants obtained using pUC19 and MOS1

| Sample transformed | Colonies |
|--|-----------------|
| No DNA control | 0 |
| Undigested pUC19 | 906 |
| Religated pUC19 | 148 |
| Linear pUC19 | 0 |
| pUC19 (<i>EcoRI/HindIII</i>) + MOS1 (<i>EcoRI/HindIII</i>) | 423 |

No recombinants were obtained from the no DNA control following transformation of competent cells in the absence of pUC19 (Table 3.2) indicating that the competent cells were not contaminated. The competent cells used in this study had a transformation efficiency of 9.06×10^8 cfu/ μ g (Table 3.2) and therefore appropriate for use in this cloning procedure. A transformation efficiency of 1.48×10^8 cfu/ μ g was obtained from the ligase efficiency control (Table 3.2), indicating the efficiency of the ligase enzyme to religate digested DNA. There were no colonies obtained following transformation of the *E. coli* JM109 competent cells with linear

pUC19 (Table 3.2), indicating complete digestion of the cloning vector. A total of 423 colonies were obtained from the MOS1 *EcoRI/HindIII* genomic DNA library (Table 3.2).

To screen the genomic library, the primers IS1133F2 and IS1133F5 (Jacobson, 2007; Table 2.3; Figure 3.11) were used in a PCR assay (2.6) on DNA obtained from pools of 10 recombinants (2.2). The primers IS1133F2 and IS1133F5 are complementary to sequences in the IS1133 portion that harbours the left inverted repeat.

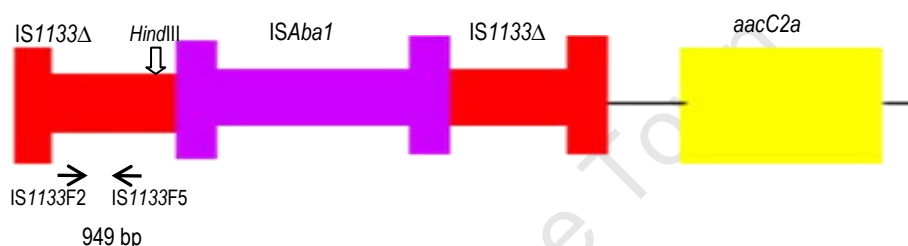


Figure 3.11: Schematic representation of the conserved genetic arrangement in *A. baumannii* strain MOS1. IS1133 (red) is interrupted by a full copy of ISAbal-1 (purple); this genetic arrangement lies upstream of an aminoglycoside resistance gene, aacC2a (yellow). A HindIII site (open arrow) lies downstream of the IS1133F5 primer. IS1133F2 and IS1133F5 primers are indicated by arrows. A 949 bp product is obtained in a PCR assay using the indicated primers. (Adapted from Jacobson, 2007).

PCR products were analysed by agarose gel electrophoresis (2.3.2; Figure 3.12). Recombinants with a positive PCR product would harbour the left inverted repeat of IS1133 and sequences flanking this region. A no DNA control was included to detect contamination. *E. coli* JM109 (pUC19), was included to ensure that IS1133F2 and IS1133F5 primers were not complementary to sequences within the *E. coli* JM109 genome or pUC19.

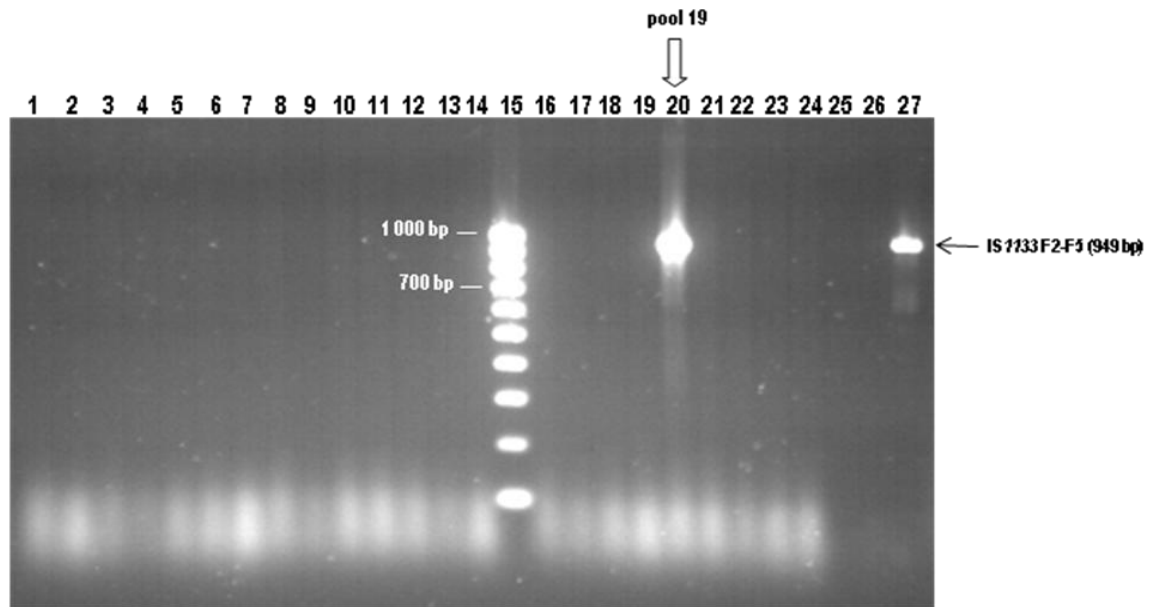


Figure 3.12: Agarose gel electrophoresis following PCR amplification of a portion of IS1133 in MOS1 EcoRI/HindIII recombinant clones. **Lane 15:** Hyperladder IV (Bioline; Appendix C). Genomic DNA extracted from transformants in pools 1-14 (**Lanes 1-14**) and pools 15-23 (**Lanes 15-24**) were included. **Lane 25:** *E. coli* JM109 (pUC19); **Lane 26:** no DNA control; **Lane 27:** MOS1. The open arrow corresponds to pool 19 in which a positive result was obtained. The closed arrow indicates the size of the product corresponding to the left hand portion of IS1133 (949 bp) and relevant marker sizes are indicated.

No product was obtained from the no DNA control (Figure 3.12; Lane 26), indicating that the PCR reagents used in this assay were not contaminated. No product was obtained from the *E. coli* JM109 (pUC19) negative control (Figure 3.12; Lane 25), indicating that there are no regions complementary to the left hand portion of IS1133 in *E. coli* JM109 or pUC19. A product of the expected size (949 bp) corresponding to the left hand portion of IS1133, was obtained from the positive control, MOS1 (Figure 3.12; Lane 27). Of the 423 transformants screened, only pool 19 (clones 181-190) yielded a 949 bp product (Figure 3.12; Lane 20).

To determine which of the ten recombinant colonies included in pool 19 resulted in the PCR product obtained above, PCR assays using primers IS1133F2 and IS1133F5 (Table 2.3; Figure

3.10) were carried out on the individual colonies in pool 19 (Figure 3.13). MOS2 was included as a negative control as it does not harbor the insertion sequence IS1133 (Jacobson, 2007).

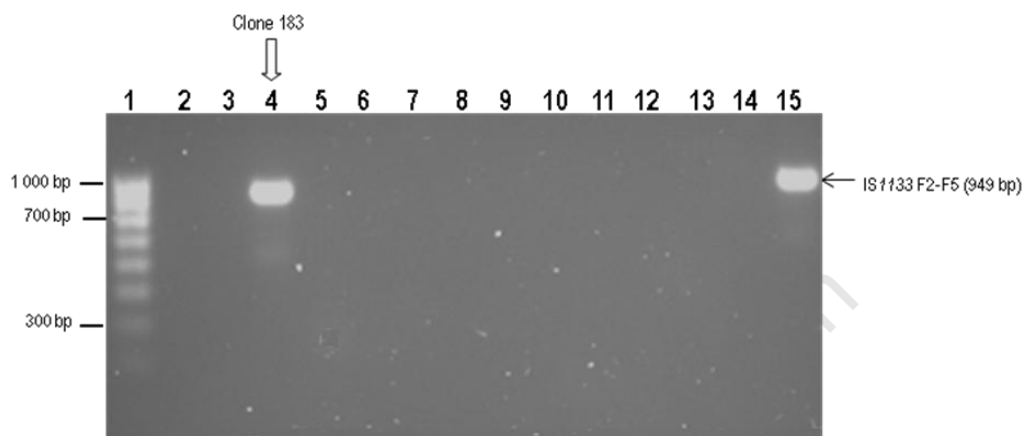


Figure 3.13: Agarose gel electrophoresis following PCR amplification of a portion of IS1133 from recombinant clones in pool 19. **Lane 1:** Hyperladder IV (Biolone; Appendix C). Genomic DNA extracted from transformants 181-190 (**Lanes 2-11**) were included. **Lane 12:** *E. coli* JM109 (pUC19); **Lane 13:** no DNA control; **Lane 14:** MOS2; **Lane 15:** MOS1. The open arrow corresponds to clone 183 in which a positive result was obtained. The closed arrow indicates the size of the product corresponding to the left hand portion of IS1133 (949 bp) and relevant marker sizes are indicated.

No product was obtained from *E. coli* JM109 (pUC19) (Figure 3.13; Lane 12), no DNA control (Figure 3.13; Lane 13) or MOS2 (Figure 3.13; Lane 14). Thus, the PCR reagents used in this assay were not contaminated and there are no sequences complementary to the primers IS1133F2 and IS1133F5 in MOS2 or *E. coli* JM109 (pUC19). A product of the expected size (949 bp) was obtained from the positive control, MOS1 (Figure 3.13; Lane 15). Only one colony (clone 183) gave a 949 bp product corresponding to the presence of the left hand portion of IS1133. The plasmid from clone 183, designated pTJ001 (Appendix B), was extracted (2.2.2) and digested with *Eco*RI and *Hind*III (2.3.1) to determine the size of the insert (Figure 3.14). The plasmid pUC19 was similarly digested to ensure pTJ001 was a pUC19 recombinant.

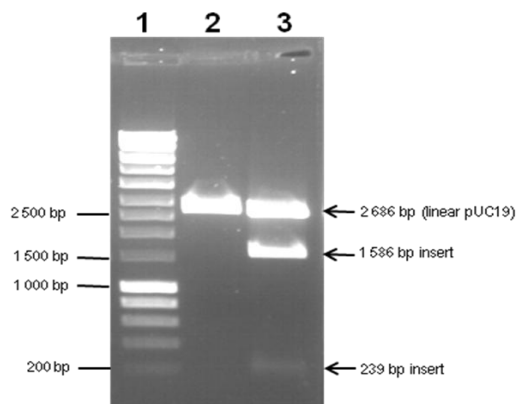


Figure 3.14: Agarose gel electrophoresis of pTJ001 digested with *EcoRI* and *HindIII*. **Lane 1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** *EcoRI/HindIII* digested pUC19; **Lane 3:** *EcoRI/HindIII* digested pTJ001. The arrows indicate fragments of pTJ001. Marker sizes are indicated.

Inserts of 1 586 bp and 239 bp were obtained (Figure 3.14) following digestion of pTJ001 with *EcoRI* and *HindIII*. Since the IS1133F2 -IS1133F5 PCR gives a 949 bp product it was likely that the 1 586 bp, not the 239 bp, insert contained a portion of IS1133. The 1 586 bp insert was therefore extracted (Figure 3.15) and purified (2.3.3).

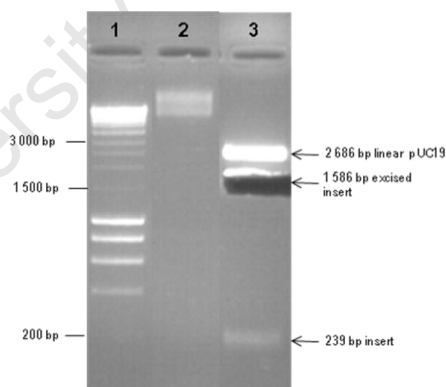


Figure 3.15: Agarose gel extracted 1 586 bp fragment from an *EcoRI/HindIII* digested pTJ001 obtained from the recombinant clone 183. **Lane1:** Hyperladder 1 (Bioline; Appendix C); **Lane 2:** undigested pTJ001; **Lane 3:** *EcoRI/HindIII* – digested pTJ001 with the 1 586 bp insert excised. The arrows indicate linear pUC19 (2 686 bp) and the two inserts excised from pTJ001 (1 586 and 200 bp).The relevant marker sizes are indicated.

Undigested pTJ001 (Figure 3.15; Lane 2) was included to check the efficiency of digestion. The undigested pTJ001 is in an open circular conformation and therefore migrates slower than the linear pUC19 on an agarose gel.

An IS1133F2 -IS1133F5 PCR (Table 2.3; Figure 3.10) was performed on the gel extracted insert to confirm the presence of the left hand portion of IS1133 on this fragment. The plasmid pUC19 was included as a negative control and pTJ001 as a positive control (Figure 3.16).

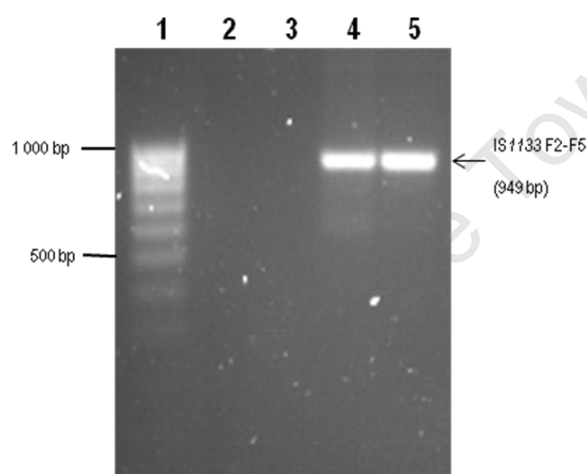


Figure 3.16: Agarose gel electrophoresis of IS1133F2 -IS1133F5 PCR on a 1 586 bp fragment obtained from an *EcoRI/HindIII* digested pTJ001. **Lane 1:** Hyperladder IV (Bioline; Appendix C); **Lane 2:** no DNA control; **Lane 3:** pUC19; **Lane 4:** pTJ001; **Lane 5:** gel extracted 1 586 bp fragment. The arrow indicates the expected product size of 949 bp. The marker sizes are indicated

No product was obtained from the no DNA control nor from pUC19 (Figure 3.16; Lane 2 and 3). A product of the expected size (949 bp) was obtained from the positive control, pTJ001 (Figure 3.16; Lane 4). A 949 bp product was obtained from the gel purified 1 586 bp *EcoRI/HindIII* fragment from pTJ001 (Figure 3.16; Lane 5), indicating that it carried a portion of IS1133.

The recombinant plasmid pTJ001 (Figure 3.17) was sequenced on both strands (2.7) using the primers M13F (5'-GTA AAA CGA CGG CCA GT-3'; Figure 3.17), M13R (Table 2.3; Figure 3.17), IS1133F2 and IS1133F5 (Table 2.3). M13F and M13R are pUC19-specific primers that lie just outside the multiple cloning site (MCS; Appendix B). Following analysis of the sequencing data obtained from pTJ001, it was determined that pTJ001 contained a concatemer of three inserts (1 586 bp, 239 bp, and 125 bp) in the MCS of pUC19.

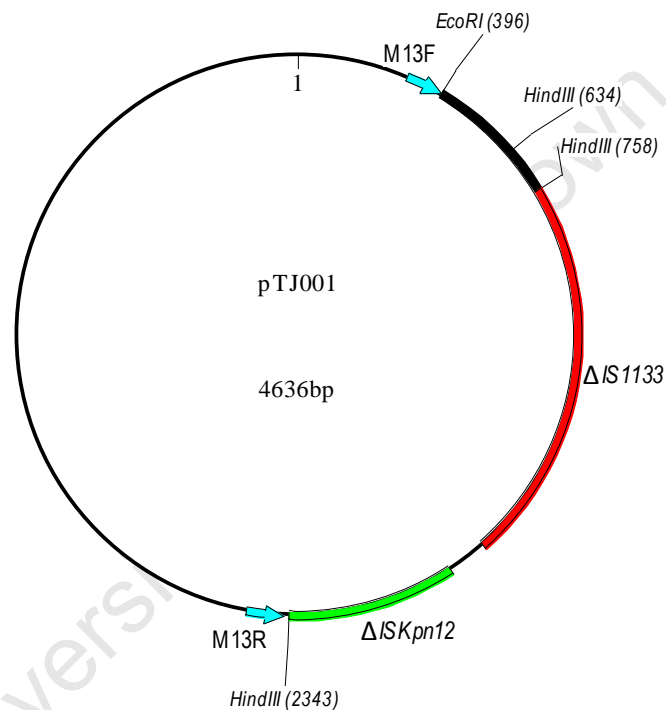


Figure 3.17: Schematic representation of pTJ001. A 1 586 bp *HindIII* fragment obtained from a MOS1 *EcoRI/HindIII* genomic DNA library is indicated in red and green. A **partial ISKpn12** gene (green) lies 108 bp downstream of an **IS1133 portion** (red). Two **other inserts on pTJ001** are represented as a black block. The pUC19-specific primers **M13F** and **M13R** are indicated in blue.

The 125 bp insert was not visible on the agarose gel (Figure 3.14, Lane 3; Figure 3.15, Lane 3) as it may have migrated off the end of the gel since it is a small fragment that would migrate faster on a 1 % agarose gel. The 239 bp and 125 bp inserts were homologous to *A. baumannii* genomic DNA encoding a hypothetical protein and a conserved region in a ferredoxin-dependent glutamase synthase protein, respectively, and are not associated with the *aacC2a*-associated genetic arrangement.

Analysis of the 1 586 bp insert of pTJ001 indicated 558 bp upstream of IS1133 (Figure 3.18). This region contained an ORF of 351 bp that showed 100 % homology to the recently published sequence of IS*Kpn12 tnpA* (Márquez *et al.*, 2008). To complete the sequence on both strands, primers complementary to the *tnpA*, IS*Kpn12F* and IS*Kpn12R* (Table 2.3; Figure 3.18), were designed and used for sequencing pTJ001.

IS*Kpn12* is an 842 bp insertion sequence (Márquez *et al.*, 2008) that belongs to group IS427 of the IS5 family of IS elements (Table 1.2; Mahillon & Chandler, 1998). IS*Kpn12* is flanked by a 10 bp inverted repeat and contains an ORF of 351 bp that encodes its *tnpA* (Figure 3.18). The presence of a full copy of IS*Kpn12* in *A. baumannii* MOS2 was investigated.

Figure 3.18. n.t seq of 1 586 bp fragment

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Figure 3.18. n.t seq of 1 586 bp fragmnt

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3.3 Characterisation of ISKpn12 in *A. baumannii* MOS1

To determine whether a full copy of ISKpn12 is present in MOS1, primers complementary to ISKpn12 (Marquez *et al.*, 2008) were designed. ISKpn12F (Table 2.3, Figure 3.18) is complementary to sequences in the ISKpn12 *tnpA*. ISKpn12R3 (Table 2.3, Figure 3.18) is complementary to the right-most end of ISKpn12 and includes part of the right inverted repeat. A PCR assay (2.6) was carried out using MOS1 and MOS2 genomic DNA as template. A no DNA control was included to detect the presence of contamination in the reagents used for the assay. Following PCR amplification, the amplicons were analysed by agarose gel electrophoresis (2.3.2; Figure 3.19).

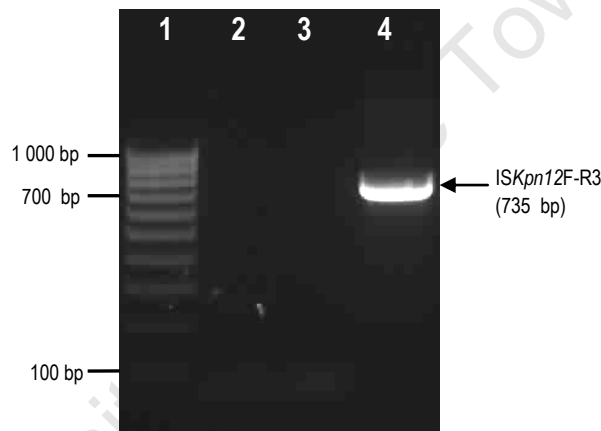


Figure 3.19: Agarose gel electrophoresis of amplicons following an ISKpn12F-ISKpn12R3 PCR. **Lane 1:** Hyperladder IV (Bioline; Appendix C); **Lane 2:** no DNA control; **Lane 3:** MOS2; **Lane 4:** MOS1. The arrow indicates the expected product size of 735 bp and relevant marker sizes are indicated.

No product was obtained from the no DNA control (Figure 3.19; Lane 2) or from MOS2 (Figure 3.19; Lane 3), indicating that this element is not present in MOS2. A 735 bp product was obtained from MOS1 (Figure 3.19; Lane 4) suggesting the presence of a complete copy of ISKpn12 in this *A. baumannii* strain. The PCR product was therefore extracted from the agarose gel (2.3.3) and sequenced (2.7) on both strands (Figure 3.20) to confirm that it corresponds to ISKpn12.

Figure 3.20: alignment of Kpn12 with MOS1

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Figure 3.20: alignment of Kpn12 with MOS1 2 pages...

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Analysis of the sequencing data obtained (Figure 3.20) indicated the presence of a full copy of *ISKpn12* in MOS1. Thus, MOS1 has a full copy of *ISKpn12* upstream of the *aacC2a*-associated conserved genetic arrangement.

ISKpn12 has only been described in an MDR *K. pneumoniae* strain 12836 from Uruguay, South America (Figure 3.21a; Márquez *et al.*, 2008). This is the first study that describes *ISKpn12* in *A.baumannii*. Interestingly, this element was part of a plasmid in an *A. baumannii* isolate, 7037 (Figure 3.21b; Poirel *et al.*, 2007), however, *ISKpn12* was not described in that study. The sequence containing *ISKpn12* was obtained when a novel β -lactamase, *bla*_{SCO-1} was identified in *A. baumannii* 7037.

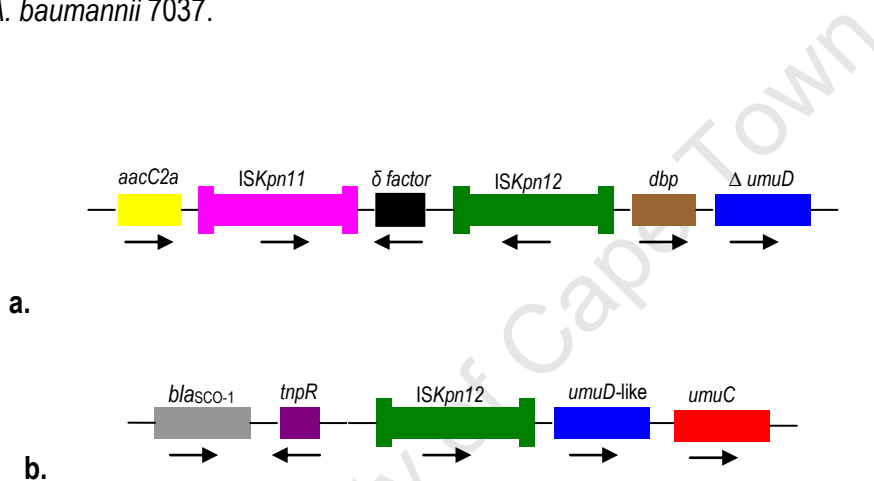


Figure 3.21: Schematic representation of the genetic environment of *ISKpn12* in **a)** *K. pneumoniae* 12836. *aacC2a*, aminoglycoside resistance gene; insertion sequence element, *ISKpn11*; δ , RNA polymerase sigma factor; insertion sequence element, *ISKpn12*; *dbp*, DNA-binding protein; Δ *umuD*, partial UV mutagenesis-encoding gene (Adapted from Márquez *et al.*, 2008); **b)** *A. baumannii* 7037. *bla*_{SCO-1}, β -lactamase resistance gene; *tnpR*, Tn21-related sequence; insertion sequence element, *ISKpn12*; *umuD*-like, gene encoding a UV mutagenesis-like protein; *umuC*, UV mutagenesis-encoding gene (Adapted from Poirel *et al.*, 2007). The direction of transcription is indicated by arrows.

Some IS elements like *ISAbal-1* in *Acinetobacter* (Segal *et al.*, 2005) and *IS2000* in *K. pneumoniae* (Aubert *et al.*, 2003) have only been described in one bacterial genera or species, suggesting that these elements are unique to these species. The identification of

ISKpn12 in *A. baumannii* MOS1 and *A. baumannii* 7037, and in *K. pneumoniae* 12836 suggests that this IS element is not confined to a particular species or genus, but rather that it is shared between different genera. IS elements may become specific to certain organisms if they are located on plasmids that are not transferable across bacterial species or genera (Segal *et al.*, 2005; Snyder & Champness, 2002). The identification of ISKpn12 in *K. pneumoniae* and *A. baumannii* suggests that this element may have been transferred on a plasmid that can replicate in both *K. pneumoniae* and *A. baumannii* and points at *K. pneumoniae* as a source of this element in *A. baumannii* (Segal & Elisha 1999).

Interestingly, *A. baumannii* 7037 that harboured ISKpn12 was also isolated from a patient in South America. IS elements including, IS1542 (UK/Ireland; Darini *et al.*, 1999), IS1251 (USA; Mackinnon *et al.*, 1997), and IS1476 (Canada; Mackinnon *et al.*, 1997), have been obtained from bacterial isolates contained within particular geographical areas. Our detection of ISKpn12 in an *A. baumannii* isolate from South Africa indicates that this mobile genetic element is not confined to South America or *K. pneumoniae*.

3.4 Prevalence and detection of ISKpn12 in *A. baumannii* clinical isolates and other clinically significant pathogens

To determine the prevalence of ISKpn12 in *A. baumannii*, a PCR assay (2.6) using ISKpn12F and ISKpn12R primers (Table 2.3; Figure 3.20) was carried out on genomic DNA from *A. baumannii* isolates obtained from hospitals in Cape Town (Table 2.1). MOS2 genomic DNA and a no DNA PCR assay were included as negative controls. MOS1 genomic DNA was included as a positive control. Following PCR amplification, the PCR products were analysed on a 2 % agarose gel (2.3.2; Figure 3.22).

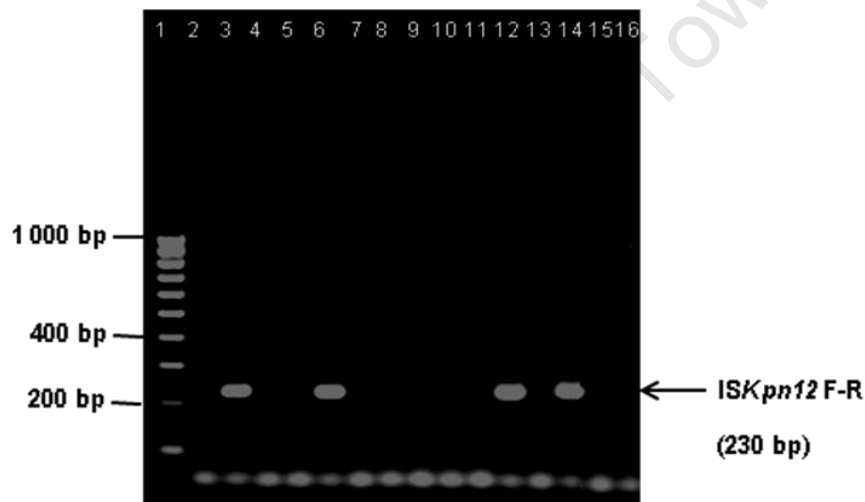


Figure 3.22: PCR amplicons following an ISKpn12F-R PCR on *A. baumannii* clinical isolates. **Lane 1:** sHyperladder IV (Bioline; Appendix C); **Lane 2:** A1; **Lane 3:** A2; **Lane 4:** A3; **Lane 5:** A4; **Lane 6:** A5; **Lane 7:** A7; **Lane 8:** A9; **Lane 9:** A10; **Lane 10:** A19; **Lane 11:** A40; **Lane 12:** A91; **Lane 13:** 794; **Lane 14:** MOS1; **Lane 15:** MOS2; **Lane 16:** no DNA control. A 230 bp product (arrow) indicates a positive result. Relevant marker sizes are indicated.

No amplicons were obtained from the no DNA control (Figure 3.22; Lane 16) and the negative control, MOS2 (Figure 3.22; Lane 15). A product of 230 bp, corresponding to ISKpn12, was obtained from the positive control, MOS1 (Figure 3.22; Lane 14). Similarly, 230 bp products were obtained from strains A2, A5, and A91 (Figure 3.22; Lanes 3, 6, and 12 respectively) indicating

the presence of the IS*Kpn12 tnpA* in these isolates. No 230 bp products were obtained for strains A3, A4, A7, A9, A10, A19, A40, and 794 (Figure 3.22; Lanes 4, 5, 7, 8, 9, 10, 11, and 13, respectively), indicating that these strains do not harbour IS*Kpn12*.

A more recent *A. baumannii* isolate, 794, was included in this assay. This isolate was obtained in 2008 from a patient at Red Cross War Memorial Children's Hospital and is resistant to all clinically useful antibiotics (Table 2.1) except colistin. The use of aminoglycosides to treat *A. baumannii* infections has decreased in hospitals. This is due to the increase of MDR isolates that can only be inhibited by other antibiotics apart from aminoglycosides. Reduced use of aminoglycosides to treat *A. baumannii* infections may be associated with the subsequent loss of aminoglycoside resistance genes (Jacobson, 2007). The absence of a 230 bp product from *A. baumannii* 794 (Figure 3.22; Lane 13) indicates the absence of the IS*Kpn12*-IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* conserved genetic arrangement. This genetic arrangement may have been lost by recent *A. baumannii* isolates due to the reduced use of aminoglycosides at hospitals in Cape Town (Jacobson, 2007).

Interestingly, isolates A2, A5, and A91 had a 230 bp PCR product (Figure 3.22). These isolates contain the IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* arrangement (Figure 3.1) and belong to the same PFGE profile as MOS1 (Jacobson, 2007; personal communication from H. Segal). The presence of a 230 bp PCR product in these isolates provides further evidence that IS*Kpn12* is part of the genetic arrangement that has been conserved since 1983 in *A. baumannii* clinical isolates from Cape Town.

The observed results indicate that a lineage of *A. baumannii* isolates from hospitals in Cape Town harbours an IS*Kpn12*-IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a*. This genetic arrangement may be responsible for resistance to gentamicin in these *A. baumannii* isolates. The absence of this genetic arrangement in MOS2 and in the more recently obtained isolate 794 suggests the use of alternative aminoglycoside-resistance mechanisms (Table 1.1; Bergogne-Berezin & Towner, 1996) like overexpression of the AdeABC multidrug efflux pump (1.3.2; Magnet *et al.*, 2001), methylation of the 16S rRNA (1.3.4; Lee *et al.*, 2006), or a combination of these mechanisms.

K. pneumoniae has a 56–58 % GC content (Murray *et al.*, 1984). The G + C content of IS*Kpn12* is 57 % as determined by sequence analysis (2.7), indicating its likely source to be *K. pneumoniae*. This value differs significantly from that of the *A. baumannii* core genome, which is 39 % (Fournier *et al.*, 2006). Foreign DNA acquired by *A. baumannii* can be determined by a deviation from the 39 % G + C content of the core genome (Fournier *et al.*, 2006; Schmidt & Hensel, 2004). The observed difference in the G + C content suggests that *A. baumannii* MOS1 may have acquired IS*Kpn12* from other pathogens, particularly *K. pneumoniae*. The presence of IS*Kpn12* in other pathogens was therefore investigated.

Genomic DNA (provided by H. Segal) from representative isolates of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *C. jejuni* (Table 2.2) isolated from patients from Cape Town hospitals were screened for the presence of IS*Kpn12*. The environmental isolates *A. lwoffii* and *A. calcoaceticus* (Table 2.2) were also included to determine the presence of this element in representative isolates from the genus *Acinetobacter*. A PCR assay (2.6) using 16S universal primers 16SF-16SR (Table 2.3) was carried out to determine the integrity of the DNA. MOS1 was included as a positive control. A no DNA control was included to determine the presence of contamination. Following PCR amplification, the amplicons were analysed by agarose gel electrophoresis (2.3.2; Figure 3.23).

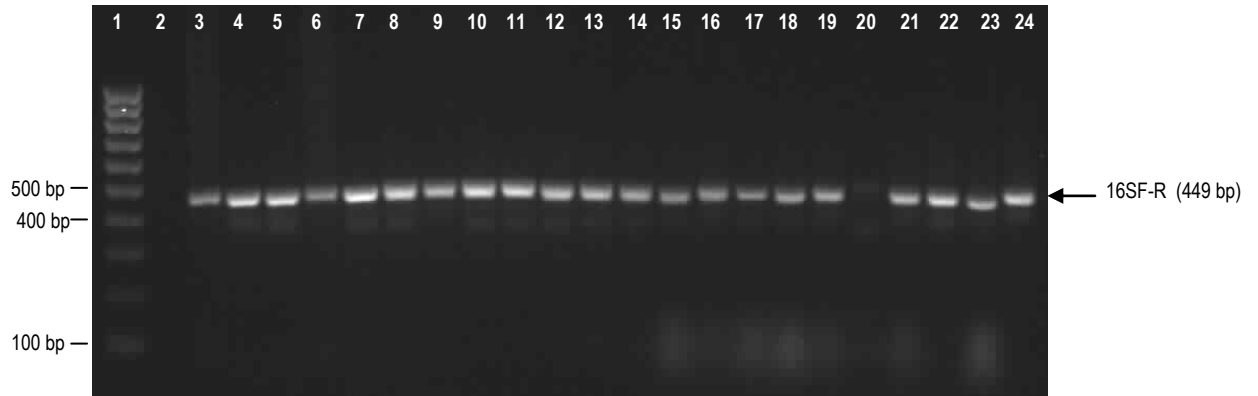


Figure 3.23: Agarose gel electrophoresis photo of 16S PCR-amplified genomic DNA from bacteria from various genera **Lane 1;** Hyperladder IV (Bioline; Appendix C); **Lane 2:** no DNA control; **Lane 3-6:** *E. coli*; **Lane 7-14:** *P. aeruginosa*; **Lane 15-19:** *K. pneumoniae*; **Lane 20:** *Campylobacter jejuni*; **Lane 21:** *A. Iwoffii*; **Lane 22-23:** *A. calcoaceticus*; **Lane 24:** MOS1. A 449 bp product (arrow) indicates a positive result. The relevant marker sizes are indicated.

No product was obtained from the no DNA control (Figure 3.23; Lane 2), indicating the absence of contamination in the reagents used for this assay. A product of 449 bp, corresponding to 16S rRNA, was obtained from the positive control, MOS1 (Figure 3.23; Lane 24). Similarly, 449 bp products were obtained from all isolates screened for the presence of 16S rRNA, except for *C. jejuni* (Figure 3.23; Lane 20), indicating that the quality of the DNA was good enough for use in PCR assays. All samples, including *C. jejuni*, were included in a PCR assay (2.6) to screen for the presence of IS*Kpn12*.

Genomic DNA from MOS1 and A91 were included as positive controls as they both contain IS*Kpn12*. A no DNA control and MOS2 genomic DNA were included as negative controls. Following PCR amplification using IS*Kpn12*F and IS*Kpn12*R primers (Table 2.3), the amplicons were analysed on a 2 % agarose gel (2.3.2; Figure 3.24).

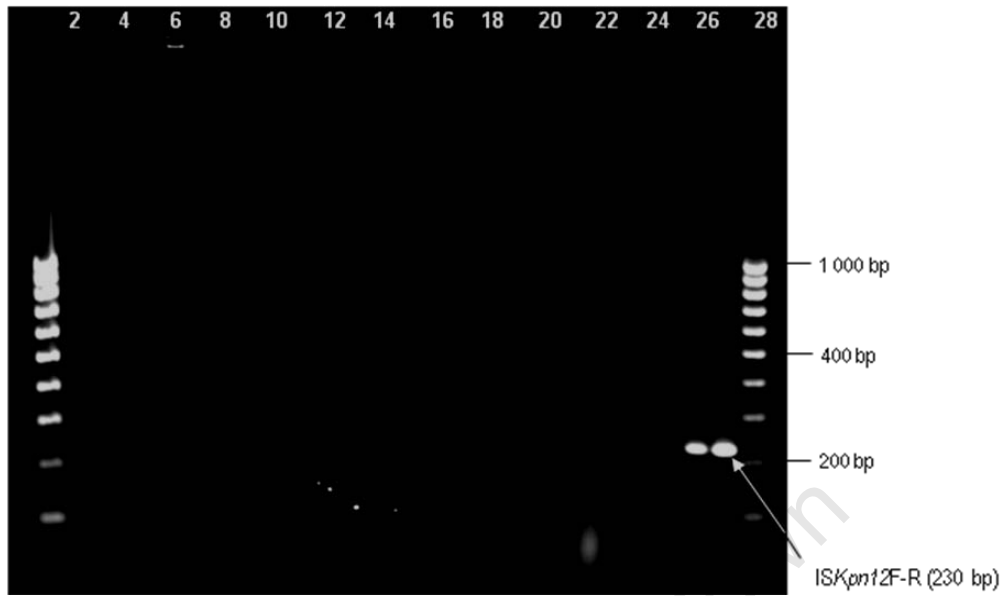


Figure 3.24: Agarose gel electrophoresis following PCR amplification of *ISKpn12 tnpA* on pathogenic isolates from various genera. **Lane 1;** Hyperladder IV (Bioline; Appendix C); **Lane 2;** no DNA control; **Lane 3-6;** *E. coli*; **Lane 7-14;** *P. aeruginosa*; **Lane 15-19;** *K. pneumoniae*; **Lane 20;** *Campylobacter jejuni*; **Lane 21;** *A. lwoffii*; **Lane 22-23;** *A. calcoaceticus*; **Lane 24;** MOS2; **Lane 25;** BLANK; **Lane 26;** MOS1. ; **Lane 27;** A91; **28;** Hyperladder IV (Bioline; Appendix C). A 230 bp product (arrow) indicates a positive result. The relevant marker sizes are indicated.

No amplicons were obtained from the no DNA control (Figure 3.24; Lane 2) and the negative control, MOS2 (Figure 3.24; Lane 24). A product of 230 bp, corresponding to *ISKpn12*, was obtained from the positive controls MOS1 and A91 (Figure 3.24; Lanes 26 and 27, respectively). A product of the expected size was not detected in *E. coli* (Figure 3.24; Lanes 3-6), *P. aeruginosa* (Figure 3.24; Lanes 7-14), *K. pneumoniae* (Figure 3.24; Lanes 15-19), *C. jejuni* (Figure 3.24; Lane 20), *A. lwoffii* (Figure 3.24; Lane 21), or *A. calcoaceticus* (Figure 3.24; Lanes 22-23). The results obtained suggest that neither of the isolates included, except for *C. jejuni*, contain *ISKpn12*. The absence of a 230 bp PCR product from *C. jejuni* (Figure 3.24; Lane 20) as well as from the 16S rRNA PCR assay (Figure 3.23; Lane 20) suggests that the DNA from this isolate was degraded, therefore, no conclusion can be made about this element in *C. jejuni*.

The first description of ISKpn12 was in a *K. pneumoniae* isolate (Figure 3.21a; Márquez *et al.*, 2008). The *K. pneumoniae* isolates screened in this study did not contain ISKpn12 (Figure 3.24; Lanes 15-19). *K. pneumoniae* may not be the reservoir for this element in our hospital setting or ISKpn12 may have been lost by horizontal gene transfer from the *K. pneumoniae* strains included in this study. However, only five *K. pneumoniae* isolates were screened for the presence of ISKpn12 in this study. It would be necessary to screen a larger population of *K. pneumoniae* isolates to determine if this pathogen might be the reservoir of ISKpn12 in our setting.

An *A. baumannii* isolate 7037 (Poirel *et al.*, 2007) contains ISKpn12 (Figure 3.21b). This isolate was obtained from a patient in a South American hospital, the same geographical region *K. pneumoniae* 12836 was obtained from. Both *K. pneumoniae* and *A. baumannii* are soil organisms and nosocomial pathogens (Bouvet & Grimont, 1986; Brown & Seidler, 1973; Glew *et al.*, 1977). It may be that in South America, ISKpn12 was transferred between *K. pneumoniae* and *A. baumannii* by horizontal gene transfer either in the soil or the hospital environment. The source of ISKpn12, however, remains to be determined.

That ISKpn12 is present in *A. baumannii* MOS1 and related strains but absent in *A. baumannii* MOS2 and related strains suggests that this element is part of the conserved genetic arrangement associated with *aacC2a* in *A. baumannii*. It is interesting to speculate about the role of ISKpn12 in the *aacC2a*-associated conserved genetic arrangement. IS elements often provide promoter sequences for antibiotic-conferring resistance genes (Corvec *et al.*, 2003; Depardieu *et al.*, 2007; Segal *et al.*, 2005). Since ISKpn12 lies upstream of the *aacC2a* antibiotic resistance gene, it may be that this IS element plays a role in the expression of *aacC2a*. However, this is unlikely as ISKpn12 is 2 645 bp upstream of the *aacC2a* gene. It is more likely that ISAbA-1, previously shown to provide promoter sequences for a number of genes, including *phaB_{AC}* (Schembri *et al.*, 1995), *bla_{OXA-23}* (Donald *et al.*, 2000; Segal *et al.*, 2007), *bla_{OXA-27}* (Afzal-Shah *et al.*, 2001), and *ampC* (Corvec *et al.*, 2003; Segal *et al.*, 2004), is the element that regulates expression of *aacC2a* in MOS1 and related strains. Primer extension analysis experiments are underway in our laboratory to detect the promoter sequences for *aacC2a* in MOS1. ISKpn12 may

play a role in stabilising the IS*Kpn12*-IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* conserved genetic arrangement in MOS1. An increased copy number of IS elements in bacterial cells suggests mobility, as determined by Segal and colleagues for IS*Aba-1* (2004). The copy number of IS*Kpn12* in MOS1 was therefore investigated.

3.5 Investigation of the role of IS*Kpn12* in *A. baumannii* strains MOS1

3.5.1 Detection of IS*Kpn12* copy number in *A. baumannii* strain MOS1

The role of IS*Kpn12* in relation to the maintenance of the IS*Kpn12*-IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* genetic arrangement in MOS1 was investigated. A Southern hybridisation (2.8) was carried out on *Eco*RI-digested MOS1 genomic DNA. Importantly, *Eco*RI does not cleave within the IS*Kpn12* nucleotide sequence. *Eco*RI-digested MOS2 genomic DNA was included as a negative control. The digested genomic DNA was electrophoresed on an agarose gel (2.3.2; Figure 3.25a) and transferred to a nitrocellulose membrane (2.8). The labeled IS*Kpn12*F-R probe (2.8.1) used for this assay was obtained following PCR amplification using MOS1 genomic DNA as template. An agarose gel extracted (2.3.3) IS*Kpn12*F-R PCR product obtained from MOS1 was included as a positive control.

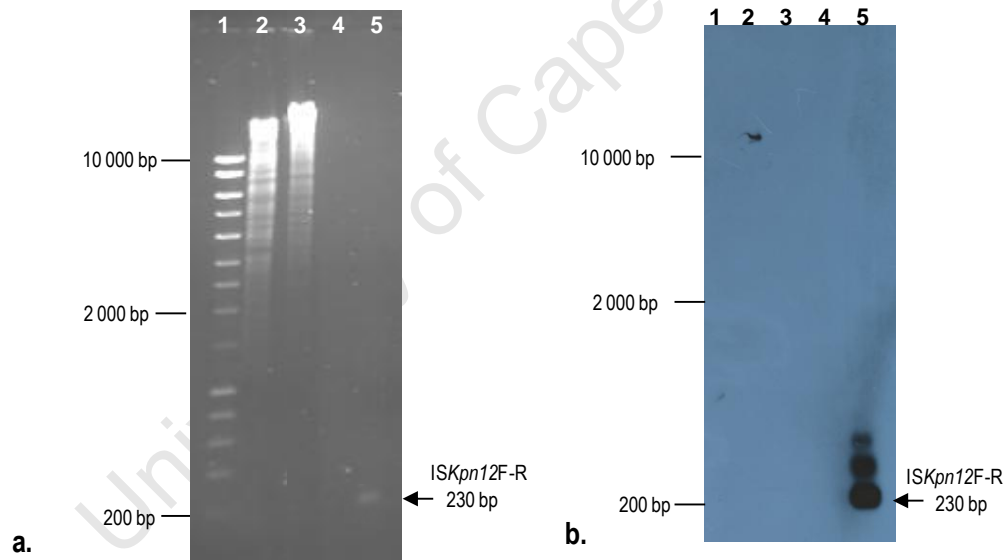


Figure 3.25: Autoradiograph displaying IS*Kpn12* copy number in *A. baumannii* MOS1. **a)** Agarose gel electrophoresis of *Eco*RI digested genomic DNA. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** MOS2; **Lane 4:** BLANK; **Lane 5:** IS*Kpn12*F-R PCR product. **b)** Autoradiograph of IS*Kpn12*F-R probe hybridised to *Eco*RI digested *A. baumannii* strains MOS1 and MOS2. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** MOS2; **Lane 4:** BLANK **Lane 5:** IS*Kpn12*F-R PCR product. Relevant marker sizes are indicated and the 230 bp PCR product indicated by an arrow.

Following Southern hybridisation (2.8) with 300 ng of labeled probe (2.8.2; Figure 3.25b), no hybridisation signal was obtained from the negative control, MOS2 (Figure 3.25b; Lane 3), providing further evidence that this strain does not contain *ISKpn12*. A product of the expected size (230 bp) corresponding to the *ISKpn12F*-*ISKpn12R* (Figure 3.20) PCR product obtained from MOS1 yielded a signal (Figure 3.25a and b; Lane 5). Following an overnight exposure of the X-ray film, non-specific signals were detected from the PCR product (Figure 3.25b; Lane 5). This may be due to the probe binding to non-specifically amplified PCR products that are not visible on the agarose gel (Figure 3.25a; Lane 5). A positive hybridisation signal was detected from strain MOS1 (Figure 3.25b, Lane 2), corresponding to a fragment greater than 10 000 bp (Figure 3.25a and b, Lane 2), indicating that MOS1 contains at least one copy of *ISKpn12* on an *EcoRI* fragment greater than 10 000 bp. It may be that more than one *EcoRI* fragment of the same size harbours this IS element, resulting in only one hybridisation signal being observed (Figure 3.25b, Lane 2). The > 10 000 bp fragment may also contain more than one copy of *ISKpn12*.

To further determine the copy number of *ISKpn12* in MOS1, a Southern hybridisation (2.8) was carried out on *EcoRI/HindIII* digested MOS1 genomic DNA (Figure 3.26). A *HindIII* site is present within *ISKpn12* (Figure 3.20). A probe was generated using *ISKpn12F*-*ISKpn12R2* primers (Table 2.3; Figure 3.20) and MOS1 genomic DNA as template. Importantly, the *ISKpn12F*-*ISKpn12R2* probe spans the *HindIII* site in *ISKpn12* (Figure 3.20).

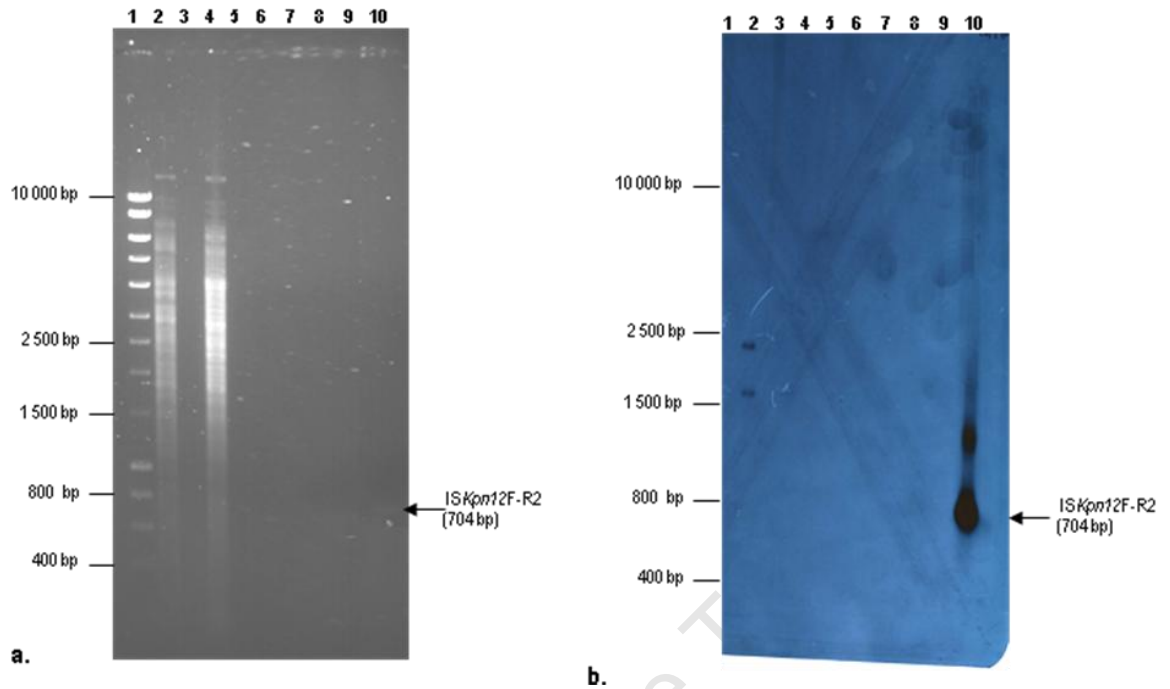


Figure 3.26: Autoradiograph of ISKpn12F-R2 probe hybridised to *EcoRI/HindIII* digested *A. baumannii* strains MOS1 and MOS2. **a)** *EcoRI/HindIII* digested genomic DNA. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** BLANK; **Lane 4:** MOS2; **Lane 5-9:** BLANK **Lane 10:** gel purified ISKpn12F-R2 PCR product. **b)** Autoradiograph of ISKpn12F-R2 probe hybridised to *EcoRI/HindIII* digested *A. baumannii* strains MOS1 and MOS2. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** BLANK; **Lane 4:** MOS2; **Lane 5-9:** BLANK **Lane 10:** gel purified ISKpn12F-R2 PCR product. Relevant marker sizes are indicated and the 704 bp product/signal indicated by an arrow.

No product was obtained from the negative control, strain MOS2 (Figure 26b; Lane 4) previously shown (Figure 3.19) not to contain a copy of ISKpn12. A 704 bp product corresponding to the region ISKpn12F- ISKpn12R2 was obtained from the PCR product from MOS1 (Figure 3.26a and b; Lane 10). Following an overnight exposure of the X-ray film, non-specific signals were detected (Figure 3.26b; Lane 10). This may be due to the probe binding to non-specifically amplified PCR products that are not visible on the agarose gel (Figure 3.26a; Lane 10). Two hybridisation signals of 1 586 kb and ~2 400 bp were detected from strain MOS1

(Figure 3.26b, Lane 2). *EcoRI/HindIII* digestion of MOS1 genomic DNA generated a 1 586 bp *HindIII* fragment (Figure 3.18), amongst many other fragments (Figure 3.26a; Lane 2). The IS*Kpn12*F-IS*Kpn12*R2 probe hybridised to the 1 586 bp *HindIII* fragment (Figure 3.26a and b; Lane 2). Another hybridisation signal was obtained on a fragment ~ 2 400 bp (Figure 3.26a and b; Lane 2). The two hybridisation signals obtained (Figure 3.26b; Lane 2) indicate the presence of one copy of IS*Kpn12* in MOS1. If the > 10 000bp fragment (Figure 3.25b; Lane 2) harboured more than one copy of IS*Kpn12*, then following digestion with *EcoRI* and *HindIII*, these elements would be on different sized fragments. This would have resulted in more than two signals being obtained with the *EcoRI/HindIII* Southern hybridisation (Figure 3.26b; Lane 2).

The presence of one copy of IS*Kpn12* suggests that this element is not mobile in the MOS1 genome. This suggests that IS*Kpn12* may not have a functional role in *A. baumannii*, even though it may be useful in *K. pneumoniae*. It is important to note that the preferred promoter sequences in *K. pneumoniae* and *E. coli* are not the preferred promoter sequences recognized by the *A. baumannii* RNA polymerase (Segal & Elisha, 1999). IS*Kpn12*, together with genes that may have been useful to MOS1 and *A. baumannii* 7037, may have therefore been acquired on one mobile genetic element. However, it was interesting to see if the *tnpA* of IS*Kpn12* was expressed in MOS1.

3.5.2 Expression of IS*Kpn12 tnpA* in *A. baumannii* MOS1

DNA sequence analysis (2.7) of pTJ001 (Figure 3.17) revealed the presence of IS*Kpn12* upstream of a portion of IS1133 in MOS1 (Figure 3.18; Figure 3.20). Southern hybridisation studies (2.8) revealed the presence of one copy of IS*Kpn12* in *A. baumannii* MOS1 (Figure 3.25; Figure 3.26). To determine whether the *tnpA* of IS*Kpn12* in MOS1 is expressed, reverse transcriptase PCR assays were carried out (2.10). RNA was extracted using the hot acid phenol method (2.9) from logarithmic and stationary phase cultures of strain MOS1 and MOS2. Any contaminating DNA was eliminated by DNase treatment (2.9).

Controls containing no cDNA and no RNA were included to detect for contamination in the reaction. The expression of the 16S rRNA gene was determined using 16S universal primers (Table 2.3) and was included as a positive control. Prokaryotic ribosomes have a 30S subunit made up of protein and 16S rRNA (Garret & Grisham, 2005). Expression of the 16S rRNA was included as a positive control for the presence of RNA since ribosomes are required for protein synthesis and are always present in bacterial cells. The primer IS*Kpn12R* (Table 2.3; Figure 3.20) anneals within the *tnpA* of IS*Kpn12* and was used for first strand synthesis of cDNA. The primers IS*Kpn12F* and IS*Kpn12R* (Table 2.3; Figure 3.20) were used for PCR amplification of *tnpA* from the reverse-transcribed cDNA. MOS2 does not contain IS*Kpn12* (Figure 3.19; Figure 3.25b; Figure 3.26b) and was used as a negative control, and MOS1 included as a positive control. Following reverse transcription and PCR amplification, the PCR products were analysed on a 2 % agarose gel (2.3.2; Figure 3.27).

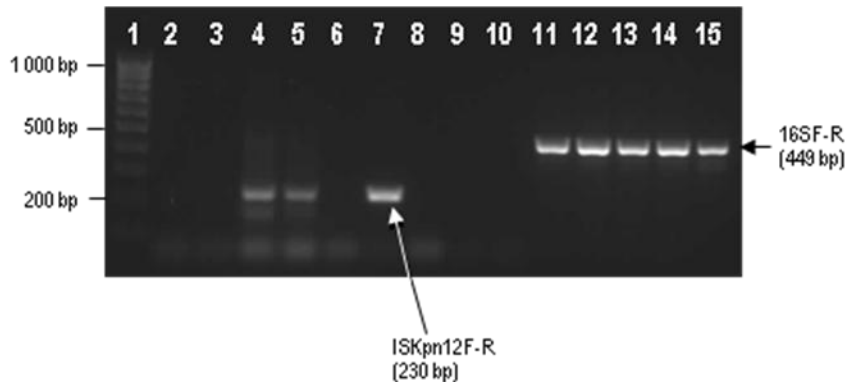


Figure 3.27: Agarose gel electrophoresis of amplicons obtained following reverse transcription PCR of *ISKpn12* and 16S rRNA in MOS1 and MOS2. **Lane 1:** Hyperladder IV (BioLine; Appendix C); **Lane 2:** no RNA control; **Lane 3:** no cDNA control; **Lane 4:** MOS1 logarithmic phase; **Lane 5:** MOS1 stationary phase; **Lane 6:** MOS2 logarithmic phase; **Lane 7:** MOS1 genomic DNA; **Lane 8:** MOS2 stationary phase; (**Lanes 9 – 15** display results for 16S Reverse transcriptase PCR); **Lane 9:** no RNA control; **Lane 10:** no cDNA control; **Lane 11:** MOS1 logarithmic phase; **Lane 12:** MOS1 stationary phase; **Lane 13:** MOS2 logarithmic phase; **Lane 14:** MOS2 stationary phase; **Lane 15:** MOS1 genomic DNA. Marker sizes are indicated. A 230 bp and 449 bp product corresponding to *ISKpn12 tnpA* and 16S PCR products, respectively are indicated with arrows.

No product was obtained from the no RNA (Figure 3.27; Lane 2 and 9) and no cDNA controls (Figure 3.27; Lane 3 and 10) indicating the absence of contamination in the reagents used in this assay. This result also indicates the absence of DNA contamination in the RNA used for reverse transcriptase PCR. An expected 449 bp product was obtained from MOS1 genomic DNA following amplification with the 16S universal primers (Figure 3.27; Lane 15). A product of 449 bp was obtained from both MOS1 and MOS2 (Figure 3.27; Lanes 11-14) following PCR amplification of the cDNA using 16S universal primers. Thus, the 16S rRNA was synthesised in the logarithmic and stationary phase cultures from both strains and the quality of the RNA was good enough to use in reverse transcriptase PCR assays to determine *ISKpn12 tnpA* expression.

An expected product (230 bp) corresponding to the *tnpA* of *ISKpn12* was obtained from MOS1 genomic DNA (Figure 3.27; Lane7). No product was obtained from the negative control, MOS2, (Figure 3.27; Lanes 6 and 8). A 230 bp product corresponding to *ISKpn12 tnpA* was obtained

from MOS1 cDNA synthesised from RNA obtained from both the logarithmic and stationary phase cultures (Figure 3.27; Lanes 4 and 5), indicating that *ISKpn12 tnpA* is expressed in both growth phases in MOS1. The PCR product obtained from the logarithmic phase culture (Figure 3.27; Lane 4) is brighter than that from the stationary phase culture (Figure 3.27; Lane 5). It may be that *ISKpn12* expression is different in the logarithmic and stationary growth phases. In the latter, an increased expression of *ISKpn12* would result in more RNA being obtained. To determine the latter, real time PCR assay could be carried out.

Unlike *ISKpn12*, *ISAbA-1* is present in *A. baumannii* isolates in multiple copies (Segal *et al.*, 2005). This element is unique to *Acinetobacter* spp. and is considered highly mobile (Segal *et al.*, 2005). Increased resistance to various antibiotics is achieved through *ISAbA-1* providing promoter sequences for expression of the antibiotic resistance genes (Schembri *et al.*, 1995; Donald *et al.*, 2000; Afzal-Shah *et al.*, 2001; Corvec *et al.*, 2003; Segal *et al.*, 2004 and 2007; Le Hello *et al.*, 2008; Niumsup *et al.*, 2009). *ISAbA-1* may be responsible for the plasticity observed in the *A. baumannii* genome (Segal *et al.*, 2005) by rearranging genes and contributing to the formation of transposons (Corvec *et al.*, 2007). Since *ISAbA-1* is associated with the *aacC2a*-associated conserved genetic arrangement in MOS1 (Figure 3.1), its role was investigated in MOS1 and MOS2.

3.6 Investigation of the role of IS*Aba-1* in *A. baumannii* strains MOS1 and MOS2

IS*Aba-1* is a 1 180 bp IS element that belongs to the IS4 family, according to the IS nomenclature proposed by Mahillon and Chandler (1998). This insertion sequence has only been described in *Acinetobacter* spp.. IS*Aba-1* encodes a transposase with similarity to a putative transposase from *Deinococcus radiodurans* (Segal *et al.*, 2003), a common soil organism. *Acinetobacter* spp. are also found in the soil and this might reflect the source of this insertion sequence (Segal *et al.*, 2003).

IS*Aba-1* is associated with a variety of antibiotic resistance genes, where it plays a role in the expression of these genes by providing alternative promoter sequences (Corvec *et al.*, 2003; Segal *et al.*, 2004; Le Hello *et al.*, 2008; Niumsup *et al.*, 2009). A copy of IS*Aba-1* interrupts IS1133 in the conserved genetic arrangement in *A. baumannii* MOS1 (Figure 3.1). Primers complementary to the IS*Aba-1* *tnpA*, HRR and HRF (Table 2.3; Figure 3.28), were used in a PCR assay to detect the presence of IS*Aba-1* in strain MOS2 (Jacobson, 2007).

Figure 3.28: Nucleotide sequence of ISAb1. The **inverted repeats** are underlined and in bold blue text. Highlighted in green are the primers HRF and HRR and their direction is indicated by the arrows. The numbers on the right hand side indicate nucleotide bases.

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This insertion sequence is present in MOS2 (Jacobson, 2007), yet MOS2 does not contain the *ISKpn12-IS1133_L/ISAb_a-1/IS1133_R-aacC2a* genetic arrangement. In MOS2, *ISAb_a-1* has been identified upstream of *bla_{OXA-23}* where it provides additional promoter sequences for expression of this β -lactamase resistance gene (Segal *et al.*, 2007). To determine whether *ISAb_a-1* is mobile in MOS1 and MOS2, reverse transcriptase PCR assays evaluating the expression of *ISAb_a-1 tnpA* were carried out (2.10). RNA was extracted using the hot acid phenol method (2.9) from logarithmic and stationary phase cultures of strain MOS1 and MOS2. Any contaminating DNA was eliminated by DNase treatment (2.9).

Controls containing no cDNA and no RNA were included to detect for contamination in the reaction. The expression of the 16S rRNA gene was determined using 16S universal primers (Table 2.3) and was included as a positive control. The primer HRR (Table 2.3; Figure 3.28) anneals within the *tnpA* of *ISAb_a-1* and was used for first strand synthesis of cDNA. The primers HRF and HRR (Table 2.3; Figure 3.28) were used for PCR amplification of *tnpA* from the reverse-transcribed cDNA. MOS1 was included as a positive control. Following reverse transcription and PCR amplification, the PCR products were analysed on a 2 % agarose gel (2.3.2; Figure 3.29).

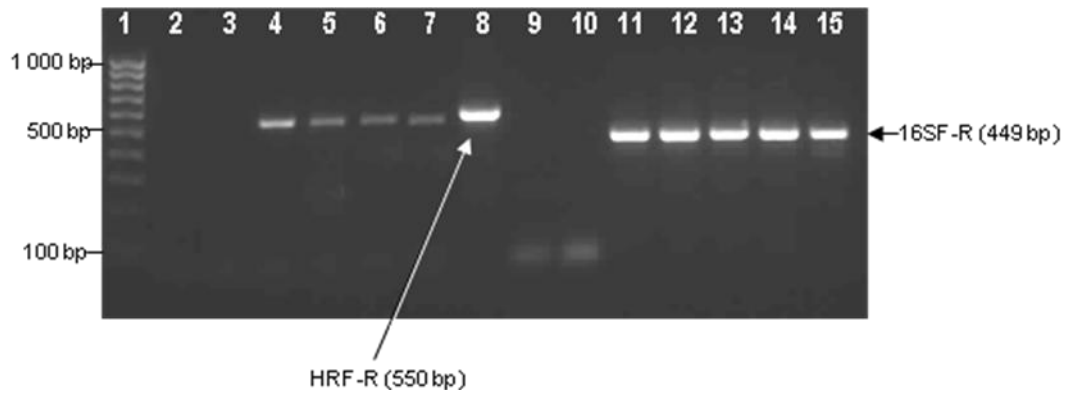


Figure 3.29: Agarose gel electrophoresis of amplicons obtained following reverse transcription PCR of *ISAbA-1* and 16S rRNA in MOS1 and MOS2. **Lane 1:** Hyperladder IV (Bioline; Appendix C); **Lane 2:** no RNA control; **Lane 3:** no cDNA control; **Lane 4:** MOS1 logarithmic phase; **Lane 5:** MOS1 stationary phase; **Lane 6:** MOS2 logarithmic phase; **Lane 7:** MOS2 stationary phase; **Lane 8:** MOS1 genomic DNA; (**Lanes 9 – 15** display results for 16S Reverse transcriptase PCR). **Lane 9:** no RNA control; **Lane 10:** no cDNA control; **Lane 11:** MOS1 logarithmic phase; **Lane 12:** MOS1 stationary phase; **Lane 13:** MOS2 logarithmic phase; **Lane 14:** MOS2 stationary phase; **Lane 15:** MOS1 genomic DNA. Marker sizes are indicated. Products of 550 bp and 449 bp corresponding to *ISAbA-1 tnpA* and 16S PCR products from positive controls, respectively are indicated with arrows.

No product was obtained from the no RNA (Figure 3.29; Lane 2 and 9) and no cDNA controls (Figure 3.29; Lane 3 and 10) indicating the absence of contamination in the reagents used in this assay. This result also indicates the absence of DNA contamination in the RNA used for reverse transcriptase PCR. An expected 449 bp product was obtained from MOS1 genomic DNA following amplification with the 16S universal primers (Figure 3.29; Lane 15). A product of 449 bp was obtained from both MOS1 and MOS2 (Figure 3.29; Lanes 11-14) following PCR amplification of the cDNA using 16S universal primers. Thus, the 16S rRNA was synthesised in the logarithmic and stationary phase cultures from both strains and the quality of the RNA was good enough to use in reverse transcriptase PCR assays.

An expected product (550 bp) corresponding to the *tnpA* of *ISAbA-1* was obtained from MOS1 genomic DNA (Figure 3.29; Lane 8). A 550 bp product corresponding to *ISAbA-1 tnpA* was

obtained from MOS1 and MOS2 cDNA synthesised from RNA obtained from both the logarithmic and stationary phase cultures (Figure 3.29; Lanes 4-7), indicating that *ISAb_a-1 tnpA* is expressed in both growth phases in these two strains. The PCR product obtained from the logarithmic phase culture of MOS1 (Figure 3.29; Lane 4) is brighter than that for MOS2 (Figure 3.29; Lane 6). It may be that there are different expression levels for *ISAb_a-1* in the growth phases of these two strains. To determine the latter, a real-time PCR, assay which accurately determines DNA expression levels, could be carried out. The observed differences in the intensity of the 550 bp product obtained may also be a result of the presence of different copy numbers in these two strains where a higher copy number would be associated with an increased expression level. The copy number of *ISAb_a-1* was therefore investigated.

It has been previously reported that an increased distribution of *ISAb_a-1* in *A. baumannii* isolates may aid creation of composite transposons (Corvec *et al.*, 2007). Since MOS1 and MOS2 may harbour large antibiotic resistance elements, determining the copy number of *ISAb_a-1* would provide further evidence for the presence of large resistance genomic regions in MOS1 and MOS2. A Southern hybridisation (2.8) was carried out on *EcoRI/BamHI*-digested MOS1 and MOS2 genomic DNA (Figure 3.30). Importantly, there are no *EcoRI* or *BamHI* restriction sites within the *ISAb_a-1* sequence. The labeled HRF- HRR probe (2.8.1) used for this assay was obtained following PCR amplification using MOS1 genomic DNA as template. An agarose gel extracted (2.3.3) HRF- HRR PCR product obtained from MOS1 was included as a positive control.

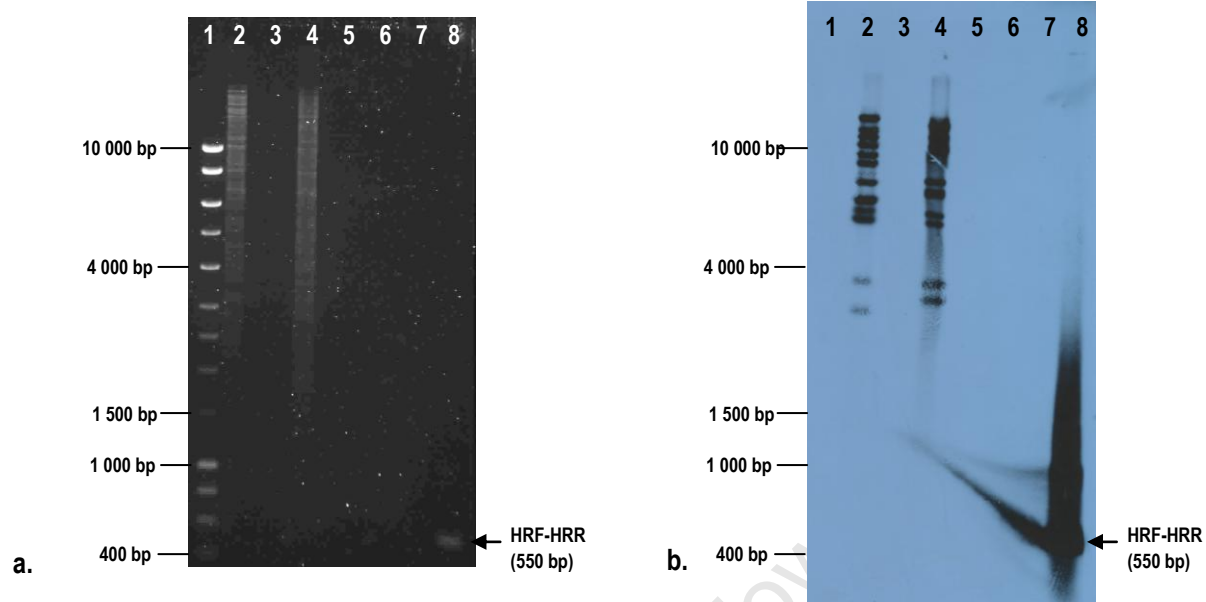


Figure 3.30: Autoradiograph displaying *ISAbA-1* copy number in *A. baumannii* MOS1 and MOS2. **a)** *EcoRI/BamHI* digested genomic DNA. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** BLANK; **Lane 4:** MOS2; **Lane 5-7:** BLANK **Lane 8:** gel purified HRF-HRR PCR product. **b)** Autoradiograph of HRF-HRR probe hybridised to *EcoRI/BamHI* digested *A. baumannii* strains MOS1 and MOS2. **Lane 1:** Hyperladder I (Bioline; Appendix C); **Lane 2:** MOS1; **Lane 3:** BLANK; **Lane 4:** MOS2; **Lane 5-7:** BLANK **Lane 8:** gel purified HRF-HRR PCR product. Relevant marker sizes are indicated and the 550 bp product/signal indicated by an arrow.

A product of the expected size (550 bp) corresponding to an HRF-HRR PCR product was obtained for the positive hybridisation control (Figure 3.30a and b; Lane 8). There were 12 hybridisation signals obtained from MOS1 (Figure 3.30b; Lane 2), indicating the presence of at least 12 copies of *ISAbA-1* in this strain. A total of 10 hybridisation signals were obtained for MOS2 (Figure 3.30b; Lane 4), indicating the presence of at least 10 copies of *ISAbA-1* in this strain. The copy number, nonetheless, may be underestimated as the strongly hybridising bands (Figure 3.30b; Lanes 2 and 4) may contain more than one copy of *ISAbA-1*. It was not surprising that MOS1, which is resistant to fewer antibiotics than MOS2, might have more copies of *ISAbA-1* since at least eight copies of this element have been observed in an *A. Iwoffii* non-resistant isolate (Segal *et al.*, 2005). Thus, *ISAbA-1* copy number in *Acinetobacter* spp. is not an indication of antibiotic resistance, but of genomic plasticity. That MOS1 may have more copies of *ISAbA-1*

than MOS2 may explain the observed difference in expression levels of this element (Figure 3.29). It may be that the more resistant *A. baumannii* isolates have more stable genomes as the IS*Aba-1* elements are integrated and associated with antibiotic resistance genes to provide promoter sequences for their expression. In addition, the susceptible isolates may have more unstable genomes, with more copies of IS*Aba-1*, as they try to adapt to the toxic hospital environment. It would be interesting, however, to determine the copy number of IS*Aba-1* in more MDR isolates, especially from various geographic regions.

Although specific to *A. baumannii*, IS*Aba-1* has a pivotal role of transposing and providing promoter sequences for antibiotic resistance genes, even those acquired from other pathogens (Corvec *et al.*, 2007). Contrary to the role it might play in mobilising genomic regions, IS*Aba-1*, together with IS*Kpn12*, may be responsible for stabilizing the IS*Kpn12*-IS1133_L/IS*Aba-1*/IS1133_R-*aacC2a* conserved genetic element present in *A. baumannii* strain MOS1 (Jacobson, 2007). Thus, IS*Aba-1* has more than one role in *A. baumannii* strain MOS1. In MOS2, IS*Aba-1* is responsible for the observed resistance to carbapenems by providing alternative promoter sequences to *bla*_{OXA-23} (Segal *et al.*, 2007). IS*Aba-1* may also be involved in mobilizing genomic regions in MOS2, however, this may be to a lesser extent than in MOS1, given the differences in expression levels (Figure 3.29) and copy number (Figure 3.30).

IS1133 was first described on a Tn5393 transposon in the plant pathogen *Erwinia amylovora* (Chiou & Jones, 1993) in America. It was later described in *A. baumannii* strains in South Africa (Segal *et al.*, 2003), *Salmonella enterica* strains obtained from animals in Italy (Pezzella *et al.*, 2004), and *Salmonella enterica* isolated from European patients (Doublet *et al.*, 2008). It is noteworthy that in the *Salmonella enterica* isolates from Europe (Pezzella *et al.*, 2004; Doublet *et al.*, 2008), IS1133 was located on Tn5393 or Tn5393-like structures. Interestingly, IS1133 from *A. baumannii* isolates obtained from South Africa is interrupted by a full copy of IS*Aba-1* and may not be associated with Tn5393, but possibly with Tn21 (Figure 3.3), as determined by sequence analysis (2.7). The role of IS1133 in *A. baumannii* MOS1 has been reported (Jacobson, 2007). This element may be involved in stabilising the *aacC2a*-associated genetic arrangement (Figure

3.1), since it is interrupted by a full copy of *ISAb_a-1* (Jacobson, 2007) and there is a T-A transversion in the right inverted repeat of this element (Segal *et al.*, 2003).

The *ISKpn12-IS1133_L/ISAb_a-1/IS1133_R-aacC2a* conserved genetic element represents an interesting mosaic structure of an aminoglycoside resistance gene associated with; *ISAb_a-1* that's unique to *A. baumannii*, *IS1133* that has been identified in plant, animal, and human pathogens, and *ISKpn12* that's been identified in clinical pathogens and may have originated from *K. pneumoniae*. In the absence of whole genome sequencing, the sequences upstream of this arrangement in MOS1 could be characterised further by constructing other genomic DNA libraries using different combinations of restriction enzymes. On the other hand, enriched cloning could be used to increase the chances of obtaining sequences upstream of *ISKpn12*. Sequences downstream of the *ISKpn12 HindIII* site (Figure 3.20) lie within a fragment ~2 400 bp (Figure 3.26b; Lane 2) as revealed by Southern hybridisation of *EcoRI/HindIII* digested MOS1 genomic DNA. This genomic fragment has a *HindIII* site on one end that lies within *ISKpn12*. The ~2 400 bp fragment has either an *EcoRI* or a *HindIII* site on the other end. Following agarose gel extraction and purification (2.3.3) of this fragment, it could be used in two separate cloning experiments using *HindIII*- and *EcoRI/HindIII*-digested pUC19. The recombinants obtained could then be screened by PCR for sequences upstream of *ISKpn12* using the primers *ISKpn12F2* and *ISKpn12R2* (Figure 3.20). Positive recombinants harbouring *ISKpn12* would contain sequences upstream of this element, and possibly a recombination site as previously proposed (3.2; Figure 3.9).

Sequencing and sequence analysis could also be used to identify sequences upstream of *ISKpn12*. Since a single copy of this element is present in MOS1 (Figure 3.25; Figure 3.26), the sequences upstream of *ISKpn12* could be characterised by sequencing MOS1 genomic DNA. Initially, the primer *ISKpn12F* or *ISKpn12F2* (Figure 3.20) could be used to sequence the genomic DNA on one strand. The presence of one copy of *ISKpn12* means that the primer used for sequencing, *ISKpn12F* or *ISKpn12F2*, will only yield sequences upstream of this element. Based on the initial sequencing results, more primers could then be designed and used to sequence the second DNA strand to determine *ISKpn12*-linked sequences.

It would be interesting however, to know if this conserved region lies on a plasmid or on the chromosomal DNA. Some portions of large resistance regions are located on plasmids or are excised from the chromosomal DNA and exist as independent units (Chen *et al.*, 2008; Iacono *et al.*, 2008; Ronan & Boyd, 2008; D' Andrea *et al.*, 2009). Genomic DNA electrophoresed on an agarose gel did not reveal the presence of plasmid DNA (Figure 3.4; Lane 2), however, additional studies need to be conducted to determine the location of this large conserved genetic arrangement. This would provide interesting clues to the origin or role of this *aacC2a*-associated conserved genetic arrangement in a particular lineage of aminoglycoside resistant *A. baumannii* isolates from Cape Town.

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SUMMARY AND CONCLUSION

A genetic arrangement, IS1133_L/ISAb_a-1/IS1133_R-aacC2_a, has been described in *A. baumannii* clinical isolates obtained from patients admitted to hospitals in Cape Town since 1983. *A. baumannii* isolates that have this genetic arrangement are resistant to gentamicin and amikacin. In 2005, a carbapenem susceptible strain, MOS1, was obtained from a patient at GSH. MOS1 harboured this conserved genetic arrangement associated with *aacC2a*. Following two weeks of treatment with meropenem, an *A. baumannii* strain MOS2 was obtained from the same patient. MOS2 did not contain the conserved genetic arrangement. However, it had an ISAb_a-1-associated *bla*_{OXA-23} gene and was resistant to imipinem and meropenem.

The IS1133_L/ISAb_a-1/IS1133_R-aacC2_a arrangement has a G+C content of 47 % compared to that of 39 % for *A. baumannii* (Fournier *et al.*, 2006). This suggests that the sequence comprises foreign DNA that may have been acquired by MOS1. This foreign DNA may well be part of a larger mobile genetic element such as a transposon or PAI. The absence of this genetic arrangement in MOS2, and the presence of ISAb_a-1-associated *bla*_{OXA-23} suggests that the former may have been replaced by the latter through a recombination event when these strains originally diverged.

The ATPase gene in *A. baumannii* may be interrupted by insertion of large genomic regions including PAIs and transposons (Fournier *et al.*, 2006; Corvec *et al.*, 2007; Iacono *et al.*, 2008; Shaikh *et al.*, 2009). The primers 42-156F and 46-358R (Fournier *et al.*, 2006) flank a 253 bp region within the ATPase gene where such large genomic regions may be inserted. To determine whether the ATPase gene was interrupted in MOS1 and MOS2, a PCR assay using these primers was carried out. Following a number of optimisations including increasing the annealing time to allow for amplification of larger products, no PCR amplicon was obtained from either strain, suggesting that the ATPase genes in these strains are disrupted, possibly by a large genomic insertion. Following a PCR assay using ATPase flanking primers, 5 of 22 MDR

A. baumannii isolates yielded no PCR products (Fournier *et al.*, 2006). The presence of an ATPase gene interrupted by large mobile genetic elements was suggested and confirmed using whole genome sequencing. In a similar study, a PCR assay on 10 MDR *A. baumannii* isolates using primers that flank the ATPase gene no PCR products were obtained (Shaikh *et al.*, 2009). However, further studies revealed the presence of an interrupted ATPase gene in eight of the 10 isolates.

Sequences downstream of the *aacC2a*-associated conserved genetic arrangement have been characterised (Segal *et al.*, 2003). Primers on either side of this region were used, in combination with the ATPase-specific primers (Fournier *et al.*, 2006), in PCR assays to identify further sequences that may be associated with putative transposons or PAIs in MOS1. Though no product was obtained, further assays using strains with both an intact and a disrupted ATPase gene with known sequence need to be carried out to determine whether these strains harbour interrupted ATPase genes and hence large genomic insertions.

In the absence of whole genome sequencing and since the sequences downstream of the IS1133/ISAb_a-1/IS1133_R-*aacC2a* conserved region in MOS1 have been characterised (Segal *et al.*, 2003), a standard cloning procedure (Sambrook *et al.*, 1989) was used to detect sequences upstream of this genetic arrangement.

When screening an *EcoRI/HindIII* genomic DNA library of *A. baumannii* MOS1, one recombinant plasmid was identified and designated pTJ001. Sequence analysis of the genomic DNA fragment in pTJ001 revealed the presence of a partial ISK_{pn12} element 558 bp upstream of the left inverted repeat of IS1133. To determine whether a full copy of this element was present in MOS1, primers were designed to amplify a full copy of ISK_{pn12}. A full copy of ISK_{pn12}, associated with the *aacC2a*-linked conserved genetic region, was identified in MOS1 and not in MOS2, suggesting that ISK_{pn12} is part of the conserved genetic arrangement associated with MOS1 and related strains from our hospitals. Detection of ISK_{pn12} in a selection of MOS1-related isolates and not in MOS2-related isolates further supports the fact that this element is associated with the *aacC2a*-associated genetic arrangement conserved in *A. baumannii* isolates conserved in Cape Town since 1983.

The first report of IS*Kpn12* was from a *K. pneumoniae* isolate obtained from Uruguay, South America (Márquez *et al.*, 2008). *In silico* genomic DNA analysis identified this element as a part of a plasmid in an *A. baumannii* isolate, 7037 obtained from a patient in Argentina, South America (Poirel *et al.*, 2007). Since IS*Kpn12* was not described in that study, this is the first report of this element in *A. baumannii*. Our detection of IS*Kpn12* in an *A. baumannii* isolate from South Africa indicates that this insertion sequence element is neither confined to South America nor to a particular genus, raising interesting questions about the origin, spread and role of this element in *A. baumannii*.

Since the G+C content of IS*Kpn12* (57 %) differs significantly from that of *A. baumannii* (39 %; Fournier *et al.*, 2006), it was likely that *A. baumannii* MOS1 and related isolates obtained this element from other pathogens. However, IS*Kpn12* was not detected in representative isolates of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. lwoffii*, and *A. calcoaceticus* as determined by PCR assays. Interestingly, the *K. pneumoniae* isolates screened in this study did not contain IS*Kpn12*, suggesting that *K. pneumoniae* might not be the reservoir for this IS element in our setting. However, a more thorough investigation comprising a larger number of *K. pneumoniae* isolates from the same time periods and hospitals as the *A. baumannii* isolates included in this study needs to be conducted to determine whether *K. pneumoniae* is the source of IS*Kpn12* in *A. baumannii*.

Since IS*Aba-1* provides alternative promoter sequences for resistance genes, it was interesting to investigate if IS*Kpn12* had the same role in *A. baumannii*. The *tnpA* of IS*Kpn12* is expressed in both the logarithmic and stationary phases of growth in MOS1. However, only one copy of this IS element was detected in MOS1, suggesting that it is not mobile and may not provide promoter sequences for the *aacC2a* gene in the conserved genetic arrangement. Besides, IS*Kpn12* is too far upstream of the *aacC2a* gene in the conserved genetic arrangement and so most likely does not play this role in *A. baumannii*. IS*Kpn12* may therefore be involved in stabilizing the *aacC2a*-associated conserved genetic arrangement in MOS1 and related strains, even though this insertion sequence may have a different role in its natural host.

Interestingly, *ISAb_a-1* in MOS1 may also be involved in stabilizing the the conserved genetic arrangement in MOS1 where it disrupts an *IS1133* element (Jacobson, 2007). In addition, *ISAb_a-1* may be responsible for genome plasticity in the less resistant *A. baumannii* isolate, MOS1, since at least 12 copies of this element were detected. *ISAb_a-1* may have a different role in MOS2 since this isolate does not contain the *ISKpn12-IS1133_L/ISAb_a-1/IS1133_R-aacC2a* genetic arrangement. It is known that in MOS2, *ISAb_a-1* is responsible for the observed resistance to carbapenems by providing alternative promoter sequences to *bla_{OXA-23}* (Segal *et al.*, 2007). The other roles of this element in MOS2 remain to be determined. However, the presence of at least ten copies, and what seemed to be a reduced expression level of this element in MOS2 compared to MOS1 suggests *ISAb_a-1* may also be responsible for genomic plasticity in MOS2, though to a lesser extent than in MOS1. The observed differences in the copy number and expression of *ISAb_a-1* in MOS1 and MOS2 may be an indication of how resistant an *Acinetobacter* spp. may be. However, that both MOS1 and MOS2 had a high copy number of *ISAb_a-1* points to the possibility that both strains harbour large resistance genomic islands (Corvec *et al.*, 2007).

ISKpn12 exists as a single copy in MOS1 and is associated with an *aacC2a*-conserved genetic region. This suggests that *ISKpn12* has no other roles in MOS1 than that of possibly stabilizing the conserved genetic region. The presence of this element, together with a highly mobile *ISAb_a-1* in the same genetic arrangement that has been conserved for over 23 years in *A. baumannii* isolates from Cape Town represents a structure that was once useful to *A. baumannii* before the development of MDR strains.

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APPENDIX A

Media, buffers and solutions

2 X Y Tryptone agar

| | |
|---------------|------|
| Yeast extract | 10 g |
| Tryptone | 16 g |
| NaCl | 5 g |
| Agar | 15 g |

Make up to 1 litre with distilled water and autoclave

2 X Tryptone broth

| | |
|---------------|------|
| Yeast extract | 10 g |
| Tryptone | 16 g |
| NaCl | 5 g |

Make up to 1 litre with distilled water and autoclave

50 X TAE buffer

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

Make up to 1 litre with distilled water

Denaturing buffer

| | |
|------|---------|
| NaCl | 87.66 g |
| NaOH | 10 g |

Make up to 1 litre with distilled water

Depurinating buffer

HCl 21.6 ml

Make up to 1 litre with distilled water

LB agar

Yeast extract 5 g

Tryptone 10 g

NaCl 10 g

Agar 15 g

Make up to 1 litre with distilled water and autoclave

LB broth

Yeast extract 5 g

Tryptone 10 g

NaCl 10 g

Make up to 1 litre with distilled water and autoclave

Neutralising buffer (pH 7.5)

NaCl 87.66 g

Tris 60.58 g

Make up to 1 litre with distilled water

Prehybridization buffer (depending on the size of membrane being used)

ECL blocking agent 5 % (w/v)

NaCl final concentration of 0.5 M

Make up to the required volume with ECL hybridisation buffer

Primary wash buffer

Urea 360 g

SDS 4 g

Make up to 1 litre with distilled water

Secondary wash buffer

20 X SSC 100 ml

Make up to 1 litre with distilled water

20 X SSC (pH 7)

Sodium citrate 88.23 g

NaCl 175.32 g

Make up to 1 litre with distilled water

SOC medium (pH 7)

Yeast extract 0.5 g

Tryptone 2.0 g

1M NaCl 1 ml

1M KCl 0.25 ml

Bring to 97 ml with distilled water, autoclave and cool to room temperature. Add Mg²⁺ stock and 2M glucose. Filter through a 0.2µm filter unit and store at 4°C

Mg²⁺ stock 1 ml

(Mg²⁺ stock: 1M MgCl₂.6H₂O, 1M MgSO₄.7H₂O: filter sterilised)

2M glucose 1 ml

TE buffer (pH 8)

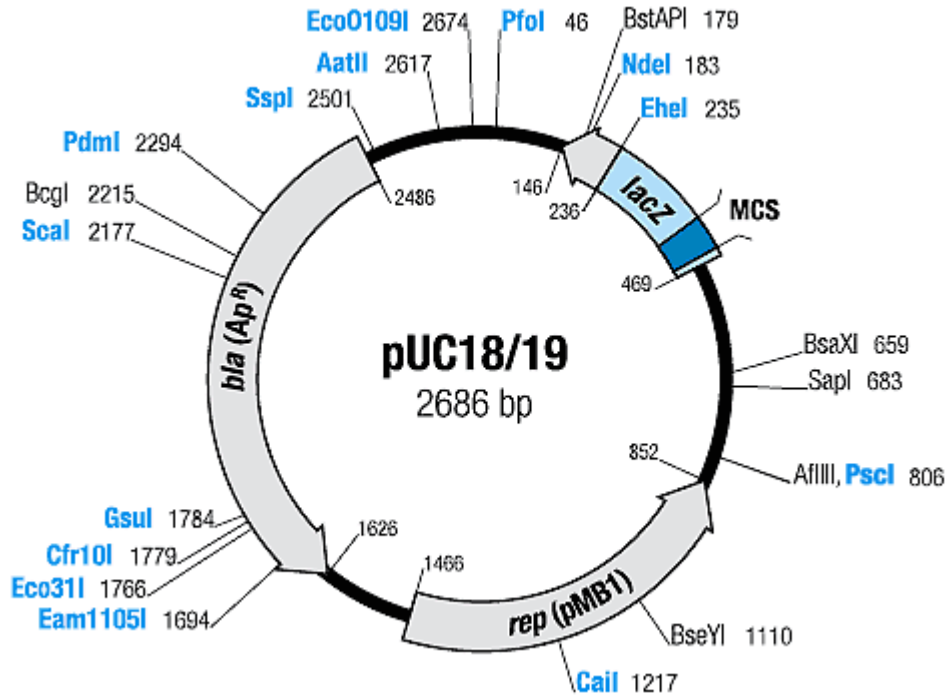
1M Tris-HCl 5 ml

1M EDTA 5 ml

Make up to 500 ml with distilled water and autoclave

APPENDIX B

pUC19



M13/pUC sequencing primer (-20), 17-mer → 396

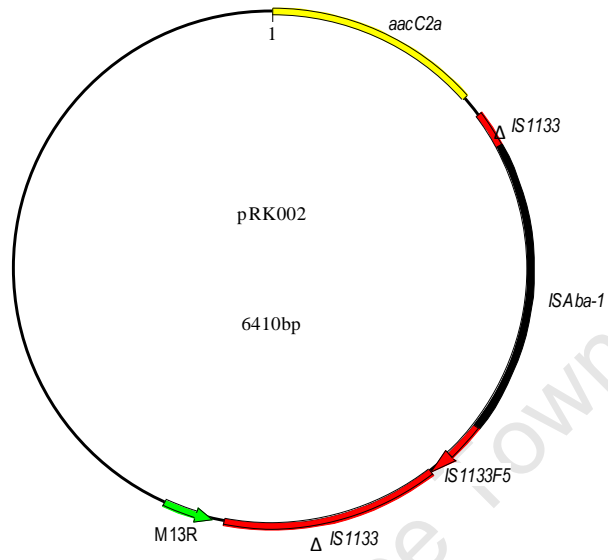
5' G TTG TAA AAC GAC GGC CAG TGA ATT CGA GCT CGG TAC CCG GGG ATC CTC TAG
 3' C AAC ATT TTG CTG CCG GTC ACT TAA GCT CGA GCC ATG GGC CCC TAG GAG ATC
 LacZ ← Leu Val Val Ala Leu Ser Asn Ser Ser Pro Val Arg Pro Asp Glu Leu

HincII PstI
 Sall BvuI SdaI PaeI HindIII 452
 XmiI

AGT CGA CCT GCA GGC ATG CAA GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG 3'
 TCA GCT GGA CGT CCG TAC GTT CGA ACC GCA TTA GTA CCA GTA TCG ACA AAG GAC 5'
 Thr Ser Arg Cys Ala His Leu Ser Pro Thr Ile Met Thr Met

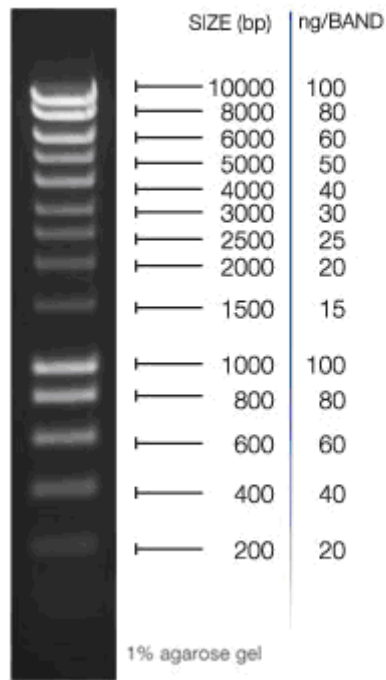
M13/pUC reverse sequencing primer (-26), 17-mer ←

pRK002

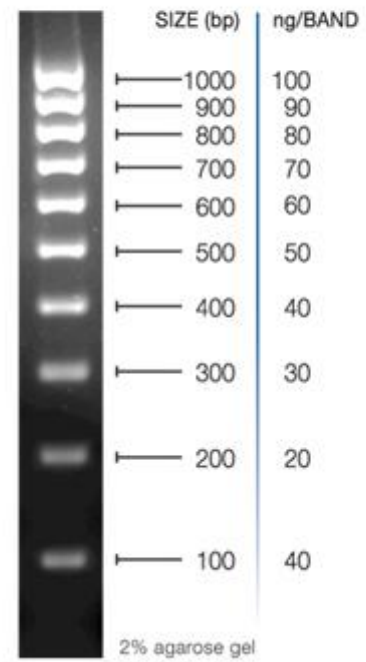


APPENDIX C

HyperLadder I



HyperLadder IV



*Hind*III
AAGCTTTCTACTGCGGAGTTATCAAAACAATTGCCTTTCCCGCTCATGGACGGCAGCAATT**GATGTTTGAGCAGTAG**CTTTTGATATTCATGAGCGCAAT 100

ATTGGCTCCCACGGTCTGTGTGTTGAATACAACCCGGTGGTGGTTTGCCTAAAGCCAACGCCATATTCAGTGCCCTTAATGCAAGATCCTGCTTTAATCG 200

ATCACCTGTTGCCAGCCAATCACGCGACGGGAATACAGGTCAAGGATAACAGCAAGATAGACCCATCCTTCTCTGGTCCAAACATAAGTGATATCGCCT 300

GCCCATTCTGGTTGGGTGCGCTTGCCTAAAGTCTTGTTTTAATAGGTTGCGTGAATGTTGAAGGTATGATGACTATCCGTTGTCCGTTTGAATTTAC 400

GCGTTCGAACAACACTGTAATGTTATTCTGGCGCATCAAACGTCCAACCCGACGCTGCCAACCTGCAGGCCAGCGCTTCAACTCTTCTGTCATACGCGG 500

CCTACCATAGCTCCCCAAACACAACCGATGCTGCTCACGTATATGCGCTAGAAGTATAAGATCACGACGCTGGCGCAGTGATGGAGGACGGCGTTTCCAT 600

← IS1133 *tnpA*
 GCACGTAAACCACGATCTGTTACGCC**CAT**CAAACGACATATGCGTGAACGTGAGAGAGAGCCACGGTAATCCGTAATAAACTGAAATCTCACAGCTTTTG 700

TACTGCGAAAAATATTGCTGCCTTTTTTAATATCTCCCTCTCCTCCCGAAGGATACGGTCTCTTTGCGTAAACGTTTCATTCTCACGCAGAAGATCAGTG 800

TCTTGGGTAGGAATTTTAGTTTCATCGGAAATTGATGCGATCCATTTCCCAAGCGTGGAAAGCCCAATACTTAAATCTGACGCAACTTGACGGCGTGTTA 900

AGCCACTAGTGAGTGCTATGCGAACTGCATCACGCTTAAATTCATCACTATGCTTTAATGACATATATGGTCTCCTTGATAACAAATAATGCT**CTCAAAA** 1000

← IS1133F2
AGACCGGAACGAAACCGAG**GCAAGTCCA**TTTATCCGCGTCGTCTCGCCGCCGCTCCGCTCCGCGCGAATAGCAAGCAGCGAAGGACCAAGAAGCTCAC 1100

GCGCATGAGCGACCGTTTTCGGCGGATGCCGTGTTTA**GGGTCAGGAC**CCATTGATTTGAACTGACGGCTGTGATTTCAGATGGGCGTATAGGAGCCCGACTG 1200

GGGTCAGGACCCATTGATTTGAACTGACGGCTGTGATTTCAGATGGGCGTATAGGAGCCCGACTG 64

IS*Kpn*12 *tnpA* → IS*Kpn*12F →
ATGAGTAATTTGTTTTGGCTGACGGACGAGCAAATGGCTCGT**CTTCAGCCCTATTTCCC**CAAGAGCCATGGCCGCCAGCGCGTTGATGATCGACGCGTTC 1300

ATGAGTAATTTGTTTTGGCTGACGGACGAGCAAATGGCTCGT**CTTCAGCCCTATTTCCC**CAAGAGCCATGGCCGCCAGCGCGTTGATGATCGACGCGTTC 164

TGAGCGGCATCATCTTCGTCAACCGCAACGGGCTGCGTTGGCGTGATGCGCCGAAGGAATATGGCCCGGCGAAGACGCTTTATAACCGCTGGAAACGCTG 1400
 TGAGCGGCATCATCTTCGTCAACCGCAACGGGCTGCGTTGGCGTGATGCGCCGAAGGAATATGGCCCGGCGAAGACGCTTTATAACCGCTGGAAACGCTG 264
 GGGCGACAAGGGCGTATTTATCCAGATGATGGAAGGTCTGGCCGTGCCTGAAGCC **GCAGAGCACAAGACCAT** ← ISKpn12R CATGATCGATGCGACCTATCTCAAGGCC 1500
 GGGCGACAAGGGCGTATTTATCCAGATGATGGAAGGTCTGGCCGTGCCTGAAGCC **GCAGAGCACAAGACCAT** CATGATCGATGCGACCTATCTCAAGGCC 364
 CACCGCACGGCTTCCAGCTTGCGGGTAAAAAAGGGGGCGCGGGCCGCC **TGA**TTGGACGCACGAAAGGCGGCATGAACACC **AAGCTT** HindIII 1586
 CACCGCACGGCTTCCAGCTTGCGGGTAAAAAAGGGGGCGCGGGCCGCC **TGA**TTGGACGCACGAAAGGCGGCATGAACACC **AAGCTT** CATGCCGTAACGGA 464
 TCGAGTGGTCGCCCCGATCAGCTTCTTCATAACGGCAGGCCAGGTCAGCGATTACACCGGTGCTGCCGCTTGCTTGATGAACTTCCCAAGGCCAAATGG 564
 TTGCTGGCCGACCGTGGCTATGATGCCGACTGGTATCGTGACGCTTTACAGGCGAAGGGGATCACTCCCTGCATTCCAGGTCGAAAATCCCGCAAGAAGA 664
 CCATCAAATACGACAAACGCCGCTACAAACGGCGCAACCGGATCGAGATCATGTTTCGGGCGTCTCAAAGACTGGCGGCGTGTGCTACGCGTTACGACCG 764
 GTGCCCAATGGCCTTTCTCTCCGCCATCGCTCTCGCTGCGACCGTTATCTTCTGGCTATG **AGCAATGAGTCTGACC** ← ISKpn12R3 IR_R 842

Figure3.18: Nucleotide sequence of a 1 586 bp **HindIII** insert in pTJ001 showing 100 % homology to *ISKpn12*. The **HindIII** sites on either side of the insert are highlighted in red, the primers **IS1133F2** and **IS1133F5** are highlighted in yellow. The **left inverted repeat of IS1133** is underlined and in pink text. The *tnpA* in IS1133 is indicated by the **start codon CAT** in red text, the stop codon lies downstream of the **HindIII** site. The published sequence of *ISKpn12* is in green text. The **left inverted repeat of ISKpn12** is underlined and in blue text. An *ISKpn12 tnpA* is indicated by the **start** and **stop codons**, **ATG** and **TGA** respectively, in red text. Highlighted in green are **ISKpn12- specific primers** used in PCR assays and for sequence analysis. The numbers on the right hand side indicate nucleotide base.

| | | | |
|---------|---|----------------|-----|
| | IR _L | ISKpn12 tnpA → | |
| ISKpn12 | GGGTCAGGACCCATTGATTTGAACTGACGGCTGTGATTCAGATGGGCGTATAGGAGCCCAGCTGATCAGTAATTTGTTTTGGCTGACGGACGAGCAAATG | | 100 |
| MOS1 | GGGTCAGGACCCATTGATTTGAACTGACGGCTGTGATTCAGATGGGCGTATAGGAGCCCAGCTGATCAGTAATTTGTTTTGGCTGACGGACGAGCAAATG | | 100 |
| | ISKpn12F → | | |
| ISKpn12 | GCTCGTCTTCAGCCCTATTTCCCAAGAGCCATGGCCGCCAGCGCTTGATGATCGACGCGTTCTGAGCGGCATCATCTTCGTCAACCGCAACGGGCTGC | | 200 |
| MOS1 | GCTCGTCTTCAGCCCTATTTCCCAAGAGCCATGGCCGCCAGCGCTTGATGATCGACGCGTTCTGAGCGGCATCATCTTCGTCAACCGCAACGGGCTGC | | 200 |
| ISKpn12 | GTTGGCGTGATGCGCCGAAGGAATATGGCCCGGCGAAGACGCTTTATAACCGCTGGAAACGCTGGGGCGACAAGGGCGTATTTATCCAGATGATGGAAGG | | 300 |
| MOS1 | GTTGGCGTGATGCGCCGAAGGAATATGGCCCGGCGAAGACGCTTTATAACCGCTGGAAACGCTGGGGCGACAAGGGCGTATTTATCCAGATGATGGAAGG | | 300 |
| | ← ISKpn12R | | |
| ISKpn12 | TCTGGCCGTGCCTGAAGCCGCAGAGCACAAAGACCATCATGATCGATGCGACCTATCTCAAGGCCACCGCACGGCTTCCAGCTTGCGGGTAAAAAAGGGG | | 400 |
| MOS1 | TCTGGCCGTGCCTGAAGCCGCAGAGCACAAAGACCATCATGATCGATGCGACCTATCTCAAGGCCACCGCACGGCTTCCAGCTTGCGGGTAAAAAAGGGG | | 400 |
| | HindIII | ISKpn12F2 → | |
| ISKpn12 | GCGCGGGCCGCCATGATTGGACGCACGAAAAGGCGGCATGAACACCAAGCTTCATGCCGTAACGGATGCGAGTGGTCGCCCCGATCAGCTTCTTCATAACGGC | | 500 |
| MOS1 | GCGCGGGCCGCCATGATTGGACGCACGAAAAGGCGGCATGAACACCAAGCTTCATGCCGTAACGGATGCGAGTGGTCGCCCCGATCAGCTTCTTCATAACGGC | | 500 |
| ISKpn12 | AGGCCAGGTCAGCGATTACACCGGTGCTGCCGCCTTGCTTGATGAACTTCCCAAGGCCAAATGGTTGCTGGCCGACCGTGGCTATGATGCCGACTGGTAT | | 600 |
| MOS1 | AGGCCAGGTCAGCGATTACACCGGTGCTGCCGCCTTGCTTGATGAACTTCCCAAGGCCAAATGGTTGCTGGCCGACCGTGGCTATGATGCCGACTGGTAT | | 600 |
| ISKpn12 | CGTGACGCTTTACAGGCGAAGGGGATCACTCCCTGCATTCAGGTCGAAAAATCCCAGCAAGAAGACCATCAAATACGACAAAACGCCGCTACAAAACGGCGCA | | 700 |
| MOS1 | CGTGACGCTTTACAGGCGAAGGGGATCACTCCCTGCATTCAGGTCGAAAAATCCCAGCAAGAAGACCATCAAATACGACAAAACGCCGCTACAAAACGGCGCA | | 700 |
| ISKpn12 | ACCGGATCGAGATCATGTTTCGGGCGTCTCAAAGACTGGCGGCGTGTGCTACGCGTTACGACCGGTGCCCAATGGCCTTTCTCTCCGCCATCGCTCTCGC | | 800 |
| MOS1 | ACCGGATCGAGATCATGTTTCGGGCGTCTCAAAGACTGGCGGCGTGTGCTACGCGTTACGACCGGTGCCCAATGGCCTTTCTCTCCGCCATCGCTCTCGC | | 800 |



Figure 3.20: Nucleotide sequence alignment of ISKpn12 from MOS1 and the published sequence. There is 100 % homology between the two sequences, indicated by the black highlighting. The **inverted repeats** are underlined and a **HindIII** site is highlighted in red. Sequences upstream of the HindIII site were part of the 1586 bp fragment obtained in pTJ001, and includes the 351 bp **tnpA** indicated by **ATG** and **TGA** in red text. The **primers** used for sequencing and PCR assays are highlighted in green. The numbers on the right hand side indicate nucleotide bases.

ISAb-1-IRL
CTCTGTACACGACAAATTTACAGAACCCTTATCCTATCAGGGTTCTGCCTTCTTAAAATTGCCAAAATTTCTTAAACTCTTCTTTTTTCCCAAACCA 100
ATTAACGCTGAATCGCCATTTGAACATAGTCTAAACCATAGCGAAATAAACTCATTGAGAGTCGTCCATGCTTCTTTATTTTTATCGCTTTTTTTTTGAT 200
CATGTTGCCATTACCCGTTAAGTAACACCAACAGAAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGGGTCTGTCAAGCGCGTATTTTC 300
AAGATTAACCCGCGTCTTTGAGACAACCTGAATAAGGTTTCAATTTCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACG 400
ACGAGTAAAAGCTCTCCATTTTCTAACTGTAGTGCACCTATATATAGTTTTACCCGACCAACCAAATCCGTCGTTTACGACATTCAATTTGACCAACTT 500
TAAGATGGCGAAATAAATCACTAATTTTATGATTCTTTCTAATGATTGGTGACAATGAAGTTTTTTTTAA **CACGAATGCAGAAGTTG**ATGTCTTGTTC 600
ATTAACCATGTAAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCAAAG 700
CAATACGCTCTTTTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATT 800
AATATTTTCGTTTTCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTTCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAA 900
TCAAATACTGACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATG 1000
TTTGCTTTAAAATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCATTTTAGATATTTGTTAAGATAAGATATAACTCATTGAG 1100
← ISAb-1 *tnpA* ← HRR
AT **GTGTCTAGTATTCGTCC**TTAGAAAACAATTATTATGACATTATTTCAATGAGTTATCTATTTTTAT CGTGTACAGAG ISAb-1-IRR 1180

Figure 3.28: Nucleotide sequence of ISAb-1. The inverted repeats are underlined and in blue text. Highlighted in green are the primers HRF and HRR and their direction is indicated by the arrows. An ISAb-1 *tnpA* is indicated by the start and stop codons, **CAT** and **CTA** respectively, in red text. The numbers on the right hand side indicate nucleotide bases.