THE ASSOCIATION OF IS*1133* WITH AN AMINOGLYCOSIDE RESISTANCE GENE, *aacC2a*, IN *ACINETOBACTER BAUMANNII* ISOLATES

Rachael Kiera Jacobson

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Dedicated with love to my mother, Judy Alison Buxton

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Declaration

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Abstract

Acinetobacter baumannii (*A. baumannii*) is a pathogenic organism, associated with nosocomial infections, especially in immuno-compromised patients. Of serious concern to clinicians are their often high levels of resistance to almost all of the clinically available antibiotics, including the aminoglycosides. In *A. baumannii*, resistance to the aminoglycosides can arise due to a number of mechanisms, with inactivation of the aminoglycoside by enzymatic modification the most common. Previous studies identified a portion of an insertion sequence, IS*1133*, sandwiched between an aminoglycoside resistance gene, *aacC2a* and a portion of IS*Aba-1*, in *A. baumannii* strain PAU (Segal *et al*, 2003). The T to A transversion in the right inverted repeat (IR_R) of the IS*1133* fragment from strain PAU was observed in a portion of IS*1133* upstream of *aacC2a* in *A. baumannii* SAK, isolated 9 years earlier (Elisha, 1991). It was suggested that this mutation may limit recognition of the IR_R by IS*1133* transposase, inhibiting transposition of this element, and therefore may play a role in the stabilization of *aacC2a* in clinical isolates of *A. baumannii*.

A total of 44 gentamicin resistant *A. baumannii* isolates as well as a gentamicin susceptible *A. baumannii* isolate, collected from patients at Groote Schuur Hospital and Red Cross War Memorial Children's Hospital from 1983 to 2006 were included in this study. The *aacC2a* gene was detected in 12 strains using PCR assays. In all 12 strains, the *aacC2a* gene was linked to the IR_R of IS*1133*. Sequencing analysis of the IR_R of IS*1133* from one strain isolated in 2001, 9 years after strain PAU, also contained the T to A transversion in the IR_R. To determine whether the *aacC2a* genes are linked to complete copies of IS*1133* rather than fragments, PCR assays using primers directed against *aacC2a* and IR_L of IS*1133* were carried out. Products of the expected size were not obtained from any of the 45 strains; instead, amplicons of 2.3kb were obtained from the 12 strains carrying *aacC2a*. The 2.3kb amplicon from strain SAK was purified and sequenced. Analysis of the sequencing data indicated that IS*1133* is disrupted by an insertion of IS*Aba-1* and that the insertion site is identical to that previously identified in strain PAU. This disruption of IS*1133* may explain the stabilization of this element linked to *aacC2a* in *A. baumannii* over a period of 23 years.

To determine whether the promoter sequences necessary for the expression of this gene were located within the IS*Aba-1* element, the IS*1133* element, or in the region immediately upstream of the *aacC2a* gene a number of experiments were carried out. RT–PCR experiments

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suggested that transcription of *aacC2a* is initiated upstream of the IS*1133* promoter sequence, therefore possibly from promoter sequences located within IS*Aba-1*.

It is interesting to speculate whether the *aacC2a* is dependant on promoter sequences within IS*Aba-1* for its expression and that disruption of IS*1133 tnpA* by IS*Aba-1*, combined with the T to A transversion in the IR_R, has stabilized IS*1133* and in turn, *aacC2a*, in *A. baumannii* strains, and that the strains containing this genetic organization have been maintained in our hospitals.

This work was presented at the 7th Annual Acinetobacter Symposium that took place in Barcelona, Spain in November 2006. A paper based on this work has also been submitted for publication in a peer reviewed journal (Jacobson *et al*, 2007).

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Abbreviations

%	percent
°C	degrees Celsius
2xYT broth/ agar	2xYeast-Tryptone broth/ agar
A. baumannii	*Acinetobacter baumannii*
AGE	agarose gel electrophoresis
Am	amikacin
AAC	aminoglycoside acetyltransferase
aacC	gene encoding 3-N-acetyltransferase
AAD	aminoglycoside adenyltransferase
APH	aminogl ycoside phosphotransferase
AME	aminoglycoside modifying enzyme
bp	base pair(s)
BSA	bovine serum albumin
$CaCl_2$	calcium cholride
cDNA	complementary deoxyribonucleic acid
CTAB	hexadecyl trimethylammonium bromide
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleoside triphosphates
DTT	dithiotrietol
DMF	dimethyl formamide
E. coli	*Escherichia coli*
EDTA	disodium ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
ESBL	extended-spectrum β-lactamase
g	gravitational acceleration
Gm	gentamicin

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GSH	Groote Schhur Hospital
H^+	proton
I	intermediate
IPTG	isopropyl-β-D-thio-galactosidase
IR	inverted repeat
IR_R	right inverted repeat
IR_L	left Inverted repeat
IS	insertion sequence
Kda	Kilodalton
kb	Kilobase(s)
Km	Kanamycin
L	litre(s)
m	milli
M	molar
mA	milli amps
MBL	metallo-β-lactamase
MDR	multi drug resistant
µg	microgram(s)
mg	milligram(s)
$MgCl_2$	magnesium chloride
MIC	Minimum Inhibitory Concentrations
µl	microlitre(s)
ml	milliliter(s)
µM	micromolar
mM	millimolar
mm	millimeter(s)
mRNA	messenger ribonucleic acid
MW	molecular weight
MWM	molecular weight marker
M-MLV-RT buffer	M-MLV reverse transcriptase buffer
NaCl	sodium chloride

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NCCLS	National Committee for Clinical Laboratory Standards
Neo	Neomycin
ng	nanogram(s)
nm	nanomoles
nt	nucleotide(s)
OD	optical density
O/N	overnight
OMP	outer membrane proteins
ORF	open reading frame
PBP	penicillin binding proteins
PCR	polymerase chain reaction
pH	percentage hydrogen
pmoles	picomoles
PIPES	1,4-piperazinediethanesulfonic acid
R	resistance
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT–PCR	reverse transcriptase-polymerase chain reaction
RCH	Red Cross War Memorial Children's hospital
SDS	sodium dodecyl sulphate
spp	Species
SSC	standard sodium citrate
Sm	streptomycin
TAE	Tris-acetate EDTA electrophoresis buffer
TE	Tris–EDTA
Tb	tobramycin
Tn	transposon
U	enzyme unit
UK	United Kingdom
UV	ultra-violet

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v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

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Figures

Figure 1.1	Schematic representation of the genetic organization of the 6,080 kb fragment cloned into pUC19	17
Figure 2.1.1a	Schematic representation of the *aacC2a* gene linked to a portion of IS*1133* as identified in *A. baumannii* strain SAK (Elisha, 1991) and *A. baumannii* strain PAU (Segal *et al*, 2003).	21
Figure 2.1.1b	Nucleotide sequence of the *aacC2a* gene linked to a portion of IS*1133*	21
Figure 2.2.1	The Qubit™ flurometer [Invitrogen] and all the reagents required	27
Figure 2.2.2	An electropherogram displaying a region of DNA sequence	29
Figure 2.2.3	Diagram indicating the transfer of DNA from the agarose gel to the positively charged membrane to using the Sothern blot technique	30
Figure 2.2.4	DNA sequence of a portion of IS*1133* interrupted by IS*Aba-1* showing the area encompassing the 730 bp probe	31
Figure 2.3.1	Agarose gel electrophoresis of amplicons following CPR amplification of *aacC2a* from *A. baumannii* strains	36
Figure 2.3.2	Alignment of *aacC2a* from *A. baumannii* strain PAU, SAK and MOS-1	40
Figure 2.3.3	Schematic representation of *aacC2a* linked to a portion of IS*1133* as identified in *A. baumannii* strain SAK (Elisha, 1991) and *A. baumannii* strain PAU (Segal *et al*, 2003).	37
Figure 2.3.4	Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* linked to *aacC2a* from *A. baumannii* strains	39
Figure 2.3.5	Alignment of *aacC2a* linked to IS*1133* in *A. baumannii* strains PAU, RAN and MOS-1	41
Figure 2.3.6	Agarose gel electrophoresis of amplicons following PCR amplification of a portion of the right end of IS*1133* in *A. baumannii* strains	43
Figure 2.3.7	Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* from *A. baumannii* strains	44
Figure 2.3.8	Schematic representation of IS*Aba-1* inserted within IS*1133*	46
Figure 2.3.9	Nucleotide sequence of the full copy of IS*1133* interrupted by IS*Aba-1* obtained from *A. baumannii* strain SAK	47

University of Cape Town

Figure 2.3.10	Autoradiograph of IS*1133* interrupted by ISAba-1 hybridised to PCR amplicons obtained from *A. baumannii* strains	48
Figure 2.3.11A	Agarose gel electrophoresis of *Bam*HI/*Hind*III digested and purified genomic DNA from *A. baumannii* strain SAK	49
Figure 2.3.11B	Agarose gel electrophoresis of *Bam*HI/*Hind*III digested and purified pUC19DNA	49
Figure 2.3.12	Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* from 1-100 colonies obtained from cloning	50
Figure 2.4.1	Schematic representation of the genetic arrangement of *aacC2a* as determined by DNA sequence analysis in *A. baumannii* strain SAK	53
Figure 3.1.1a	Schematic representation of the genetic arrangement of the *aacC2a* gene indicating the regions where three possible sets of promoter sequences could be located for expression of the *aacC2a* gene	58
Figure 3.1.1b	Nucleotide sequence of *aacC2a* linked by 115 bp to IS*1133* which has been interrupted by IS*Aba-1*	58
Figure 3.2.1	Agarose gel electrophoresis of amplicons following PCR amplification of the *aacC2a* linked to a portion of IS*1133* interrupted by IS*Aba-1* from *A. baumannii* strain SAK	59
Figure 3.2.2	Agarose gel electrophoresis of the restriction enzyme digested *E. coli* JM109 (pRK002) clone	60
Figure 3.2.3	Growth curve for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in 2xYT with gentamicin selection (5 µg/ml)	61
Figure 3.3.3	Electrophoresis of the extracted RNA obtained from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) during mid exponential and stationary growth phase in the presence of gentamicin selection	66
Figure 3.3.4	Agarose gel electrophoresis of amplicons following PCR amplification of *aacC2a* and 16s RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)	67
Figure 3.3.5	Schematic representation of the genetic arrangement of *aacC2a* in *A. baumannii* strain SAK	68
Figure 3.3.6	Agarose gel electrophoresis of PCR assays with primers, HRF and aacC2aR2, using cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) as templates	69

University of Cape Town

Figure 3.3.7 Agarose gel electrophoresis of PCR assays using primer, IS1133R and aacC2aR, with cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) as template 70

Figure 4.3.a Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* 82

Figure 4.3.b Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* PFGE from *A. baumannii* 82

Figure 4.3.c Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* PFGE from *A. baumannii* 83

Figure 4.3.d Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* PFGE from *A. baumannii* 84

Figure 4.3.e Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* PFGE from *A. baumannii* 84

Figure 4.3.f Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* PFGE from *A. baumannii* 85

University of Cape Town

Tables

Table 1.1 Ambler classification scheme for the β-lactamase in *A. baumannii* 7

Table 1.2 Aminoglycoside modifying enzymes in *Acinetobacter* spp 12

Table 2.2.1 Antibiogram of the 44 aminoglycoside resistant *A. baumannii* aminoglycoside resistant *A. baumannii* strains included in this study 23

Table 2.2.2 Primers used in this study 25

Table 3.3.1 Aminoglycoside antibiotic MICs for *E. coli* JM109, *E. coli* JM109 (pRK002) and *A. baumannii* strain SAK 65

Table 4.3.2 PFGE profiles of the 45 *A. baumannii* strains included in this study 86

Contents

Declaration i

Acknowledgments ii

Abstract iii

Abbreviations v

Figures and tables xiii

Chapter one: Literature review

1.1 **Emergence of *Acinetobacter baumannii* as an important nosocomial pathogens** 1

1.2 Mechanisms contributing to antibiotic resistance in *A. baumannii* 2

1.2.2 Decrease in membrane permeability 3

1.2.2 Expulsion of antimicrobial agents 4

1.2.3 Alteration of antimicrobial binding sites 5

1.2.4 Enzymatic inactivation 6

1.2.4.1 β – lactamases 6

1.2.4.2 Aminoglycoside modifying enzymes 10

1.2.4.2.1 Aminoglycoside phosphotransferases 12

1.2.4.2.2 Aminoglycoside nucleotidyltransferases 13

1.2.4.2.3 Aminoglycoside acetyltransferases 14

1.3 The genetic environment and spread of antibiotic resistance genes in *A. baumannii* 14

1.4 The aim of this study 18

University of Cape Town

Chapter two: Genetic environment of an aminoglycoside resistance gene, *aacC2a*, in clinical isolates of *A. baumannii* from GSH and RCH

2.1 Introduction 19
2.2 Experimental protocol 22
2.2.1 Bacterial strains, growth conditions and antibiotic susceptibility testing 22
2.2.2 Large scale genomic DNA preparation 23
2.2.3 Polymerase chain reaction 24
2.2.4 Agarose gel electrophoresis and nucleic acid visualization 25
2.2.5 DNA purification 26
2.2.5.1 DNA in an agarose gel 26
2.2.5.2 DNA in aqueous phase 27
2.2.5.3 DNA quantification 28
2.2.6 DNA sequencing and analysis 28
2.2.7 Southern Blotting 29
2.2.7.1 Southern transfer 29
2.2.7.2 Preparation of the probe 31
2.2.7.3 Hybridisation and post-hybridisation washes 32
2.2.7.4 Signal detection and washes 32
2.2.8 Preparation of a DNA library from *A. baumannii* strain SAK 33
2.2.8.1 Preparation of genomic DNA 33
2.2.8.2 Preparation of pUC19 vector DNA 33
2.2.8.3 Preparation of competent *E. coli* JM109 cells 33
2.2.8.4 Ligation and transformation 34
2.2.9 Small-scale plasmid preparation 35
2.3 Results
2.3.1 Detection of *aacC2a* in gentamicin resistant clinical *A. baumannii* isolates 36
2.3.2 Genetic arrangement of *aacC2a* in clinical *A. baumannii* isolates 37
2.3.3 Detection of a portion of IS*1133* in clinical *A. baumannii* isolates 40
2.3.4 Detection of a full copy of IS*1133* in clinical *A. baumannii* isolates 43

University of Cape Town

2.3.5 DNA-DNA hybridization studies to confirm IS*1133* interrupted by a full copy of ISAba-1 in *A. baumannii* isolates 45
2.3.6 Investigation of the genetic environment of IS*1133* in clinical *A. baumannii* isolates 48
2.4 Discussion 51

Chapter three: The expression of an aminoglycoside resistance gene, *aacC2a* in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

3.1 Introduction 55
3.2 Experimental protocol
3.2.1 Preparation of *E. coli* JM109 (pRK002) 59
3.2.2 The plotting of a growth curve for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) 60
3.2.3 Preparation of total cellular RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) using hot acid phenol 61
3.2.4 DNase treatment of recovered RNA 62
3.2.5 Reverse transcriptase-polymerase chain reaction 62
3.2.6 Primer extension studies 63
3.3 Results
3.3.1 Expression of *aacC2a* in *E. coli* JM109 (pRK002) 65
3.3.2 Extraction of total cellular RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) 65
3.3.3 Expression of the *aacC2a* gene in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) 66
3.3.4 Investigation of the transcriptional start site of *aacC2a* gene in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) 68
3.4 Discussion 72

University of Cape Town

Chapter four: Investigation into the clonal relatedness amongst *A. baumannii* isolates from GSH and RCH

4.1 Introduction 76

4.2 Experimental procedure

4.2.1 PFGE 79

4.2.1.1 Plug preparation 79

4.2.1.2 Lysis of plug 79

4.2.1.3 Restriction digestion 79

4.3 Results 81

4.4 Discussion 87

Chapter five: Final discussion and concluding remarks

References 93

Appendix A 104

Appendix B 108

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Chapter one

Literature review

1.1 The emergence of *Acinetobacter baumannii* as an important nosocomial pathogen

One of the greatest achievements of modern medicine is the discovery and extensive use of antimicrobial agents. However, the emergence and spread of antibiotic resistant organisms has been taking place since the introduction of these antimicrobial therapies over 50 years ago. Unfortunately, the past two decades have seen a marked decline in the discovery and development of novel antibiotics coupled with a dramatic increase in resistance to those currently available, and concern is growing that we may be at the end of antibiotic era. An increase in levels of antibiotic resistance has been particularly prevalent in the hospital environment where acquired nosocomial pathogens; such as vancomycin-resistant *Enterococci* (Harbeth *et al*, 2002), *Pseudomonas aeruginosa* (Aubert *et al*, 2003) and methicillin-resistant *Staphylococcus aureus* (Edmond *et al*, 1999) are becoming quite troublesome to treat.

One such bacterial pathogen and the main focus of this study is *Acinetobacter baumannii*. The *Acinetobacter* genus, belonging to the family *Moraxellaceae* (Bergogne-Bérézin & Towner, 1996), is composed of pleomorphic, Gram-negative, aerobic bacteria (Forster *et al*, 1998). *Acinetobacters* are naturally ubiquitous and have been isolated from a variety of sources, including water, soil, sewage and the skin of humans (Bergogne-Bérézin & Towner, 1996; Forster *et al*, 1998; Da Silva *et al*, 1999).There are at least 32 *Acinetobacter* species described thus far, but of these *A. baumannii* is the best characterised. Over the past 30 years, *A. baumannii* has become one of the most recognized opportunistic pathogens affecting immuno-compromised patients and numerous outbreaks of nosocomial infections due to *A. baumannii* have been reported worldwide (Bou *et al*, 2000; Brown *et al*, 2005).

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The most common site of infection is the respiratory tract but *A. baumannii* has been isolated from numerous other infection sites, including septicaemia, pneumonia, endocarditis, meningitis, skin and wound infections and urinary tract infections (Bergogne-Bérézin & Towner, 1996; Forster *et al*, 1998). A number of factors have been implicated in the spread of these bacteria such as the length of ICU stay, contamination of medical equipment, direct contact between infected individuals and the overuse of certain antibiotics (Bergogne-Bérézin & Towner, 1996, Lee *et al*, 2004). Recently, multi-drug resistant (MDR) *A. baumannii* have emerged at high incidence from bloodstream infections in US army soldiers injured in the war in Iraq (Turton *et al*, 2006 (a)). These organisms are resistant to many of the clinically used antibiotics, including the aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline and the β-lactams antibiotics excluding imipenem (Turton *et al*, 2006 (a)).

Recently, whole genome sequencing was carried out to compare the DNA content of a MDR *A. baumannii* strain AYE, endemic in France and a fully susceptible *A. baumannii* strain SDF, associated with human body lice (Fournier *et al*, 2006). Surprisingly, most of the genes responsible for conferring resistance to the currently used clinical antibiotics including the β-lactams, aminoglycosides, chloramphenicol and tetracycline antibiotics, were all clustered in an 86-kb region in the genome of *A. baumannii* AYE (Fournier *et al*, 2006).

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1.2 Mechanisms contributing to antibiotic resistance in *A. baumannii*

There are four major mechanisms contributing to antibiotic resistance in *A. baumannii*, and a single mechanism or a combination of mechanisms can give rise to resistance.

These include (i) a decrease in outer membrane permeability, due to a reduced expression or loss of outer membrane proteins (OMPs) (Clark *et al*, 1996; Livermore, 2003; Mussi *et al*, 2005), (ii) antibiotic expulsion from the cell by efflux pumps (Marchand *et al*, 2004) (iii) alteration of antimicrobial target sites (Gehrlein *et al*, 1991; Fernandez-Cuenca *et al*, 2003), (iv) and the enzymatic hydrolysis or inactivation of the antibiotic (Afzal-Shah *et al*, 2001; Chu *et al*, 2001; Brown & Amyes, 2005). These mechanisms can usually arise due to the acquisition of new genes on transferable mobile genetic elements (Gombac *et al*, 2002; Segal *et al*, 2004; Corvec *et al*, 2007) or as a result of mutation(s) that have arisen in the existing genome.

1.2.1 Decrease in outer membrane permeability

A decrease in the permeability of the outer membrane due to a loss or reduced expression of an OMP, limits the entry of antibiotic into the bacterial cell and subsequent access to its target, the PBPs, therefore resulting in resistance (Gehrlein *et al*, 1991). Antibiotic resistance due to a decreased expression of an outer membrane protein has previously been shown among a number of Gram-negative bacteria including *Pseudomonas aeruginosa* (Llamas *et al*, 2003), *Enterobacter* and *Proteus* spp (Rasmussen *et al*, 1997) however, knowledge concerning the permeability of antibiotics through the outer-membrane of *A. baumannii* is limited. Costa *et al* (2000), investigated the *in-vivo* selection of carbapenem resistant *A. baumannii* isolates and determined that in these isolates resistance was due to the acquisition of two β-lactamases as well as the subsequent loss in the expression of an outer membrane protein of 31 - 36 kDa (Costa *et al*, 2000).

Imipenem resistance in *A. baumannii* can be associated with the loss of a 29 kDa OMP in clinical isolates which displayed no carbapenemases (Limansky *et al*, 2002). Subsequently, both imipenem and meropenem resistance have also been attributed to the loss of a heat-modifiable, constitutively expressed 29 kDa OMP, designated carbapenem resistance associated outer membrane protein' (CarO) (Mussi *et al*, 2005). This study determined that *carO* is present as a single copy located on the chromosome and encodes a polypeptide that consists of 247 amino acid residues, has an N-terminal signal sequence and a β-barrel topology (Mussi *et al*, 2005). The lack of CarO in a variety of different carbapenem resistant clinical isolates of *A. baumannii* was determined to have resulted from the disruption of *carO* by distinct insertion elements resulting in loss gene expression (Mussi *et al*, 2005). This result supports the hypothesis that CarO participates in the influx of carbapenem antibiotics into *A. baumannii*. Consequently, Siroy *et al* (2005) investigated the role of CarO further and found that although CarO displays pore forming properties, it contains no specific binding sites for imipenem and therefore may allow for the entry of non-specific substrates (Siroy *et al*, 2005).

It has also been reported that resistance to the carbapenems may be associated with the reduced expression of a 22 and a 33 kDa protein, as was shown in a MDR *A. baumannii* isolate that also produced a *bla*$_{OXA-24}$ (Bou *et al*, 2000). This result suggests that both the expression

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of carbapenemases and a decrease in membrane permeability may be contributing to the high-levels of resistance observed in this isolate (Bou *et al*, 2000).

Interestingly, Del mar tomas *et al* (2005) investigated a clinical *A. baumannii* isolate that displayed no carbapenemase activity but still exhibited high-levels of resistance to carbapenems. Further investigation of this strain's mechanism of resistance revealed the loss of a 33-36 kDa OMP, which after subsequent complementation assays resulted in restoration of the lost OMPs functionality and a decrease in carbapenem resistance (Del mar tomas *et al*, 2005).

1.2.2 Expulsion of antimicrobial compounds

The expulsion of various compounds, including antibiotics, by efflux systems is a common mechanism of resistance in a number of bacterial species. An efflux pump comprised of a trimer of proteins was described in the outer membrane of *A. baumannii* strain BM4454 (Magent *et al*, 2001). The AdeABC efflux pump belonging to the resistance-nodulation-cell-division (RND) family of efflux systems has been shown to allow expulsion of a number of structurally different compounds, including most of the clinically used antibiotics (Magnet *et al*, 2001). Consequently, the overexpression or activation of this pump contributes to high levels of resistance to the aminoglycosides, as well as a decreased susceptibility to a number of other drugs including the fluoroquinolones, chloramphenicol, cefotaxime, erythromycin (Magnet *et al*, 2001) and recently, the tigecyclines (Peleg *et al*, 2007). The *adeABC* operon is chromosomally encoded and preceded by an *adeRS* gene cluster with sequence homology to a two component regulatory system and is transcribed in the opposite direction to the *adeABC* operon (Marchand *et al*, 2004). When the *adeRS* regulatory system is inactivated, the AdeABC efflux system is constitutively expressed and a two-fold increase in the MICs of both imipenem and meropenem is observed, suggesting that the AdeABC efflux system is involved in contributing to carbapenem resistance in clinical *A. baumannii* isolates (Héritier *et al*, 2005).

Another multi-drug efflux pump belonging to the multi drug and toxic compound extrusion (MATE) family of multi-drug transporters, designated AbeM, has been described (Su *et al* 2005). The *abeM* gene encodes an efflux pump that is a unique member of the MATE family and requires proton coupling in order to function effectively (Su *et al*, 2005). The AbeM efflux

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and requires proton coupling in order to function effectively (Su *et al*, 2005). The AbeM efflux pump, was shown to have substrate specificity for a number of clinically used antibiotics including the aminoglycosides, fluoroquinolones and trimethoprim (Su *et al*, 2005).

1.2.3 Alteration of antimicrobial binding sites

Limited research into the alterations of PBPs in *A. baumannii* has been conducted. A study by Gehrlein *et al* (1991) compared the PBP profiles of a carbapenem susceptible isolate and a derived resistant mutant obtained *in vitro*. The mutant strain exhibited a diminished expression of six PBPs, each distinguishable by their different molecular weight and an increased expression of a seventh, 24 kDa PBP, when compared to the imipenem susceptible *A. baumannii* strain (Gehrlein *et al*, 1991). Analysis of the hyper-produced 24 kDa PBP revealed an increased expression in conjunction with resistance to imipenem, which could not be saturated by increasing the concentration of this antibiotic suggesting, that resistance to imipenem arises due to low affinity of this compound for the PBPs (Gehrlein *et al*, 1991). Interestingly, not only was an increase in resistance to imipenem observed but also an increase in resistance to ampicillin when there was a diminished expression of the other six PBPs (Gehrlein *et al*, 1991). How these alterations to the target PBPs arose in *A. baumannii* is still under investigation, although it has been suggested that the 24 kD PBP is the possible precursor for the other six PBPs (Gehrlein *et al*, 1991). Therefore, a mutation in the transcriptional sequences or post-translational modification of this precursor could lead to decrease in the expression of the other six PBPs thus contributing to an increase in the 24 kDa PBP precursor (Gehrlein *et al*, 1991).

Another study has described the existence of 12 PBP patterns among a collection of *A. baumannii* isolates which displayed variable β-lactam resistance profiles (Femandez-Cuenca *et al*, 2003). Isolates with imipenem MICs > 4 mg/L, were shown to have an absence of the 73.2 kDa PBP which in conjunction with the production of a β-lactamase, could contribute to carbapenem resistance in these strains (Femandez-Cuenca *et al*, 2003).

The alteration of certain target sites can result in resistance to the quinolone antibiotics. This can be caused by a mutational change occurring in either the *gyrA* or *parC* genes. A mutation occurring in either the *gyrA* or *parC* genes results in structural changes occurring in DNA

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gyrase and DNA topoisomerase, respectively (Dever & dermondy, 1991). A mutation or mutations occurring in either *gyrA* or *parC* can result in a compromised functionality of DNA replication and folding. In *Acinetobacter* low levels of resistance to ciprofloxacin and high levels of resistance to the quinolones has been shown to be associated with a mutation occurring in both *gyrA* and *parC* (Spence & Towner, 2003). A mutational change occurring at amino acids Ser-83 and Gly-81 in GyrA and mutations occurring in Ser-80 and Glu-84 in ParC are the most commonly reported mutational changes identified in *A. baumannii* (Bergogne-Bérézin & Towner, 1996; Spence & Towner, 2003). Notably, a reduced expression of outer membrane proteins coupled with an up-regulation of efflux pump expression, can account for an increased resistance to the quinolones (Bergogne-Bérézin & Towner, 1996; Van Looveren *et al*, 2004).

1.2.4 Enzymatic inactivation

The expression of antibiotic resistance genes which produce enzymes capable of enzymatic hydrolysis of certain antibiotics, have been described numerously in clinical isolates of *A. baumannii*. Enzymatic inactivation of the clinically important antibiotics, the β-lactams and aminoglycosides, is commonly reported in strains of *A. baumannii* worldwide (Bou *et al*, 2000; Poirel *et al*, 2003; Lee *et al*, 2005; Segal *et al*, 2007).

1.2.4.1 β - lactamases

Initially the β–lactam antibiotics were effective in the fight against *A. baumannii* infections but resistance to this class of antibiotics is widely reported. All β–lactamases described thus far have been organised according to two distinct classification systems based on their substrate profile and inhibition properties. The Ambler-classification system separates these enzymes into 4 Ambler classes A – D based on the enzymes substrate profile and gene sequences (Table 1.1). The Bush classification scheme separates the β-lactam hydrolysing enzymes into groups I, II, III, based on their substrate profiles only (Bush *et al*, 1995). In this review, the sequence based classification system derived by Ambler will be used to discuss the β-lactamases in *A. baumannii*.

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Table1.1: Ambler Classification scheme for β-lactamases in *A. baumannii*

Class	Name	Representative enzymes
A	* Extended spectrum β-lactmases (ESBLs) * Broad-spectrum β-lactmases	TEM-5, TEM-10, SHV, PER, VEB, CTX-M, CARB TEM
B	Metallo-β-lactamases	IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, IMP-11, VIM-1 and VIM-2, SIM
C	AmpC chromosomal β-lactmases	AmpC
D	Oxacillinases	OXA-10, OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, OXA-51-like, OXA-58

* ESBLs and broad-spectrum β-lactamases both belong in Ambler class A

Class A: Extended spectrum-β-lactamases

There is no consensus on the precise definition for the extended spectrum β-latamases (ESBLs), however, ESBLs are β-lactamases capable of conferring bacterial resistance to a number of classes of antibiotics including, penicillins, the first, second and third-generation cephalosporins and aztreonam, but not the cephamycins or carbapenems (Poirel *et al*, 1999). The hydrolyzing action of these enzymes is inhibited by β-lactamase inhibitors such as clavulanic acid (Poirel *et al*, 1999). The first β-lactamase identified in *Acinetobacter* spp. was a plasmid mediated TEM-1 enzyme (Goldstein *et al*, 1983; Bou *et al*, 2000). A Later study revealed the presence of a TEM-2 and CARB-5 β-lactamase which were plasmid encoded (Devaud *et al*, 1982). The bla_{PER-1} gene first described in *Pseudomonas* spp is now widespread amongst *Acinetobacter* spp from Turkey, France and Korea (Vahaboglu *et al*, 1997; Poirel *et al*, 1999). Both the CTX-M-2 and CTX-M-5 enzymes displaying high activity against cefotaxime have been described in *A. baumannii* (Nagano *et al*, 2004).

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Another ESBL to be described in a MDR *A. baumannii* was the plasmid-mediated SHV-12, first characterized by Huang *et al* (2004). In another study, VEB-1 initially identified on an integron *P. aeruginosa* has subsequently been identified in a nosocomial isolate of *A. baumannii* (Poirel *et al*, 2003). Interestingly, ESBLs production is rare in *Acinetobacter* spp when compared to Ambler class B, C or D enzymes.

Class B: Metallo-ß-lactamases

Previously, the metallo-ß-lactamase (MBL) enzymes, which require a bivalent metal ion for activity, have been reported in scattered isolates of *Pseudomonas aeuriginosa, Serratia marcescens* and *Klebsiella pneumonia* (Rasmussen *et al*, 1997), however, this group is increasingly being reported in clinical isolates of *A. baumannii* worldwide. Of the five groups of acquired MBLS identified to date IMP, VIM and SIM have all been identified in *A. baumannii* (Ricco *et al*, 2000; Gales *et al*, 2003; Lee *et al*, 2005) The IMP and VIM variants are responsible for conferring high levels of resistance to all of the ß-lactam and carbapenem antibiotics, except for aztreonam. Conversely, isolates containing the SIM variants confer a much lower level of resistance to the ß-lactam and carbapenem antibiotics (Lee *et al*, 2005). To date a total of six IMPs from the 19 variants already described have been identified in clinical isolates of *A. baumannii*. These six include; IMP-1 and IMP-2 in Italy (Riccio *et al*, 2000), IMP-4 in Hong Kong, which displays 95.6% homology to IMP-1 and 89.3% homology to IMP-2 (Chu *et al*, 2001), IMP-5 in Portugal (Da silvia *et al*, 2002), IMP-6 in Brazil (Gales *et al*, 2003) and IMP-11 in Japan (Walsh *et al*, 2005).

Both the VIM and SIM enzymes have been identified very rarely in *A. baumannii*, and are mostly seen in clinical isolates of *A. baumannii* from South Korea (Lee *et al*, 2005). Much research has gone into elucidating the genetic environment of MBLs and it has been discovered most of the genes encoding MBLs form gene cassettes in class-1 integrons (Yum *et al*, 2002; Poirel & Nordmann, 2002).

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Class C: AmpC

AmpC β-lactamases are usually inducible by β-lactam antibiotics and are encoded by genes chromosomally located in Gram-negative bacteria. These enzymes confer resistance to cephamycins as well as to oxyimino-β-lactams and are resistant to inhibition by clavulanic acid (Corvec *et al*, 2003). An intrinsically expressed AmpC-type cephalosporinase is produced at basal levels in all clinical *A. baumannii* isolates but does not confer resistance to extended-spectrum cephalosporins in these strains (Bou *et al*, 2000). Resistance to this group of antibiotics can arise due to the introduction of an insertion element upstream of the *ampC* (Corvec *et al*, 2003). The most commonly described IS element associated with *ampC* is IS*Aba-1*, which inserts upstream of the gene and provides the promoter sequences necessary gene expression (Segal *et al*, 2004).

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Class D: Oxacillinases

The first description of a carbapenem-hydrolyzing enzyme in *A. baumannii* was from an isolate obtained from Edinburgh, Scotland in 1985. This enzyme designated ARI-1 encodes resistance to imipenem and is carried on a 45 kb plasmid, pUK1356, which is self-transferable between *Acinetobacter* and other bacterial spp (Scaife *et al*, 1995). The ARI-1 gene was later re-named bla_{OXA-23} (Donald *et al*, 2000), after biochemical analysis determined it shares 56% amino acid identity to the OXA-51/69 variant (Poirel & Nordmann, 2006 (b)). All of the oxacillinase enzymes classified thus far fall into three subgroups. The first subgroup consists of OXA-23 (Scotland) (Donald *et al*, 2000), OXA-27 (Singapore) and OXA-49 (China) (Afzal-Shah *et al*, 2001), as theses three enzymes share 99% amino acid sequence identity with one another. The bla_{OXA-23} has frequently been identified in a number of carbapenem resistant clones spread throughout most of the United Kingdom (Turton *et al*, 2005). Interestingly, the bla_{OXA-23} has also been identified in clinical isolates from South Africa (Segal *et al*, 2007) and Brazil (Dalla-Costa *et al*, 2003).

The second subgroup, mostly epidemic in Spain (Dalla-Costa *et al*, 2003) differs from the first sub-group by one or two amino-acid changes and consists of OXA-24, OXA-25, OXA-26, and OXA-40 (Lopez-Otsoa *et al*, 2002). OXA-40 shares 63% and 60% amino acid identity with OXA51/69 and OXA-23, respectively (Lopez-Otsoa *et al*, 2002). There is a 60% amino acid

similarity between these two subgroups, but all of these enzymes possess a tyrosine to phenylalanine substitution occurring in the conserved Y-G-N motif (Brown *et al*, 2005).
A. baumannii also produces another intrinsic β – lactamase which is expressed at relatively low levels and is represented by OXA-51/69 variants (Héritier *et al*, 2005; Brown & Amyes 2005). Oxacillinases are able to hydrolyse the following antibiotics; amoxicillin, methicillin, oxacillin and cephalridine (Poirel & Nordmann, 2006 (b)). Brown *et al* (2005) identified several OXA-type β-lactamases that have been placed into a third subgroup based on their relatedness to OXA-51, this subgroup includes OXA-51, OXA-65, OXA-66, OXA-69, OXA-64, OXA-70, and OXA-71. These enzymes have been isolated from strains from a variety of continents worldwide, and subgroup three now represents the most widely found class D subgroup of carbapenemases in *A. baumannii* (Brown *et al*, 2005; Brown & Amyes, 2005).

The acquisition of oxacillinase genes is still under investigation because although the *bla*$_{OXA-23}$ and the *bla*$_{OXA-58}$ are both carried on plasmids, genes belonging to the OXA-24 subgroup seem to be chromosomally encoded. Interestingly, both *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ are often associated with insertion sequences and studies have shown that promoter sequences in the adjacent IS element are responsible for an increase in gene expression (Poirel & Nordmann, 2006 (a); Corvec *et al*, 2007; Segal *et al*, 2007).

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1.2.4.2 Aminoglycoside modifying enzymes

The first aminoglycoside antibiotic, streptomycin, was used clinically in 1944 (Vakulenko & Mobashery, 2003). Generally the newer aminoglycoside antibiotics such as gentamicin, tobramycin and amikacin have a broader range of antimicrobial activity against Gram-negative pathogens than the older compounds such as streptomycin and kanamycin. Importantly, the aminoglycoside antibiotics exhibit activity against a wide variety of clinically important Gram-negative bacilli including *Escherichia coli*, *Salmonella* spp, *Pseudomonas* spp, as well as *Acinetobacter* spp (Vakulenko & Mobashery, 2003). Aminoglycosides act by binding to the bacterial ribosome thus inhibiting protein synthesis however, the precise mechanism of binding is not that well understood, as the ribosome is a complex structure, comprising three RNA molecules and over 50 proteins (Vakulenko & Mobashery, 2003). Although all aminoglycosides bind to the 30S ribosomal subunit, the exact mechanism of action varies depending on the different class of aminoglycoside antibiotic.

In *A. baumannii*, aminoglycoside resistance can result from one of three possibilities; alteration of the antibiotic target site, a diminished uptake of the antibiotic resulting in low levels of broad spectrum resistance or via the enzymatic modification of the aminoglycosides which then binds poorly to the ribosome and allows the bacteria to survive in the presence of the drug (Fournier *et al*, 2006). However, aminoglycoside resistance in clinical isolates of *A. baumannii* most often arises due to enzymatic modification by aminoglycoside modifying enzymes (AMEs). Three classes of AMEs have been described (Foster, 1983, Philips & Shannon, 1984, Mingeot-Leclercq *et al*, 1999) and these enzymes are classified according to their site of action and substrate modification. The three classes of AMEs include; the O-nucleotidyltransferases (ANT), enzymes that allow for adenylation of the antibiotic, O-phophotransferases (APH), which bring about phosphorylation of hydroxyl groups and finally the N-acetyltransferases (AAC), which catalyses acetylation of the amino acid groups inactivating the antibiotic.

The three classes of AMEs have all been described in *Acinetobacter* spp as indicated in Table 1.2. Interestingly, an aminoglycoside antibiotic can be inactivated by more than one AME. Geographic variation is apparent amongst the acquisition of particular genes and it has been shown that certain strains may harbour as many as 6 different aminoglycoside resistance genes (Shaw *et al*, 1993) carriedon plasmids, transposons or associated with integron-type structures (Murray *et al*, 1980, Shaw *et al*, 1993).

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Table 1.2: Aminoglycoside modifying enzymes in *Acinetobacter* spp

AME	Reference
Phosphotransferases	
APH(3')I	Bergogne-Bérézin & Towner, 1996
APH(3')II	Bergogne-Bérézin & Towner, 1996
APH(3')III	Seward *et al*, 1998
APH(3')VI	Vakulenko & Mobashery, 2003
Adenylases	
AAD(3")I	Bergogne-Bérézin & Towner, 1996
AAD(2')I	Seward *et al*, 1998
Acetyltransferases	
AAC(6')	Bergogne-Bérézin & Towner, 1996
AAC(2')I	Bergogne-Bérézin & Towner, 1996
AAC(3)I	Bergogne-Bérézin & Towner, 1996
AAC(3)II	Seward *et al*, 1998
AAC(3)IV	Seward *et al*, 1998

Modification of table taken from Bergogne-Bérézin & Towner, 1996

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1.2.4.2.1 Aminoglycoside phosphotransferases

The APHs act as kinase by using ATP as a cofactor and are able to phosphorylate specific hydroxyl groups on all classes of aminoglycoside antibiotics (Vakulenko & Mobashery, 2003). This group of phosphotransferases are separated into seven classes of enzymes; APH(3'), APH(2"), APH(3"), APH(4), APH(7"), APH(6) AND APH(9) (Vakulenko & Mobashery, 2003). The largest class of enzymes in this group are the phosphotranferases that modify the 3' position of the hydroxyl group on the aminoglycoside molecule.

Within this class of enzymes seven different subgroups of aminoglycoside phosphotransferases comprising APH(3')-I to APH(3')-VII, have been identified in all Gram-negative bacteria (Bergogne-Bérézin & Towner, 1996). The gene encoding APH(3')-VI has been reported to be primarily associated with aminoglycoside resistance in clinical *A. baumannii* isolates conferring amikacin resistance (Martin *et al*, 1988; Shaw *et al*, 1993).

1.2.4.2.2 Aminoglycoside nucleotidyltransferases

The aminoglycoside nucleotidyltransferases (ANTs) act by transferring AMP to their hydroxyl group using ATP resulting in structural changes occurring to the antibiotic (Vakulenko & Mobashery, 2003). The ANTs comprises five groups; ANT(2"), ANT(3"), ANT(4'), ANT(6) and ANT(9). This group of enzymes have been identified in many clinical isolates of *A. baumannii*, however both ANT(2") and ANT(3") are more commonly described in *A. baumannii* (Bergogne-Bérézin & Towner, 1996; Seward *et al*, 1998).

The investigation of 24 aminoglycoside resistant *A. baumannii* isolates revealed that each isolate harboured at least one aminoglycoside modifying enzyme. The ANT(3")-I enzyme was by far the most common in combination with another class of enzyme and it is quite common for an aminoglycoside resistant *A. baumannii* to contain more than one AME gene (Seward *et al*, 1998). An aminoglycoside resistant *A. baumannii* isolate, designated strain SAK was shown to carry two aminoglycoside resistance genes coding the gene products AAD(2") and AAC(3) (Elisha & Steyn, 1991). Only the AAC(3) gene in strain SAK was shown to be expressed and therefore responsible for conferring resistance to the aminoglycosides in this strain (Elisha & Steyn, 1991). Cloning and expression of AAD(2") in an *E. coli* host demonstrated that the structural component of this gene remains intact and perhaps the absence of gene expression is due to changes occurring in the regulatory elements of this gene in *A. baumannii* strain SAK (Elisha & Steyn, 1991).

The expression of AMEs can occur either from promoter sequences upstream of the gene or from promoter sequences located in mobile genetic elements (Elisha, 1991; Allmansberger *et al*, 1985). The aminoglycoside resistance gene *aadB* which forms part of a gene cassette in

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A. baumannii strain SUN was integrated at a secondary site on a 6.0kb plasmid designated pRAY and the expression of this gene was shown to be dependant from promoter sequences on pRAY (Segal & Elisha, 1997; Segal & Elisha, 1999).

1.2.4.2.3 Aminoglycoside acetyltransferases

Aminoglycoside acetyltransferases act by using a coenzyme, acetyl-coenzyme A, in the modification of the aminoglycoside molecule. This group comprises; AAC(1), AAC(3), AAC(2') and AAC(6') (Vakulenko & Mobashery, 2003). The AAC(3) group of enzymes are widely distributed among different bacterial genera and they constitute the second largest group of AMEs after the AAC(6')-I enzymes. Representatives from each one of the above mentioned groups have all been isolated either singly or in conjunction with another AME in clinical *A. baumannii* strains (Shaw *et al*, 1993; Elisha & Steyn, 1994; Seward *et al*, 1998). The AAC(3)-IIa enzyme, encoded by the *aacC2a* gene, modifies the clinically important antibiotics, gentamicin and tobramycin, and the gene encoding this enzyme, has been identified in clinical and environmental isolates of *A. baumannii* (Seward *et al*, 1998; Nemec et *al*, 2004; Diaz *et al*, 2006). A number of acetyltransferases have been found on gene cassettes in variable regions of class 1 integrons in *A. baumannii* (Nemec *et al*, 2004). A novel aminoglycoside resistance gene *aac(6') –lad*, which encodes an aminoglycoside 6'-N-acetyltransferase in *A. baumannii* strains endemic to Japan, is believed to be responsible for the increase in amikacin resistance in this area (Doi *et al*, 2004).

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1.3 Genetic environment and expression of antibiotic resistance genes

Mobile elements including plasmids, transposons, integrons and insertion sequences all provide vehicles of transport for the transfer of resistance genes from one bacterium to another. In addition, integrons and insertion sequences often provide promoter sequences necessary for the expression of the associated resistance genes (Segal *et a*l, 2004; Segal *et al*, 2005; Poirel *et al*, 2006; Segal *et al*, 2007; Corvec *et al*, 2007).

Mahillon & Chandler (1998) define insertion sequences as "small, < 2.5 kb phenotypically cryptic segments of DNA that have a simple genetic structure and are capable of inserting at multiple sites within a target molecule" (Mahillon & Chandler, 1998). IS elements are typically

between 800 and 2500 bp long and contain two distinguishing components, a single transposase gene, *tnpA*, (typically 250 – 400 amino acids in length) necessary for its mobility and an inverted repeat (IR) structure at the termini (Mahillon & Chandler, 1998; Lee *et al*, 2005). IS elements have been shown to cause spontaneous mutations resulting in the alteration of gene expression (Segal *et al*, 2003; Segal *et al*, 2005; Segal *et al*, 2007) and can cause the genetic re-arrangement of a bacterial chromosome (Corvec *et al*, 2007).

The extended spectrum β-lactamase PER-1 is widespread in *Acinetobacter* spp (Vahaboglu *et al*, 1997) and has been located downstream of an IS element, IS*Pa12*, in *A. baumannii* AMA-1 (Poirel *et al*, 2005). Further analysis of the regions located downstream of this chromosomally encoded *bla*$_{PER-1}$, in the same *A. baumannii* isolate, revealed that *bla*$_{PER-1}$ forms part of a composite transposon Tn*1213* and that downstream of *bla*$_{PER-1}$ is another IS element, IS*Pa13* (Poirel *et al*, 2005). IS*Pa12* and IS*Pa13* belong to the IS*4* family of insertion sequences (Poirel *et al*, 2005).

The β-lactamase resistance gene *bla*$_{OXA-58}$ is often plasmid located and is believed to be the main cause of carbapenem resistance in *A. baumannii* worldwide (Poirel & Nordmann, 2006 (a)). In a study comparing the genetic environment and expression of the *bla*$_{OXA-58}$ gene from various European *A. baumannii* isolates, it was determined that expression of *bla*$_{OXA-58}$ was dependant on promoter sequences provided by a number of different insertion sequence elements (Poirel & Nordmann, 2006 (a)). An IS*Aba3*-like element, as well as IS*Aba2* and IS*18*, were identified upstream of *bla*$_{OXA-58}$, providing promoter sequences necessary for transcription of this gene (Poirel & Nordmann, 2006 (a)). Insertion sequence IS*18* is a member of the IS*30* family and encodes a 320 amino acid transposase (Rudant *et al*, 1998). In an earlier study it was determined that the transposition of IS*18* upstream of a silent aminoglycoside resistance gene *aac(6')-Ij* in a clinical *Acinetobacter* spp 13 strain was responsible for expression of the previously silent aminoglycoside resistance *aac(6)-Ij* gene (Rudant *et al*, 1998). In a similar study, Rudant *et al* (1997) showed that the loss of an intrinsic aminoglycoside resistance gene in *Acinetobacter haemolyticus* was in part due to the insertion of a 1049 bp fragment, 50 bp downstream of the initiation codon of *aac(6')-Ig*. This sequence, designated IS*17*, displayed similarity to sequences within the IS*903* family of insertion elements (Rudant *et al*, 1997). The insertion of IS*17* in *aac(6')-Ig* lead to inhibition of expression of *aac(6')-Ig* and resulted in a reversion to aminoglycoside susceptibility (Rudant *et al*, 1997).

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IS*Aba-1*, a member of the IS*4* family of insertion elements, is 1180 bp long and contains two ORFs of 189 and 178 amino acids (Héritier *et al* 2006). This element contains a 16bp inverted repeat and generates a 9 bp duplication of the target sequence upon insertion (Héritier *et al* 2006). IS*Aba-1* was first described as a "homologous region" upstream of the aminoglycoside modifying enzyme *aacC2a* in *A. baumannii* strain PAU (Segal *et al*, 2003). It has since been located upstream of an *ampC* gene in clinical *A. baumannii* isolates where it was thought to provide promoter sequences for the over-expression of this gene (Corvec *et al*, 2003). When *ampC* was cloned from a clinical *A. baumannii* isolate strain RAN, the transcription of the gene was shown to be dependant on promoter sequences within IS*Aba-1* (Segal *et al,* 2004). The expression of β-lactamase resistance genes, bla_{OXA-23} and bla_{OXA-27}, as well as *sulII* from *A. baumannii* are similarly dependant on the same promoter sequences in IS*Aba-1* (Segal *et al*, 2003; Segal *et al*, 2005; Segal *et al*, 2007; Poirel *et al*, 2007). Recently, the bla_{OXA-23} gene in a clinical *A. baumannii* isolate was shown to be flanked by two copies of IS*Aba-1*, located in opposite orientations (Corvec *et al*, 2007). Therefore, it is possible that IS elements play a role in recombination events, which may contribute to acquisition and provide information on the possible origin of certain β-lactamase genes in *A. baumannii* (Corvec *et al*, 2007).

The high copy number of IS*Aba-1* and its location upstream of a number of antibiotic resistance genes in A. *baumannii* perhaps reflects the mobility of this genetic element (Segal *et al*, 2005). Interestingly, IS*Aba-1* has never been characterised outside of *Acinetobacter* spp (Segal *et al*, 2005). The authors postulate that IS*Aba-1* may be exclusive to *Acinetobacter* and may provide the bacterium with a selective advantage when survival is dependant on the efficient expression of antibiotic resistance genes (Segal *et al*, 2005).

IS*1133*, belonging to the IS*3* family of IS elements, was identified in *Erwinia amylovora*, a plant pathogen which causes fire blight in apple trees (Chiou *et al*, 1993). In *E. amyolvora* the IS*1133* element is part of transposon Tn*5393* and is associated with two phosphotransferase genes, *aph(6)-Ia* and *aph(6)-Id* (Chiou *et al*, 1993). The expression of theses two aminoglycoside resistance genes was shown to be dependant on promoter sequences within the IS*1133* element (Chiou *et al*, 1993). IS*1133* has also been identified on plasmids and in the chromosome of *Salmonella* spp isolated from poultry in Italy, and was thought to have been acquired through genetic transfer from pathogens in contaminated animal feeds (Pezzella *et al*, 2004).

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IS*1133* has also been identified in a clinical *A. baumannii* strain SAK, isolated in 1983 (Elisha, 1991). In this strain a portion of IS*1133* was located upstream of an aminoglycoside resistance gene, *aacC2a* (Elisha, 1991). Subsequently, a portion of IS*1133* was identified upstream of *aacC2a* in *A. baumannii* strain PAU, isolated 9 years later (Segal *et al*, 2003). To investigate the genetic environment of the portion of IS*1133* linked to *aacC2a* in *A. baumannii* strain PAU, a 6.08 kb *Hin*dIII fragment was cloned into the *Hin*dIII site of the vector pUC19 and sequenced (Segal *et al*, 2003). DNA sequence analysis determined that the functional *aacC2a* gene, linked to a portion of IS*1133*, is associated with a hypothetical protein gene (*hpg*) at its 5' end (Figure 1.1) (Segal *et al*, 2003). The 3' end of the portion of IS*1133* is linked to sequence designated ORF1 and ORF 2, with homology to *tnpA*. ORF 1 and ORF 2 were later characterised as a portion of the IS element, *ISAba-1* (Segal *et al*, 2003).

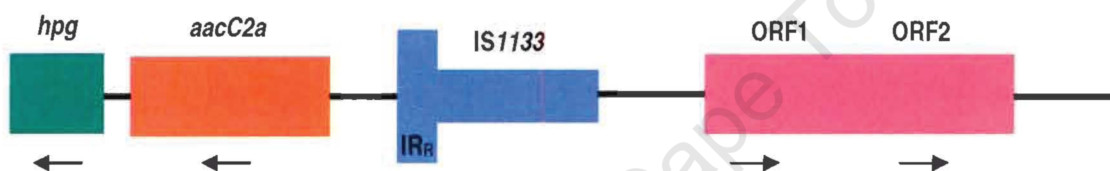

Figure 1.1: Schematic representation of the genetic organisation of the 6, 08 kb fragment cloned from *A. baumannii* strain PAU into pUC19. The arrows indicate direction of transcription; hpg, hypothetical protein gene; *aacC2a*, aminoglycoside resistance gene; ORF 1and ORF 2, portion of IS*Aba-1*.

Comparison of this portion of IS*1133* with the IS*1133* sequence already described (Chiou *et al*, 1993) revealed a T to A transversion in the fourth base at the right end of the IR (Segal *et al*, 2003). The same T to A transversion was also present in the fourth base at the right end of the IR in *A. baumannii* strain SAK, isolated 9 years earlier. Interestingly, Makarova *et al* (1999) suggested that in small fragments of insertion element IS*2621* identified in *Deinococcus Radiodurans,* a transversion occurring in the IR may prevent excision of this IS as the IR can no longer be recognised by the transposases, thus inhibiting further insertion events (Makarova *et al*, 1991). The arrangement of the *aacC2a* linked to a portion of IS*1133* has been maintained in two *A. baumannii* strains isolated 9 years apart and since the IS*1133* contains a T to A transversion in the IR_R of both strains, this transversion may account for the stable integration of the *aacC2a* gene in these *A. baumannii* isolates.

University of Cape Town

1.4 The aim of this study

Previous studies have shown the aminoglycoside resistance gene *aacC2a* linked to a portion of IS*1133* in two *A. baumannii* strains isolated 9 years apart (Elisha, 1991; Segal *et al*, 2003). It is interesting that this arrangement has been maintained over time, especially as the use of aminoglycoside antibiotics has somewhat decreased in the last decade. Accordingly, it would be interesting to investigate whether this genetic arrangement has been maintained in more recent *A. baumannii* strains.

The aim of this study was therefore to screen gentamicin resistant *A. baumannii* isolates, collected over a 24 year time period, for the presence of *aacC2a*. Consequently, those strains that contained *aacC2a* were further investigated to determine whether the *aacC2a* gene was linked to a portion of IS*1133* as previously described and to investigate the role of different promoters associated with *aacC2a* expression. Strains where this arrangement had been maintained were then genotyped to detect whether these strains are genetically related.

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Chapter two

Genetic environment of an aminoglycoside resistance gene, *aacC2a*, in clinical isolates *of A. baumannii* from GSH and RCH

2.1 Introduction

With the emergence and spread of increasing numbers of MDR pathogens, it has become necessary to examine antibiotic resistance at a genetic level. Investigations into the genetic location, expression and regulation of antibiotic resistance genes in *A. baumannii* have been considered over the past 30 years. Resistance to the aminoglycoside antibiotics in *Acinetobacter* spp most often involves the production of an AME and previous studies have reported these genes in association with plasmids, transposons and integrons (Elisha & Steyn, 1991; Seward *et al*, 1998; Nemec *et al*, 2004).

Three classes of aminoglycoside-modifying enzymes have been described and all classes have been characterised in *Acinetobacter* spp (Bergogne-Bérézin & Towner, 1996). These enzymes include the ANT and APH classes, which catalyse the adenylation and phosphorylation of hydroxyl groups, respectively. The AAC class of AMEs acetylates the amino acid groups on the aminoglycosides (Shaw *et al*, 1993). The *aacC2a* gene, belonging to the AAC class of AMEs, was first identified on pWP-related plasmids from *Serratia marcescens* and *Klebsiella pneumoniae* (Allmansberger *et al*, 1985). The *aacC2a* gene encodes 3-N-acetyltransferease activity and confers resistance to gentamicin, tobramycin and netilimicin but not to kanamycin (Shaw *et al*, 1993). In a study carried out by Shaw *et al* (1993), the frequency of the *aacC2a* gene was shown to vary greatly amongst different genera of Gram-negative bacteria; 18% of *Pseudomonas* spp, 32.4% of *Serratia* spp, and 21.3% of *Acinetobacter* spp (Shaw *et al*, 1993).

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The *aacC2a* gene was also identified in a clinical *A. baumannii* isolate, strain SAK, isolated from a tracheal aspirate, from a patient in a South African hospital (Elisha, 1991). In *A. baumannii* strain SAK, *aacC2a* has both a plasmid and a chromosomal locus (Elisha, 1991). The 858 nucleotide ORF encodes a 30,5 Kda protein (Elisha, 1991). The ORF is preceded by a ribosome binding site (RBS - AGGAG) and two regions (TATAGT and TAGAGT) (Figure 2.1.1a & 2.1.1b) with homology to the -10 consensus sequence (TATAAT) of *E. coli* promoters (Hawley & McClure, 1983). Interestingly, no optimally spaced sequences with good homology to the -35 hexamer (TTGACA) for *E. coli* promoters was identified relative to either of these putative -10 promoter regions. Upstream of the region containing the promoter sequences of the *aacC2a* gene is a 115 bp nucleotide sequence with homology to the right end of IS*1133* (Elisha & Steyn, 1991).

In a more recent study, an *aacC2a* gene with the same genetic arrangement as that described in *A. baumannii* strain SAK was identified in another *A. baumannii* strain PAU, isolated 9 years later (Segal *et al*, 2003) (Figure 2.1.1a & Figure 2.1.1b). As the genetic arrangement of the *aacC2a* gene has been maintained in two *A. baumannii* strains isolated 9 years apart, it is interesting to consider whether this arrangement has been maintained and is present in more recent aminoglycoside resistant *A. baumannii* isolates.

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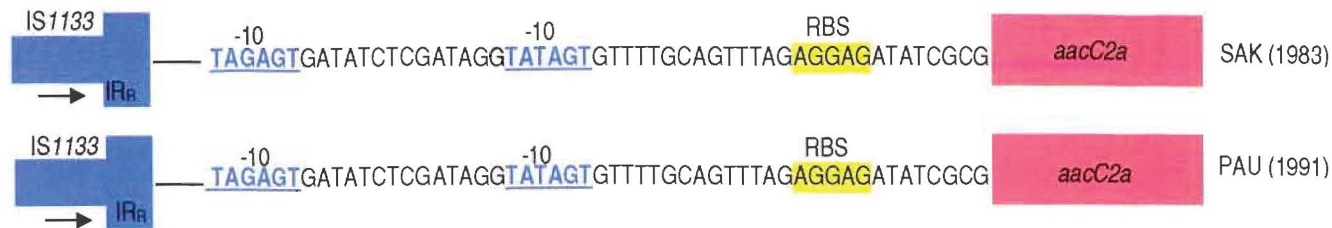

Figure 2.1.1a: Schematic representation of the *aacC2a* gene linked to a portion of IS*1133* as identified in *A. baumannii* strain SAK (Elisha, 1991) and *A. baumannii* strain PAU (Segal *et al*, 2003).

IS1133

aacC2a

```
IS1133R →
GCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACACA
← IS1133F
ACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGGTAGAGTGATATCTCGATAGGTAT
       IRR                                          aacC2aF →
AGTGTTTTGCAGTTTAGAGGAGATATCGCGATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATGG
                       ← aacC2aR2
TGCATGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGATGGG
ATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACG
GCCGGGACTTACCGTGGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTCGCGGTTG
GTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCT
GCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATG
CTTGGAAGAAACGGTGAAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGCGGTCG
AAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTT
       ← aacC2aR
CGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA
```

Figure 2.1.1b: Nucleotide sequence of the *aacC2a* gene linked to a portion of IS*1133* (AY138987) as described in *A. baumannii* strain SAK (Elisha, 1991) and PAU (Segal *et al*, 2003). The primers used are blocked in yellow. The start and stop codon for the *aacC2a* gene are indicated in bold type. The IR_R of IS*1133* is in bold type and underlined. The T to A transversion is indicated in blue. Arrows indicate the 3' end of the primer from which primer elongation proceeds.

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2.2 Experimental protocol

2.2.1 Bacterial strains, growth conditions and antibiotic susceptibility testing

A total of 44 gentamicin (GM) resistant *A. baumannii* isolates collected from patients at Groote Schuur Hospital (GSH) (n = 34) and Red Cross War Memorial Children's Hospital (RCH) (n = 10) from 1983 to 2006, were included in this study. A GM susceptible isolate, strain TOM, was included as a control when necessary (Table 2.2.1.)

Disc susceptibility testing (Table 2.2.1) and MIC testing on all 45 strains was carried out in the National Health Laboratory Service Laboratory, Groote Schuur Hospital, Cape Town, South Africa, according to the guidelines stated by the National Committee for Clinical Laboratory Standards (NCCLS).

All strains were grown overnight at 37°C on two times Yeast-Tryptone (2xYT) agar (Appendix A) or at 37°C with shaking in 2xYT broth (Appendix A), with or without antibiotic selection. Ampicillin (100mg/ml) [Ranbaxy, South Africa] or gentamicin (5ug/ml) [Bodene pty (Ltd)] was added to the growth media when necessary.

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Table 2.2.1: Aminoglycoside Antibiogram of the 45 aminoglycoside resistant *A. baumannii* strains collected from Groote Schuur Hospital and Red Cross War Memorial Children's Hospital.

Strain	Year	Hospital	Gm	Am	Tb	Strain	Year	Hospital	Gm	Am	Tb
SAK	1983	GSH	**R**	S	**R**	**A9**	2006	GSH	**R**	**R**	S
PAU	1992	GSH	**R**	S	**R**	**A10**	2006	GSH	**R**	S	S
REI	1996	GSH	**R**	S	S	**A11**	2006	RCH	**R**	**R**	S
RAN	2001	GSH	**R**	**R**	S	**A12**	2006	GSH	**R**	**R**	S
MOS-1	2005	GSH	**R**	**R**	S	**A13**	2006	GSH	**R**	S	**R**
A2	2006	RCH	**R**	S	**R**	**A14**	2006	RCH	**R**	**R**	S
A5	2006	GSH	**R**	**R**	I	**A17**	2006	GSH	**R**	**R**	S
A6	2006	RCH	**R**	S	I	**A19**	2006	GSH	**R**	**R**	R
A22	2006	RCH	**R**	**R**	S	**A20**	2006	GSH	**R**	**R**	**R**
A23	2006	RCH	**R**	**R**	**R**	**A25**	2006	RCH	**R**	S	S
A26	2006	GSH	**R**	**R**	**R**	**A27**	2006	GSH	**R**	**R**	S
A31	2006	RCH	**R**	S	**R**	**A28**	2006	GSH	**R**	**R**	S
A34	2006	RCH	**R**	I	I	**A35**	2006	GSH	**R**	S	**R**
A36	2006	GSH	**R**	**R**	S	**A39**	2006	GSH	**R**	**R**	**R**
APL	1996	GSH	**R**	S	**R**	**A40**	2006	GSH	**R**	S	S
RAM	2003	GSH	**R**	**R**	S	**A42**	2006	GSH	**R**	I	S
MOS-2	2005	GSH	**R**	**R**	S	**A46**	2006	GSH	**R**	**R**	**R**
TOM	2005	GSH	S	S	**R**	**A59**	2006	GSH	**R**	S	S
A1	2005	GSH	**R**	**R**	**R**	**A60**	2006	GSH	**R**	**R**	S
A3	2006	GSH	**R**	**R**	**R**	**A64**	2006	GSH	**R**	**R**	**R**
A4	2006	GSH	**R**	**R**	**R**	**A67**	2006	GSH	**R**	**R**	S
A7	2006	GSH	**R**	**R**	**R**	**A68**	2006	GSH	**R**	I	S
A8	2006	RCH	**R**	**R**	S						

Gm, gentamicin; Am, amikacin and Tb, tobramycin; GSH, Groote Schuur Hospital and RCH, Red Cross War Memorial Children's Hospital

2.2.2 Large scale genomic DNA preparation

Genomic DNA was extracted from each *A. baumannii* strain using the phenol/chloroform ethanol precipitation method (Ausubel *et al*, 1987). *A. baumannii* strains were grown overnight at 37°C in

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50ml of 2xYT broth. The total volume of each culture was centrifuged at 5000g for 5 minutes at 4°C in a Beckman J2- 21.

Following resuspension of the pellet in 18ml TE buffer (Appendix A), 0.5% sodium dodecyl sulphate (SDS) [BDH Chemicals], proteinase K (20mg/ml) [Boehringer Mannheim] and lysosyme (20 mg/ml) [Roche] was added to the cells and incubated for 1 hour at 37°C to denature the cellular proteins released following cell lysis. The genomic DNA was then separated from the cell debris by the addition of a CTAB/NaCl solution (Appendix A) (Ausubel *et al*, 1987). An equal volume of chloroform/isoamyl alcohol (24:1) was added to the solution and microfuged for 5 minutes to remove CTAB–complexes and any residual cell debris from the solution. The aqueous, viscous supernatant was recovered and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and microfuged for 5 minutes at 14 000g. Following this, 0.6 volume of isopropanol was added to the recovered supernatant and centrifuged for 2 minutes at 14 000rpm. The precipitated DNA was washed with 70% and the pellet was air dried at room temperature for 5 minutes, before been finally resuspended in 100µl TE buffer and stored at 4°C. Extracted DNA was electrophoresed on 1% (w/v) agarose gel (2.2.4) and quantitated by comparison with known concentrations of λ (lambda) DNA [Boehringer Mannheim].

2.2.3 Polymerase chain reaction

The polymerase chain reaction is a biochemical technique that is commonly used to amplify very small quantities of a specific DNA fragment from an often complex mix of DNA sequences, in order to provide ultimate material that can subsequently be analysed further. Primers that anneal to complementary regions of the DNA fragment of interest were designed. Primer synthesis was carried out at the Synthetic DNA Laboratory of the University of Cape Town, using a Beckman 1000m DNA synthesis machine set on the high purity program. All primers used in this study are indicated in Table 2.2.2.

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Table 2.2.2: The primers used in PCR assays

Target gene	Primers	Sequence (5'-3')	Expected product (bp)
aacC2a	aacC2aF aacC2aR	GCT TCT CAA GAT AGG TG CGC GGA AGG CAA TAA C	786
IS*1133*	IS1133F IS1133R	TGG TCT TGC ACC GGT TG GGC TTT TAT AAT CCA CG	115
IS*1133*	IS1133F IS1133R2	TGG TCT TGC ACC GGT TG GTT CCG GTC TTT TTG AG	2404
IS*1133-aacC2a*	aacC2aF IS1133R	GCT TCT CAA GAT AGG TG GGC TTT TAT AAT CCA CG	1023

PCR assays were performed using a GeneAmp PCR system 2400 [Perkin Elmer] thermocycler. Typically, the reaction mix consisted of 2.5mM of each dNTP, 20 pmoles of each primer (forward and reverse), 25mM $MgCl_2$, and 5U/ul of GoTaq Flexi DNA Polymerase [Promega] in the buffer supplied, with distilled water to a final reaction volume of 50µl. A 100ng of template DNA was added to each reaction. A PCR reaction mix with no DNA template added was always included as a negative water control, to detect possible contamination of any of the reagents used in the PCR mix. Typically a PCR cycle consisted of an initial dentauration at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 45 seconds, primer annealing at 52°C for 45 seconds, and an elongation of the template at 72°C for 90 seconds. A final elongation step carried out at 72°C for 5 minutes completed the reaction. The PCR products were analysed by gel electrophoresis (2.2.4).

2.2.4 Agarose gel electrophoresis and nucleic acid visualisation

Agarose gel electrophoresis was performed in order to separate DNA fragments on the basis of their size, in the presence of an electric field. DNA carries a negative charge and therefore upon the application of an electric field, smaller DNA fragments will migrate through the pores in an agarose gel more rapidly than larger fragments, towards the positive electrode. Separation and visualization of all nucleic acid products was performed by electrophoresis on gels made up of 1% (w/v) agarose [Hispanagar] for the efficient separation of DNA fragments.

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Aliquots of DNA were loaded with gel tracking dye (Appendix A), as the loading buffer increases the density of the DNA, and this enables the sample to drop evenly into the bottom of the well thereby allowing for easy migration of the sample during migration (Sambrook *et al,* 1989).

A molecular weight marker, Hyperladder I (Bioline, Celtic Molecular) (Appendix B) was loaded alongside DNA samples for comparison of DNA fragment sizes and concentrations. Hyperladder I is both a molecular weight marker and a quantitative marker with each band corresponding to a known concentration of DNA. Electrophoresis was performed at 90–100 Volts for 1-2 hours depending on the size of the gel. The agarose was dissolved in 1x Tris Acetate EDTA (TAE) buffer (Appendix A). Ethidium Bromide (EtBr) [Boehringer Mannheim] was added to the agarose, to a final concentration of 10ng/µl. EtBr is used in the visualisation of separated nucleic acid products, by agarose gel electrophoresis, as it intercalates between the DNA bases and fluoresces once exposed to ultra violet light (UV) light at 302nm. Visualization was achieved using a fotodyne Inc. UV light box and the results were captured using a Kodak EDAS 290 Camera (New York).

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2.2.5 DNA purification

2.2.5.1 DNA in an agarose gel

DNA was extracted from agarose gels and purified using a MiniElute® Gel Extraction Kit [Qiagen]. Briefly, 3 volumes of buffer QG were added to 1 volume of the excised gel slice and incubated at 50°C for 10 minutes. An equal volume of isopropanol was added in order to facilitate the precipitation of the DNA. Each sample was applied to a MiniElute® column and centrifuged for 1 minute at 12 000g to bind the DNA to the matrix of the column. A further 500µl of buffer QG was added to the column and subsequently, centrifuged for 1 minute at 12 000g. A volume of 750µl of buffer PE was applied to the column and centrifuged for a further 1 minute at 12 000g in order to wash the bound DNA. The residual flow-through was discarded and the column was re-centrifuged to remove any remaining ethanol. The bound DNA was eluted by the addition of 10µl of distilled water applied directly to the centre of the column which was then centrifuged for a final 1 minute.

Purified DNA samples were stored at 4°C and electrophoresed on an agarose gel alongside Hyperladder I (Bioline, Celtic Molecular) for quantification purposes.

2.2.5.2 DNA in aqueous solutions

The sample containing the DNA of interest was made up to a final volume of 100µl and an equal volume of phenol/chloroform/isoamyl (25:24:1) was added according to the protocol described by Ausubel *et al* (1987). The solution was then vortexed for a couple of seconds to ensure mixing before centrifuging [Eppendorf centrifuge 5417C] briefly for 30 seconds at room temperature. The top aqueous phase was removed and the above step was repeated until no white protein precipitate was visible at the aqueous/organic interface. A volume of 200µl 70% ethanol was added to the recovered aqueous phase and incubated at -70°C for 1 hour. Subsequently, the mixture was microfuged for 2 minutes, the recovered pellet was air dried and reconstituted in 10µl of ddH_20.

2.2.5.3 DNA quantification

DNA obtained from either genomic DNA extractions or DNA purifications was quantified using the Qubit™ fluorometer [Invitrogen] (Figure 2.2.1). This device can calculate the specific concentration of the DNA present in the sample by measuring the amount of fluorescent dye that has intercalated in the DNA backbone and comparing that relative to the relationship obtained between the two standards. The reading obtained is specific for DNA and is not affected by RNA or protein in the sample.

Figure 2.2.1: The Qubit™ fluorometer [Invitrogen] and all the reagents required.

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Briefly, 10µl of the standard solution 1 and 2, plus 1µl of the fluorescent dye are added to 190µl of the buffer supplied and incubated for 2 minutes at room temperature before reading the fluorescence using the Qubit™ fluorometer [Invitrogen]. Once the standards are set, 1µl of the sample DNA is added to 199µl of the supplied buffer with the fluorescent dye already added. This mixture is then incubated at room temperature for 2 minutes before being placed in the Qubit™ fluorometer [Invitrogen]. A concentration in µg/ml is then displayed on the screen. An aliquot of each of the DNA samples tested was also subjected to agarose gel electrophoresis (2.2.4) alongside an appropriate quantitative marker in order to visualise the purity and integrity of the DNA sample. RNA samples were also quantified using the Qubit™ fluorometer [Invitrogen] (Figure 2.2.1). Buffer and fluorescent dye specific for RNA was used, as supplied with the Qubit™ fluorometer [Invitrogen].

2.2.6 DNA sequencing and analysis

Sequencing of purified PCR amplified products was performed using the ABI Prism® BigDye™ Primer Cycle Sequencing Ready Reaction Kit [Applied Bio systems] according to the manufacture's instructions using the Sanger dideoxy sequence reaction protocol (Zimmerman *et al*, 1988).

Thermas aquaticus DNA polymerase was used in the sequencing reaction as it has a mutation allowing for the incorporatation of the dideoxynucleotides. The dideoxynucleotides are each labelled with a dye, displaying specific emission spectra and as the chain grows the sequence of the product can be determined based on the emission spectra obtained. DNA and primers were provided as specified in accordance with the recommended concentrations. Automated sequencing was either carried out by the sequencing division of GeneCare Molecular Genetics, Cape Town or at the Core Sequencing Facility at the University of Stellenbosch, using the Sanger dideoxy sequence reaction protocol (Zimmerman *et al*, 1988) (Figure 2.2.2).

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Figure 2.2.2: An electropherogram displaying a region of DNA sequence. Chain elongation is terminated by the addition of differentially labelled dideoxynucleotides (ddNTPs) which allows the exact sequence to be determined. Each ddNTP its own emission spectra and is therefore clearly identifiable by the colour it admits. Black – guanine (G); yellow – adenosine (A); red – thymidine (T) and blue – cytosine (C).

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Results obtained were analysed using DNAMAN [version 4.0, Lynnon Biosoft] and Chromas 2.31 [Techelysium Pty Ltd]. Analysed sequences were then compared with corresponding sequences in the NCBI database (www.ncbi.nlm.nih.gov/BLAST).

2.2.7 Southern blotting

2.2.7.1 Southern transfer

Southern blotting is a process that involves the transfer of negatively charged DNA onto a positively charged membrane in order to detect a specific sequence within the DNA by the addition and hybridisation of a labelled homologous probe. This process was first described by Edward Southern in 1975. Products obtained using PCR (2.2.3) from selected strains were electrophoresed on a 1% agarose gel for 3 hours at 40 Volts (2.2.4). The gel was then depurinated in 250mM HCL for 15 minutes at room temperature with gentle agitation (The Belly Dancer, Stovall Life Science, USA). This process is carried out to remove purines (A & G bases) thereby making the transfer of larger fragments onto the membrane more efficient. The gel was placed in denaturing buffer (Appendix A) and incubated for 25 minutes in 1.5 M NaCl and 0.5 NaOH.

The denaturing step facilitates the conversion of the DNA from its natural double-stranded form to a more transferable single-stranded form and was repeated twice. Neutralisation was achieved by incubating the gel in 1.5 NaCl and 0.5 M Tris-Cl (Appendix A) for 30 minutes at room temperature with gentle agitation. This step is important to neutralise the alkalinity of the denaturing buffer, and was repeated twice.

The DNA was transferred from the gel to a Hybond™- N^+ membrane (Amersham Biosciences, UK) via capillary blotting with 20xSSC buffer (Appendix A) for 18 hours at room temperature. The agarose gel was placed on top of three sheets of Whatman 3MM paper that had been soaked in 5xSSC with ends that protruded into a container containing 20xSSC. A positively charged nylon membrane, which had been soaked in 5xSSC, was placed on top of the gel and a further three sheets of Whatman 3MM paper soaked in 20xSSC were placed on top of the membrane. A layer of absorbent towel, approximately 5cm thick was placed above the 3MM paper, and a weight of 0.5 - 1kg was placed above this. The whole apparatus was covered in cling wrap to prevent evaporation of the transfer 20XSSC and left overnight (Figure 2.2.3). The blot was rinsed in 6xSSC for 1 minute at room temperature and the DNA was fixed to the blot by cross linking with an UV cross-linker [Hoefer Scientific Instruments (California, USA)] for 30 seconds at a wavelength of 254nm. The membrane was wrapped in cling film and stored at 4°C until required.

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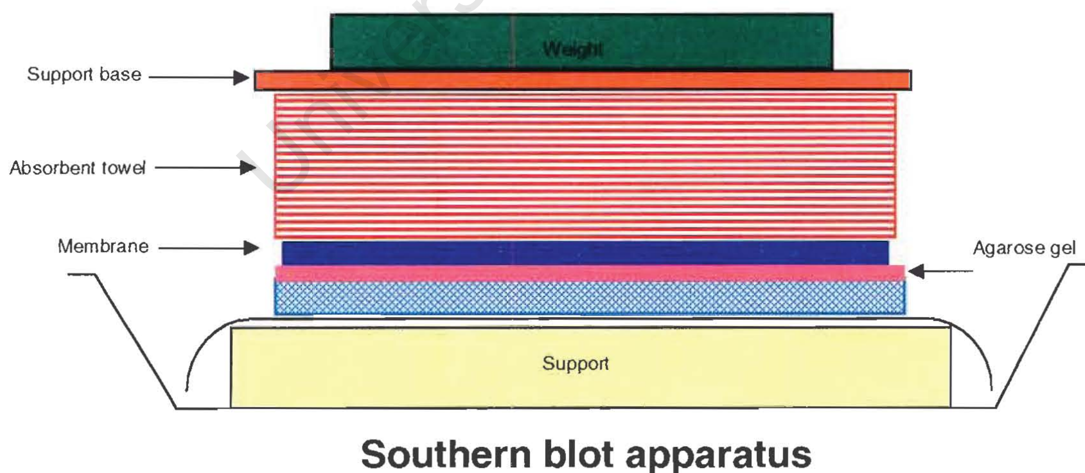

Figure 2.2.3: Diagram indicating the transfer of the DNA from the agarose gel to the positively charged membrane using the Southern blot technique.

2.2.7.2 Preparation of the probe

Primers IS1133F (5'- TGGTCTTGCACCGGTTG- 3') and HRF (5'- CACGAATGCAGAAGTTG- 3') (gifts from Dr Heidi Segal) were used to PCR amplify a 730bp fragment of IS*1133* - IS*Aba-1* using *A. baumannii* strain SAK genomic DNA as the template. The PCR product was separated by AGE (2.2.4) and a band of the expected size was excised from the gel and purified (2.2.5.1). The fragment (100 ng) was labelled using the ECL Direct Nucleic Acid Labelling Detection System (Amersham Biosciences, UK) according to the manufacturers' instructions.

Figure 2.2.4: DNA sequence of a portion of the IS*1133* interrupted by IS*Aba-1* showing the area encompassing the 730bp PCR probe. The two primers, IS1133F and HRF are blocked in yellow on the sequence and the arrows indicate direction of extension. The IR_L of IS*Aba-1* is underlined and in bold red type. The 9 bp target duplication (AAATGGCTT) flanking the copy of IS*Aba-1* in IS*1133* is blocked in red.

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2.2.7.3 Hybridisation and post-hybridisation washes

Once the DNA from the PCR products had been transferred onto the positively charged membrane, it was soaked in 6xSSC prior to pre-hybridisation. The hybridisation solution (Appendix A) was prepared according to the manufacturers' instructions in the ECL Direct Nucleic Acid Labelling Detection System Kit. The pre-hybridisation and hybridisation steps were carried out in a tube using the Techne Hybridisation oven (Techne Hybridisation HB 1D, England). Pre-hybridisation was carried out in 50ml hybridisation buffer for 1 hour at 42°C, after which the labelled probe was added to the hybridisation buffer and hybridisation was allowed to proceed overnight at 42°C. The membrane was then washed with 5xSSC for 10 minutes at 55°C and then with 100ml primary wash buffer for 10 minutes at 55°C. Two further washes, with primary wash buffer for 5 minutes and 3 minutes were carried out. The membrane was then rinsed twice with 2xSSC for 5 minutes at room temperature with gentle agitation

2.2.7.4 Signal detection and washes

After the hybridisation washes the membrane was transferred to a cling film surface, an equal volume of detection reagents 1 and 2 (0.125ml/cm^2) was added to the membrane and incubated for 1 minute at room temperature. Excess detection reagent was drained off the membrane onto paper towel and the membrane was sealed between two sheets of cling film. Detection reagent 1 decays hydrogen peroxide, the substrate for peroxidise, with which the probe was labelled (2.2.7.2). Reduction of the hydrogen peroxide by peroxidise is coupled to a light producing reaction catalysed by detection reagent 2. This reagent contains luminol and produces a blue light upon oxidation. An enhancer prolongs and increases the light output which can be detected on a blue-light sensitive film. The membrane was then exposed to an X-ray film (AGFA, CP-BU, medical X-ray film, 100NIF, 180 x 210 mm) for 1 minute, 5 minutes and 1 hour. The signal obtained on the membrane was visualised by incubating the X-ray Film in G128 Developer (AGFA, Belgium) for 3 minutes followed by a 2 minute incubation in G333C Rapid fixer (AGFA, Belgium), rinsed in water for 5 minutes and then allowed to air dry.

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2.2.8 Preparation of a DNA library from *A. baumannii* strain MOS-1

2.2.8.1Preparation of genomic DNA

To determine the genetic environment of IS*1133* linked to the *aacC2a*, genomic DNA (5μg) from strain MOS-1 was digested with 10U of *Hin*dIII [Roche] and 10U *Bam*HI [Roche] in the appropriate buffer, in a final volume of 20μl. The reaction was carried out at 37°C overnight. Subsequently, the DNA fragments obtained were purified with phenol/chloroform/ isoamyl alcohol and precipitated with 100% ethanol (2.2.5.2). The DNA pellet was washed with 70% ethanol and resuspended in 10μl ddH_2O.

2.2.8.2 Preparation of pUC19 vector DNA

The vector, pUC19 (Appendix B) was obtained by culturing *E. coli* JM109 containing pUC19 cells in 2xYT broth containing Ampicillin (100 mg/ml) [Ranbaxy, South Africa] at 37°C overnight. A QIAprep® plasmid mini kit [Germany] was used according to the manufacture's instructions to isolate pUC19 DNA (2.2.9). Two micrograms of pUC19 DNA was linearized with 10U *Hind*III [Roche] and 10U *Bam*HI [Roche] in the appropriate restriction enzyme buffer (1x), in a final reaction volume of 20μl at 37°C for 3 hours. The sample was then electrophoresed (2.2.4) and the linearized pUC19 DNA was excised and purified using the MiniElute® Gel Extraction Kit (2.2.5.1).

2.2.8.3 Preparation of competent *E. coli* JM109 cells

Competent cells were prepared from *E. coli* JM109 cells using the calcium chloride shock procedure as described by Dagert and Ehrlich (1979). This treatment induces a state of competence in the bacteria during which they are able to allow naked strands of DNA from the surroundings to enter the cell. A 5ml overnight broth culture of the *E. coli* JM109 strain was grown in 2xYT broth at 37°C and subsequently, diluted 1/100 into a 50ml 2xYT broth in flasks 5 - 10 times greater than the culture volume.

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When the culture volume reached logarithmic phase (OD_{600} = 0.2 – 0.4) the cells were harvested by centrifugation at 4000g for 10 minutes at 4°C in a Beckman J2-21 centrifuge. The pellet was resuspended in 0.5 volume ice-cold $CaCl_2$ (0.1M), incubated on ice for 1 hour and centrifuged at 5000rpm for 5 minutes at 4°C. The pellet was resuspended in 1/10 volume ice-cold $CaCl_2$ (0.1M). A final concentration of 10% (v/v) sterile glycerol was added to the cells and aliquots of 200µl were stored at – 70°C.

2.2.8.4 Ligation and Transformation

A 100ng of *Bam*HI/*Hind*III digested pUC19 DNA was ligated to *Bam*HI/*Hind*III digested *A. baumannii* MOS-1 genomic DNA in a final volume of 10µl, containing 1U T4 DNA ligase [Roche], in 1x ligation buffer [Roche]. Following incubation overnight at 4°C, 5µl of the ligation mix was added to 100µl competent *E. coli* JM109 cells and incubated on ice for 30 minutes. The cells were then induced to transform by the application of heat shock at 42°C for 45 seconds.

Subsequently, the mix was incubated with 0.9ml 2xYT broth for 1 hour at 37°C to allow expression of the antibiotic resistance markers. In order to select for transformants, aliquots of the transformation mixture were spread onto 2xYT agar plates. A 220ml aliquot of 2xYT was melted and 200µl of Ampicillin (100 mg/ml) [Ranbaxy, South Africa], 100µl of IPTG (200 mg/ml) [Roche] and 1ml of X-Gal (40 mg/ml, Appendix A) [Roche] were added. A 20ml aliquot of the 2xYT containing selection was poured into a petri dish and allowed to set for 20 minutes. Generally 50µl, 100µl and 200µl aliquots of the transformation mix were plated onto 2xYT plates containing selection and incubated overnight at 37°C. Clones appear white due to the interruption of the lacZ gene, as the α-complementation process is impaired. White colonies were subcultured onto master plates containing Ampicillin (100 mg/ml) [Ranbaxy, South Africa] and incubated overnight at 37°C.

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2.2.9 Small-scale plasmid preparation

The Qiagen miniprep system is based on the protocol published by Birnboim and Doly. (Birnboim & Doly, 1979). This method was carried out according to the manufactures instructions. White colonies obtained were inoculated into 5ml 2xYT broth grown and incubated overnight at 37°C with shaking. The cells were pelleted at 12 000rpm for 2 minutes resuspended in 250µl P1 buffer, containing RNase A. Subsequently, 250 µl of P2 lysis buffer and 350µl of N3 neutralisation buffer were added to the mixture. This was then microfuged at 12 000rpm for 10 minutes. The recovered supernatant was applied to a QIAprep® Spin Column and centrifuged. The column containing the plasmid DNA bound to the matrix was subsequently washed with 0.5ml PB wash buffer and centrifuged for 60 seconds to remove any trace nuclease activity. The column was washed with 0.75ml PE wash buffer and centrifuged for 60 seconds, after which, the column was centrifuged or a further 60 seconds so as to remove any residual wash buffer. The column was then placed in a new 1.5ml eppendorf, 50µl of distilled water was added to the centre of the QIAprep® spin column and centrifuged for 2 minutes at 12 000rpm to elute the plasmid DNA. The DNA was stored at 4°C.

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2.3 Results

2.3.1 Detection of *aacC2a* in gentamicin resistant clinical *A. baumannii* isolates

Genomic DNA isolated (2.2.2) from the 44 Gm resistant *A. baumannii* strains as well as the Gm susceptible isolate TOM (Table 2.1.1) was used as template DNA in PCR assays to screen for the presence of *aacC2a*. Primers aacC2aF and aacC2aR, designed to anneal to complementary sequences within the *aacC2a* structural gene were used in this assay (Figure 2.1.1b).

The PCR reaction mix (2.2.3) containing genomic DNA was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles 95°C for 45 seconds, 51°C for 45 seconds, and 72°C for 60 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour. Amplicons of the anticipated size (786 bp) were obtained from 6/23 and 6/10 isolates from GSH and RCH, respectively (Figure 2.3.1).

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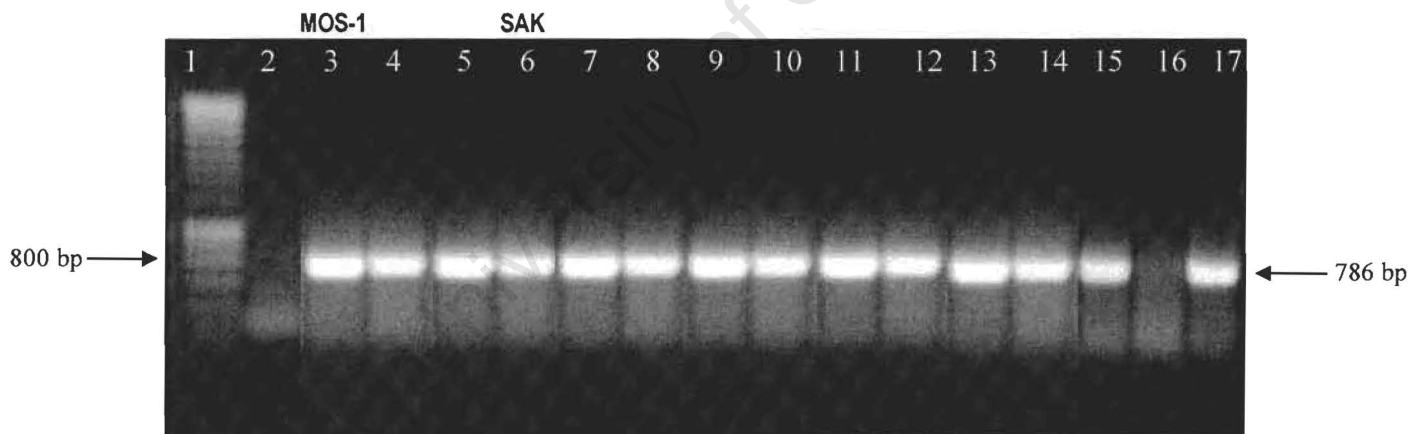

Figure 2.3.1: Agarose gel electrophoresis of amplicons following PCR amplification of *aacC2a* from *A. baumannii* strains. Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: MOS-1; Lane 4: RAN; Lane 5: PAU; Lane 6: SAK; Lane 7: REI; Lane 8: A2; Lane 9: A5; Lane 10: A6; Lane 11: A22; Lane 12: A23; Lane 13: 26; Lane 14: A31; Lane 15: A34; Lane 16: TOM; Lane 17: A36. Strain TOM was included as a negative control.

No amplification product was obtained from the water control, nor from the susceptible control strain TOM (Figure 2.3.1). A PCR product of 786 bp was obtained from strains MOS-1, RAN, REI, A2, A5, A6, A22, A23, A26, A31, A34, A36, indicating that these 12 strains contain the *aacC2a* gene. *A. baumannii* strains PAU and SAK, known to contain the *aacC2a*, were included as positive controls (Elisha, 1991; Segal *et al*, 2003). The PCR amplicons obtained from strains, MOS-1 and SAK (Lane 3 and 6, respectively) were excised and purified (2.2.5.1). The purified products were quantified by gel electrophoresis (2.2.4) and sequenced on both strands (2.2.6).

Analysis of the sequencing data obtained from both MOS-1 and SAK revealed 100% homology to *aacC2a* (Figure 2.3.2). The *aacC2a* gene was not detected in the remaining 30 Gm resistant *A. baumannii* isolates included in this study (data not shown), indicating that these strains do not harbour an *aacC2a* gene.

2.3.2 Genetic arrangement of *aacC2a* in clinical *A. baumannii* isolates

The *aacC2a* gene in *A. baumannii* strain SAK is linked to a portion of IS*1133* (Elisha, 1991) as is the *aacC2a* gene in *A. baumannii* strain PAU (Segal *et al*, 2003) (Figure 2.3.3). The association of *aacC2a* with the IR_R of IS*1133* over a 9 year period is thought to be due to the T to A transversion occurring in the IR_R of IS*1133* (Segal *et al*, 2003).

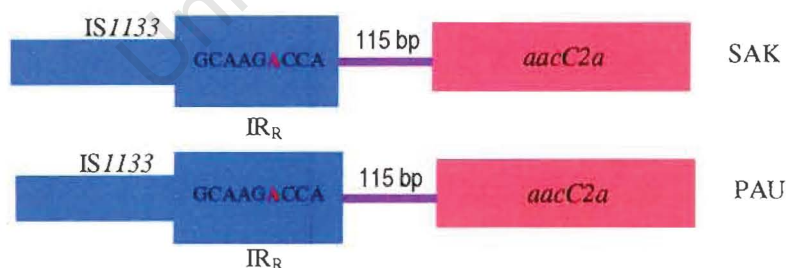

Figure 2.3.3: Schematic representation of the *aacC2a* gene linked to a portion of IS*1133* as identified in *A. baumannii* strain SAK (Elisha, 1991) and *A. baumannii* strain PAU (Segal *et al*, 2003). The T to A transversion occurring at the fourth base of the IR_R of IS*1133* is indicated in red.

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```
PAU    ATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTCCGG  100
SAK    ATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTCCGG  100
MOS-1  ATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTCCGG  100

PAU    TCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGATGGGATACGCGTCGTGGGACCGATCACCCTACGA  200
SAK    TCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGATGGGATACGCGTCGTGGGACCGATCACCCTACGA  200
MOS-1  TCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGATGGGATACGCGTCGTGGGACCGATCACCCTACGA  200

PAU    GGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTG  300
SAK    GGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTG  300
MOS-1  GGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTG  300

PAU    AATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTC  400
SAK    AATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTC  400
MOS-1  AATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTC  400

PAU    ACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTAC  500
SAK    ACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTAC  500
MOS-1  ACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTAC  500

PAU    CGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAA  600
SAK    CGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAA  600
MOS-1  CGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAA  600

PAU    ACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCG  700
SAK    ACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCG  700
MOS-1  ACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCG  700

PAU    GTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGG  800
SAK    GTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGG  800
MOS-1  GTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGG  800

PAU    AGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA
SAK    AGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA
MOS-1  AGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA
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Figure 2.3.2: Alignment of *aacC2a* gene sequence from *A. baumannii* strain PAU (AY138987; Segal *et al*, 2003) and strains SAK and MOS-1, indicating 100% homology.

To determine whether the IS*1133* - *aacC2a* genetic arrangement has been maintained in more recent *A. baumannii* isolates, PCR assays were carried out. A primer designed to anneal within the structural *aacC2a* gene (aacC2aR) and a primer that anneals upstream of the IS*1133* IR_R (IS1133R) were used (Figure 2.1.1b) in PCR assays.

The PCR reaction mix (2.2.3) containing genomic DNA was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles 95°C for 45 seconds, 51°C for 45 seconds, and 72°C for 80 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour. All 12 strains which were shown to harbour the *aacC2a* gene were screened for the linkage as previously descried between IS*1133* and *aacC2a* (Elisha, 1991; Segal *et al*, 2003). Amplicons of the anticipated size (1023 bp) were obtained from 6/23 and 6/10 isolates from GSH and RCH, respectively (Figure 2.3.4).

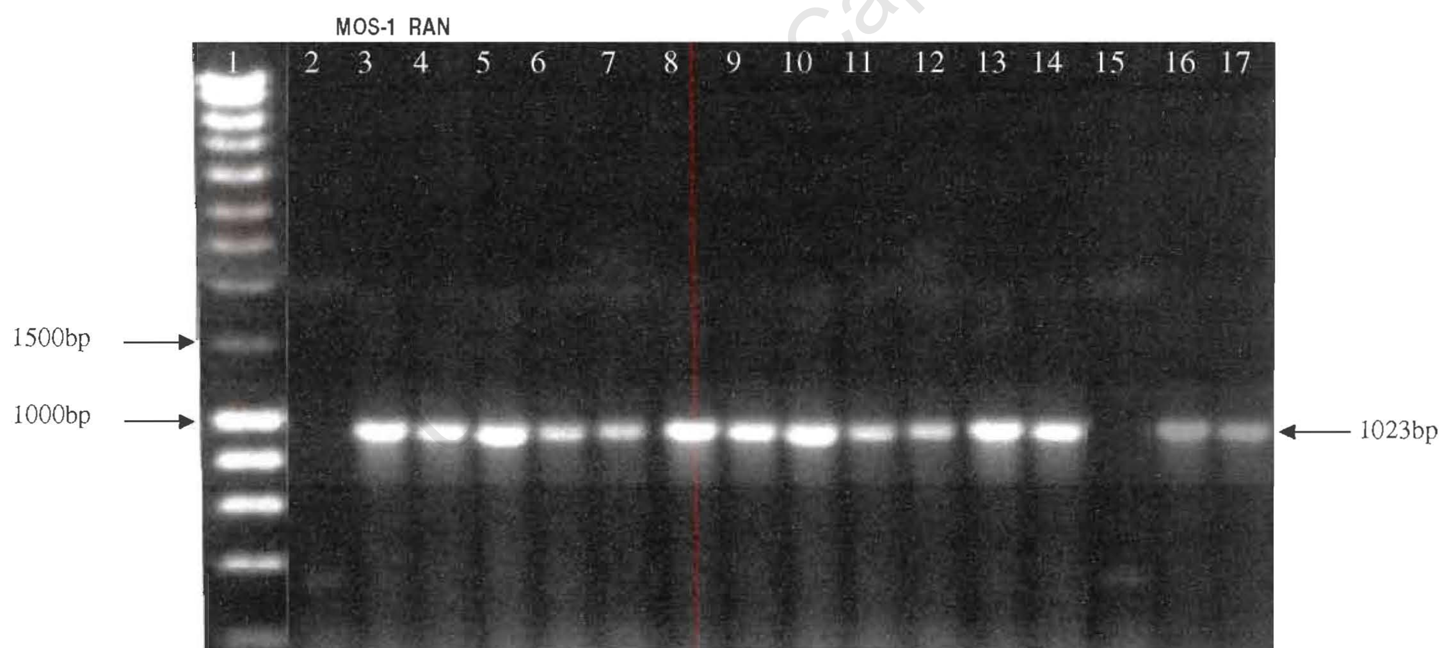

Figure 2.3.4: Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* linked to *aacC2a* from *A. baumannii* strains. Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: MOS-1; Lane 4: RAN; Lane 5: REI; Lane 6: PAU; Lane 7: SAK; Lane 8: A2; Lane 9: A5; Lane 10: A6; Lane 11: A22; Lane 12: A23; Lane 13: 26; Lane 14: A31; Lane 15: TOM; Lane 16: A34; Lane 17: A36. Strain TOM was included as a susceptible control.

No amplification product was obtained from the water control, nor from the susceptible strain TOM (Figure 2.3.4). A PCR product of 1023 bp was obtained from strains MOS-1, RAN, REI, A2, A5, A6, A22, A23, A26, A31, A34, A36, indicating that these 12 strains contain *aacC2a* linked to a portion of IS*1133*. *A. baumannii* strains SAK and PAU known to contain the *aacC2a* linked to a portion of IS*1133* were included as positive controls (Elisha, 1991; Segal *et al*, 2003).

The PCR amplicons obtained from MOS-1(isolated in 2005) and RAN (isolated in 2001) (Figure 2.3.4; lanes 3 and 4, respectively) were excised and purified (2.2.5.1). The purified products were quantified by gel electrophoresis (2.2.4) and sequenced on both strands (2.2.6).

Analysis of the sequencing data obtained from both MOS-1 and RAN revealed 100% homology to the *aacC2a* gene linked to a portion of IS*1113* (Figure 2.3.5) and the T to A transversion occurring at the fourth base of the IR_R of IS*1133* has been maintained in both MOS-1 and RAN.

2.3.3 Detection of the portion of IS*1133* in clinical *A. baumannii* isolates

It is interesting to consider whether the remaining 30 Gm *A. baumannii* isolates that did not contain *aacC2a*, harboured a portion of IS*1133*. To screen for IS*1133* sequence in the 30 *A. baumannii* isolates, PCR assays were carried out. Primers IS1133F and IS1133R, designed to anneal to complementary sequences within the IS*1133 tnpA* structural gene, were used in the assay (Figure 2.1.1b).

The PCR reaction mix (2.2.3) containing genomic DNA was subjected to an initial denaturation at 95˚C for 5 minutes, followed by 35 cycles 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 30 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 2% agarose gel at 90V for 1 hour. Amplicons of the anticipated size (115 bp) were not obtained for any of the remaining 30 isolates screened, indicating that these strains do not harbour a portion of IS*1133* (Figure 2.3.6). Strains SAK, PAU, MOS-1 and RAN were included as positive controls.

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PAU    GCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACACA   100
MOS-1  GCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACACA   100
RAN    GCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACACA   100
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IR_R IS*1133*

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PAU    ACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGGTAGAGTGATATCTCGATAGGTA   200
MOS-1  ACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGGTAGAGTGATATCTCGATAGGTA   200
RAN    ACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGGTAGAGTGATATCTCGATAGGTA   200
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aacC2a

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PAU    TAGTGTTTTGCAGTTTAGAGGAGATATCGCGATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATG   300
MOS-1  TAGTGTTTTGCAGTTTAGAGGAGATATCGCGATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATG   300
RAN    TAGTGTTTTGCAGTTTAGAGGAGATATCGCGATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATG   300

PAU    GTGCATGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGAT   400
MOS-1  GTGCATGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGAT   400
RAN    GTGCATGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGAT   400

PAU    GGGATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCG   500
MOS-1  GGGATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCG   500
RAN    GGGATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGGTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCG   500

PAU    CAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTC   600
MOS-1  CAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTC   600
RAN    CAACGGCCGGGACTTACCGTGGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGGTC   600

PAU    GCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGG   700
MOS-1  GCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGG   700
RAN    GCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGG   700

PAU    GAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGA   800
MOS-1  GAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGA   800
RAN    GAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCCCAACAAACGATGGGTGACGTATGA   800
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PAU    GATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCG   900
MOS-1  GATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCG   900
RAN    GATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCG   900

PAU    GATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACA   1000
MOS-1  GATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACA   1000
RAN    GATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACA   1000

PAU    TCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA   1100
MOS-1  TCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA   1100
RAN    TCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACACGAAGCCGCCCAGCGCTCTTGCGAGCCTTCCGGTTA   1100
```

Figure 2.3.5: Alignment of the *aacC2a* gene linked to a portion of IS*1133* from *A. baumannii* strain PAU (AY138987; Segal *et al*, 2003) and *aacC2a* linked to a portion of IS*1133* sequence obtained for *A. baumannii* strains MOS-1 and RAN, showing 100% homology.

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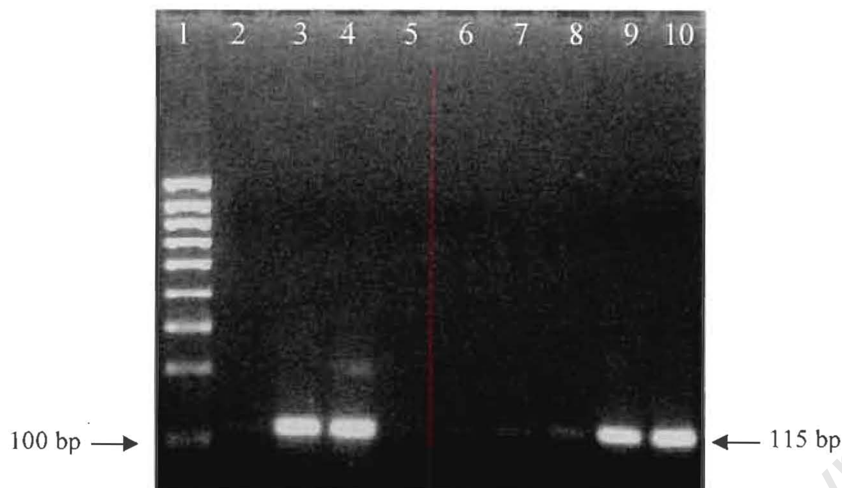

Figure 2.3.6: Agarose gel electrophoresis of amplicons following PCR amplification of a portion of the right end of IS*1133* in *A. baumannii* strains. Lane 1: Hyperladder IV (Bioline); Lane 2: Water control; Lane 3: SAK; Lane 4: PAU; Lane 5: APL; Lane 6: RAM; Lane 7: MOS-2; Lane8: TOM; Lane 9: MOS-1; Lane 10: RAN. Strain Tom was included as a susceptible control.

A PCR product of 115 bp was obtained from strains SAK, PAU, MOS-1 and RAN, indicating that these strains contain the portion of IS*1133*. No amplification product was obtained from the water control, nor from the susceptible strain TOM. No product was obtained from strains APL, RAM, and MOS-2 (Figure 2.3.6). The portion of IS*1133* was not detected in any of the remaining 26 strains which do not harbour *aacC2a*.

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2.3.4 Detection of a full copy of IS*1133* in clinical *A. baumannii* isolates

The first description of a full copy of IS*1133* was in the plant pathogen *E. amylovora* which causes fire blight in apple trees (Chiou *et al*, 1993). This IS element forms part of the transposon Tn*5393* and is located upstream of the aminoglycoside resistance genes *aph(6)-Ia* and *aph(6)-Id* (Chiou *et al*, 1993). In this study it was shown that the expression of these two aminoglycoside resistance genes is dependant on promoter sequences located with IS*1133* (Chiou *et al*, 1993).

To determine whether *aacC2a* is linked to a full copy of this element, PCR assays were carried out using primers IS1133F2 and IS1133F, designed to anneal to the left and right ends of IS*1133* (Figure 2.3.7).

The PCR reaction mix (2.2.3) containing genomic DNA was subjected to an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 minute and 40 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour.

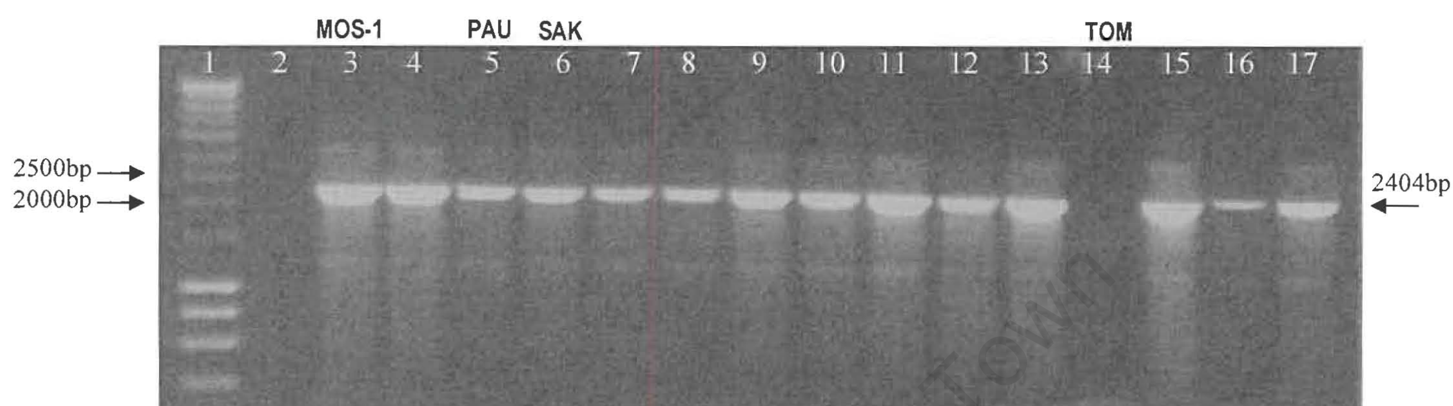

Figure 2.3.7: Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* from *A. baumannii* strains. Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: MOS-1; Lane 4: RAN; Lane 5: PAU; Lane 6: SAK; Lane 7: REI; Lane 8: A2; Lane 9: A5; Lane 10: A6; Lane 11: A22; Lane 12: A23; Lane 13: 26; Lane 14: TOM; Lane 15: A31; Lane 16: A34; Lane 17: A36. Strain TOM was included as a negative control.

No amplification product was obtained from the water control, nor from the susceptible strain TOM (Figure 2.3.7). An anticipated PCR product of 1214 bp was not obtained for the 14 strains known to harbour the *aacC2a* linked to a portion of IS*1133*. Instead a much larger PCR product of 2404 bp was obtained from strains, MOS-1, RAN, PAU, SAK, REI, A2, A5, A6, A22, A23, A26, A31, A34, and A36. This suggests that these 14 strains may contain IS*1133* with an insertion of 1207 bp. The PCR amplicon from strain SAK was excised and purified (2.2.5.1). The purified products were quantified by gel electrophoresis (2.2.4) and sequenced on both strands (2.2.6).

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Analysis of the sequencing data obtained from strain SAK indicated that a full copy of IS*1133* was disrupted by the insertion of IS*Aba-1* at nucleotide position 1121 in the *tnpA* of IS*1133* (Figure 2.3.8 & Figure 2.3.9). A 9 bp target duplication (AAATGGCTT) flanks the copy of IS*Aba-1* in IS*1133* in strains PAU and SAK. The same site of insertion and target duplication was identified in a recent isolate from GSH (MOS-1) and RCH (A2).

2.3.5 DNA-DNA hybridisation studies to confirm IS*1133* interrupted by a full copy of IS*Aba-1* in *A. baumannii* isolates.

Sequencing analysis revealed that in *A. baumannii* strain SAK contained a full copy of IS*1133* interrupted by a full copy of IS*Aba-1*. To confirm that the IS*Aba-1* element had disrupted IS*1133* in the remaining 13 *A. baumannii* strains, DNA-DNA hybridisation studies were carried out using a probe that anneals to both IS*1133* and IS*Aba-1*. Initially, PCR assays were carried out using primers IS1133F2 and IS1133F, designed to anneal to the left and right ends of IS*1133* (Figure 2.3.9).

The PCR reaction mix (2.2.3), containing genomic DNA from the 14 *A. baumannii* isolates as template, was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 minute and 40 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoreses (2.2.4) on a 1% agarose gel at 90V for 1 hour. Subsequently, the gel was placed on a positively charged membrane and DNA-DNA hybridisation studies were carried out (2.2.7) (Figure 2.3.10).

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IS1133

AAATGGCTT

ISAba-1

IS1133

AAATGGCTT

aacC2a

Figure 2.3.8: Schematic representation of IS*Aba-1* inserted within IS*1133* at nucleotide position 1121, creating a 9-bp target duplication (AAATGGCTT), and linked to *aacC2a* from *A. baumannii* strain SAK. Arrows indicate direction of IS*Aba-1*, IS*1133 tnpA* and *aacC2a* transcription. Right (IR_R) and left (IR_L) terminal inverted repeats are indicated.

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IS1133

IR_L IS*1133* **IS1133F2 →**

TGGACTTGCCTCGGTTTCGTTCCGGTCTTTTTGAGAGCATTATTTGTTATCAAGGAGACCATATATGTCATTAAAGCATAGTGATGAATTTAA

GCGTGATGCAGTTCGCATAGCACTCACTAGTGGCTTAACACGCCGTCAAGTTGCGTCAGATTTAAGTATTGGGCTTTCCACGCTTGGGAAATG

GATCGCATCAATTTCCGATGAAACTAAAATTCCTACCCAAGACACTGATCTTCTGCGTGAGAATGAACGTTTACGCAAAGAGAACCGTATCCT

TCGGGAGGAGAGGGAGATATTAAAAAGGCAGCAATATTTTTCGCAGTACAAAAGCTGTGAGATTTCAGTTTATTACGGATTACCGTGGCTCT

tnpA →

CTCTCACGTTCACGCATATGTCGTTTGATGGGCGTAACAGATCGTGGTTTACGTGCATGGAAACGCCGTCCTCCATCACTGCGCCAGCGTCGT

GATCTTATACTTCTAGCGCATATACGTGAGCAGCATCGGTTGTGTTTGGGGAGCTATGGTAGGCCGCGTATGACAGAAGAGTTGAAAGCGCTG

GGCCTGCAGGTTGGGCAGCGTCGGGTTGGACGTTTGATGCGCCAGAATAACATTACAGTTGTTCGAACGCGTAAATTCAAACGGACAACGGAT

AGTCATCATACCTTCAACATTGCACCGAACCTATTAAAACAAGACTTTAGCGCAAGCGCACCCAACCAGAAATGGGCAGGCGATATCACTTAT

GTTTGGACCAGAGAAGGATGGGTCTATCTTGCTGTTATCCTTGACCTGTATTCCCGTCGCGTGATTGGCTGGGCAACAGGTGATCGATTAAAG

CAGGATCTTGCATTAAGGGCACTGAATATGGCGTTGGCTTTACGCAAACCACCACCGGGTTGTATTCAACACACAGACCGTGGGAGCCAATAT

TGCGCTCATGAATATCAAAAGCTACTGCTCAAACATCAATTGCTGCCGTCCATGAGCGGGAAAGGCAATTGTTTTGATAACTCCGCAGTAGAA

AGCTTCTTTAAATCATTAAAGGCTGAGTTGATTTGGCGCAGACACTGGCAAACAAGGCGAGATATTGAGATTGCAATCTTCGAATATAT**AAAT**

IR_R IS*Aba-1*

GGCTTCTCTGTACACGACAAATTTCACAGAACCCTTATCCTATCAGGGTTCTGCCTTCTTAAAATTGCCAAAATTTCCTTAAACTCTTCTTTT

Figure 2.3.9: Nucleotide sequence (100 bp per line) of the full copy of IS*1133* interrupted by IS*Aba-1* in *A. baumannii* strain SAK. The primers used in PCR assays are blocked in yellow. The inverted repeats for IS*Aba-1* and IS*1133* are in bold type and underlined, the T to A transversion in IR_R of IS*1133* is indicated in blue. A 9 bp (AAATGGCTT) target sequence duplication at the site of insertion of IS*Aba-1* is indicated in bold, red type. Arrows indicate the 3' end of the primer from which elongation proceeds. The ATG for the *tnpA* of IS*1133* is highlighted in red. The arrows indicate direction of transcription of *tnpA* of IS*1133* and *aacC2a*.

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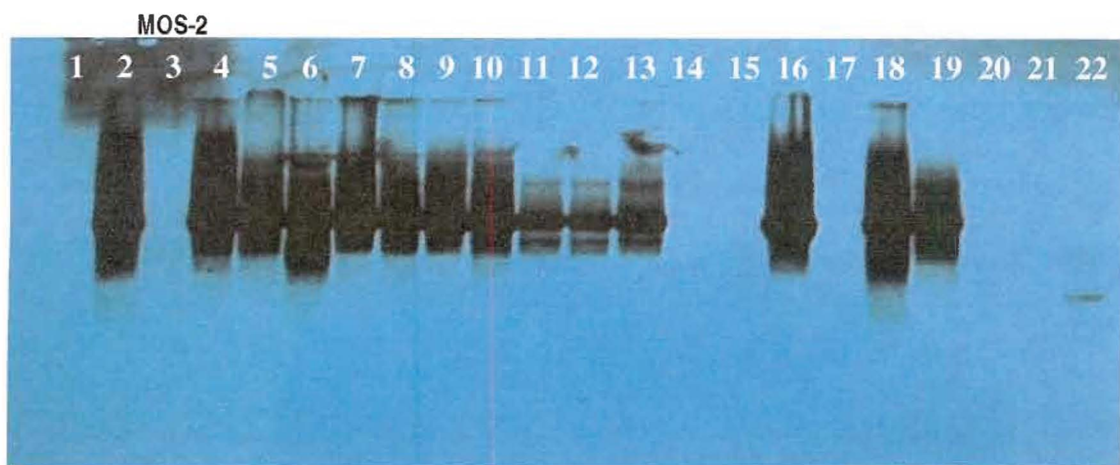

Figure 2.3.10: Autoradiograph of IS*1133* interrupted by IS*Aba-1* probe hybridised to PCR amplicons obtained from *A. baumannii* strains. Lane 1: Hyperladder I (Bioline); Lane 2: MOS-1; Lane 3: MOS-2; Lane 4: RAN; Lane 5: RAN; Lane 6: PAU; Lane 7: SAK; Lane 8: A2; Lane 9: A5; Lane 10: A6; Lane 11: A22; Lane 12: A23; Lane 13: A26; Lane 14: A28; Lane 15: TOM; Lane 16: A31; Lane 17: TOM; Lane 18: A34, Lane 19: A36; Lane 20: Water control; Lane 21:Blank; Lane 22: A portion of IS*1133* linked to a portion IS*Aba-1* (PCR product using primers HRF and IS1133F corresponding to IS*1133* linked to IS*Aba-1*).

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No hybridisation of the probe occurred with the water control, nor with *A. baumannii* strain A28 or the susceptible strain TOM (Figure 2.3.10). *A. baumannii* strain MOS-2 was included as a control known to contain a full copy of IS*Aba-1* but no IS*1133*. No signal was detected from strain MOS-2. A positive hybridisation signal was obtained for strain MOS-1, RAN, PAU, SAK, REI, A2, A5, A6, A22, A23, A26, A31, A34 and A36, indicating that in these strains, IS*1133* is associated with IS*Aba-1*.

2.3.6 Investigation of genetic environment of IS*1133* in a clinical *A. baumannii* isolate

IS*1133* has been associated with composite transposons and plasmids (Chiou *et al*, 1993; Pezzella *et al*, 2004). The 3' end of IS*1133* in *A. baumannii* strains PAU and SAK is linked to *aacC2a* (Elisha, 1991; Segal *et al*, 2003); however, the regions upstream of IS*1133* are unknown. To investigate this further a DNA library of *A. baumannii* strain MOS-1 (5µg) was prepared (2.2.8.1). Genomic DNA from *A. baumannii* strain MOS-1, as well as pUC19, was digested with *Bam*HI & *Hind*III for 3 hours at 37°C. pUC19 DNA was electrophoresed on a 1% agarose gel at 90V for 1hour (2.2.4), purified (2.2.5.1) and quantified by gel electrophoresis (2.2.4) (Figure 2.3.11A). A total of 50% of the purified genomic MOS-1 DNA (Figure 2.3.11B) was added to 100ng of pUC19 in a ligation reaction and incubated overnight at 4°C (2.2.8.4).

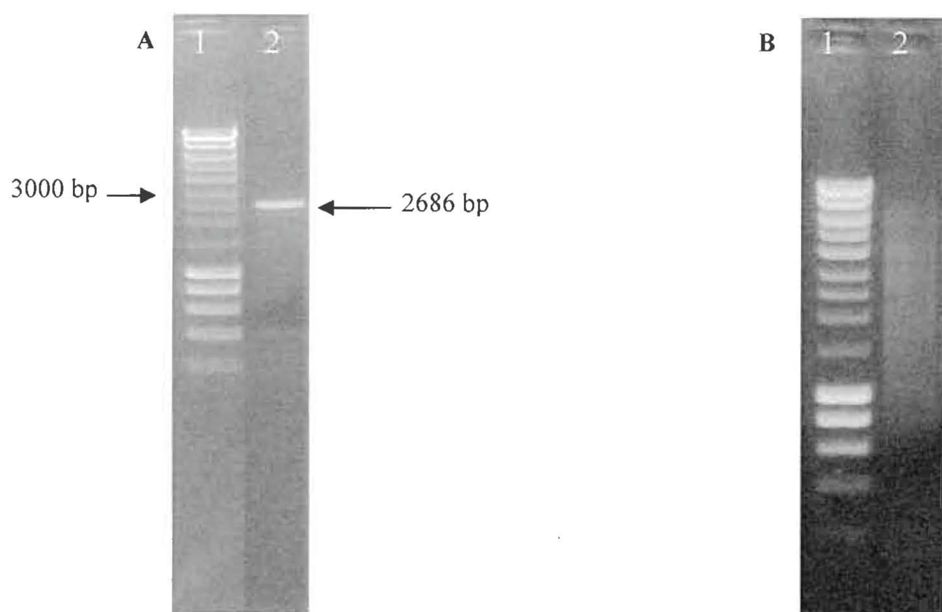

Figure 2.3.11A: Agarose gel electrophoresis of *Bam*HI/*Hind*III digested and purified pUC19 DNA. Lane 1: Hyperladder I (Bioline); Lane 2: digested and purified pUC19 DNA.
Figure 2.3.11B: Agarose gel electrophoresis of *Bam*HI/*Hind*III digested and purified genomic DNA from *A. baumannii strain* MOS-1. Lane 1: Hyperladder I (Bioline); Lane 2: *A. baumannii* MOS-1 digested genomic DNA.

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The ligation mix was transformed into competent *E. coli* JM109 cells, plated on selective media and incubated overnight (2.2.8.4). A total of 840 white colonies were obtained and screened for the presence of a portion of IS*1133*. Ten colonies were inoculated into 20µl of ddH_2O and boiled for 10 minutes to release the DNA from the cells. A 2µl aliquot was used as template in a PCR assay. Primers IS1133F2 and IS1133F5, designed to anneal to the left and right ends of IS*1133* were used in a PCR assay (Figure 2.3.9). Using these primers a PCR product of 956 bp was obtained. The amplicon was excised, purified and ligated to pGEM®-TEasy vector [Promega] overnight (2.2.8D). The ligation mix was then transformed into *E. coli* JM109 cells (2.2.8.4). The recombinant plasmid containing the 956 bp IS*1133* PCR product was designated pTED and was used as a positive control during the boiling and PCR steps when screening for recombinants from *A. baumannii* strain MOS-1.

The PCR reaction mix (2.2.3) containing DNA released from the boiled colonies was subjected to an initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of 95ºC for 45 seconds, 52ºC for 45 seconds, and 72ºC for 1 minute. A final elongation at 72ºC for 5 minutes completed the reaction. PCR products were analysed by gel electrophoreses (2.2.4) on a 1% agarose gel at 90V for 1 hour.

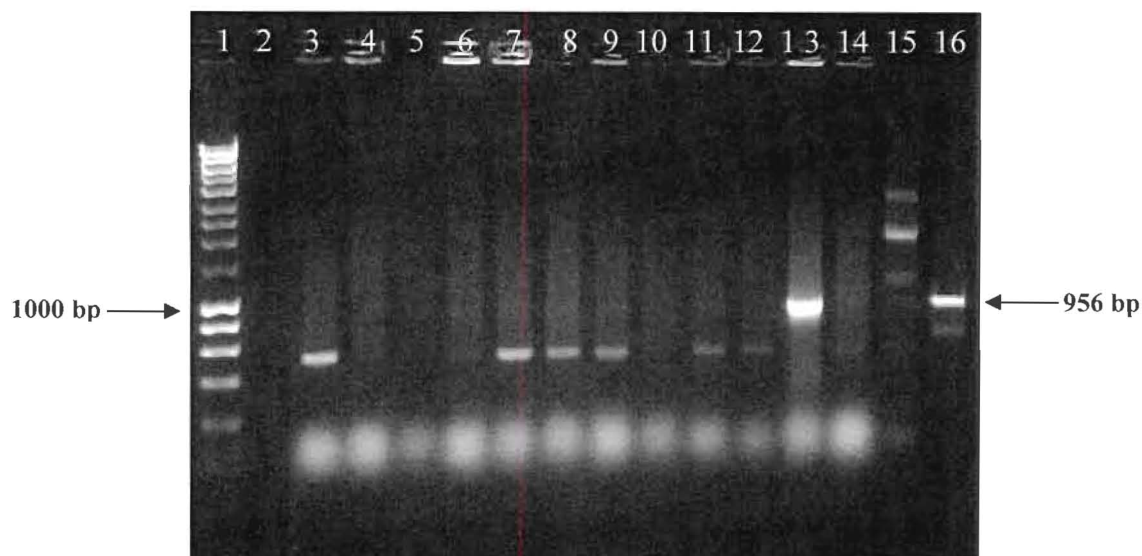

Figure 2.3.12: Agarose gel electrophoresis of amplicons following PCR amplification of IS*1133* from 10 pools of 10 colonies following shotgun cloning of *A. baumannii* strain MOS-1. Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: pool 1; Lane 4: pool 2; Lane 5: pool 3; Lane 6: pool 4; Lane 7: pool 5; Lane 8: pool 6; Lane 9: pool 7 ; Lane 10: pool 8; Lane 11: pool 9; Lane 12: pool 10; Lane 13: *A. baumannii* DNA from strain MOS-1; Lane 14: *E. coli* JM109 colony; Lane 15: *E. coli* JM109 genomic DNA; Lane 16: *E. coli* JM109 (pTED).

No amplification product was obtained from the water control, the *E. coli* JM109 colony or the *E. coli* JM109 genomic DNA (Figure 2.3.12). A PCR product of the 956 bp, obtained from both *A. baumannii* strain MOS-1 genomic DNA and *E. coli* JM109 pTED, corresponds to IS*1133* sequence in these positive controls. Since no amplification product was obtained from any of the 840 recombinant colonies screened, the left portion of IS*1133* was not cloned from strain MOS-1.

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To optimise the cloning procedure the competency of the *E. coli* JM109 cells was tested using pUC19 and shown to be in the order of 1 x 10^{-7}. To test the T4 DNA ligase, 10 pools of 10 recombinant clones obtained were screened, using PCR assays for IS*Aba-1*, present as multiple copies in *A. baumannii* (Segal *et al*, 2005; Segal *et al*, 2007). The PCR products obtained for these 10 pools were all positive for IS*Aba-1*, suggesting that genomic DNA from *A. baumannii* strain MOS-1 was cloned into pUC19. It may be that the size of the resultant *Bam*HI/*Hind*III fragments containing IS*1133* is too large to be taken up by pUC19 which is known to not readily take up larger fragments. Another cloning vector such as pBR322 needs to be considered.

2.4 Discussion

Aminoglycoside resistance in *A. baumannii* can result due to the production of an AME (Shaw *et al*, 1993), a reduced uptake of the antibiotic (Fournier *et al*, 2006), expulsion of the antibiotic through a efflux pumps (Magnet *et al*, 2001; Su *et al*, 2005) or a combination of these mechanisms. The *aacC2a* gene has been described on plasmids in *S. marcescens* and *K. pneumoniae*, as well as linked to a portion of an IS element, IS*1133* in *A. baumannii* strain SAK (Allmansberger *et al*, 1985; Elisha, 1991). Another *A. baumannii* isolate strain PAU isolated 9 years later, was shown to harbour the same *aacC2a* genetic arrangement as described in *A. baumannii* strain SAK (Segal *et al*, 2003).

A total of 42 Gm resistant *A. baumannii* strains collected from two hospitals in Cape Town, as well as the susceptible *A. baumannii* strain TOM were screened for the presence of *aacC2a*. *A. baumannii* strains SAK and PAU, known to contain *aacC2a*, were included as positive controls (Elisha, 1991; Segal *et al*, 2003). A product of the expected size (786 bp) was obtained from 6/23 and 6/10 isolates from GSH and RCH, respectively. Accordingly, these 12 strains harbour an *aacC2a* gene. The remaining 30 isolates screened for the presence of an *aacC2a* gene were negative. As these remaining 30 isolates are Gm resistant, resistance to this antibiotic could be due to the expression of another AME. Studies have shown that the most common AMEs which can contribute to Gm resistance in *A. baumannii* are AAC(3)-Ia (gentamicin and fortimicin resistance) forming a gene cassette in several integrons of *A. baumannii* epidemic isolates (Gombac *et al*, 2002; Zarrilli *et al*, 2004; Turton *et al*, 2005; Fournier *et al*, 2006) and the gene encoding ANT(2") aminoglycoside nucleotidyltransferase (gentamicin, tobramycin and kanamycin resistance.) (Seward *et al*, 1991). In addition to the expression of an AME, a reduced uptake of the antibiotic, and the presence or overexpression of the AdeABC efflux pump (Magnet *et al*, 2001; Nemec *et al*, 2007) could also be contributing to aminoglycoside resistance in these isolates, this was however not investigated further.

To determine whether the *aacC2a* identified in 12 of *A. baumannii* isolates was associated with the right end of IS*1133*, PCR assays were carried out. A product of the expected size (1023 bp) was obtained from each of the 12 isolates, indicating that the *aacC2a* is linked to IS*1133* in these strains.

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DNA sequence analysis of two more recent strains RAN and MOS-1, isolated in 2001 and 2005, respectively, indicated that both these strains have the same T to A transversion at the fourth base of the IR_R, as described in *A. baumannii* strain SAK and PAU (Segal *et al*, 2003). Markarova *et al* (1999) identified an A to T substitution occurring near the 5'-terminal repeats in small fragments of IS*2621* from *Deinococcus radiodurans* and it was suggested that this substitution may result in the stabilisation of the IS element by preventing its excision and subsequent insertion events (Markarova *et al.*, 1999). Similarly, the T to A substitution occurring at the fourth base in the IR_R of IS*1133* may have played a role in supporting the stabilisation of the *aacC2a* genetic arrangement. This has been suggested as two *A. baumannii* strains PAU and SAK, which harbour the *aacC2a* gene cluster linked to a portion of IS*1133,* were isolated 9 years apart (Segal *et al*, 2003). This substitution, in conjunction with the *aacC2a* linked to a portion of IS*1133* has been shown to be maintained in two more recent *A. baumannii* isolates, RAN and MOS-1. This genetic arrangement has been maintained over a period of 23 years.

Interestingly, using PCR assays, IS*1133* sequence could not be detected in the 30 strains that did not contain *aacC2a*. This suggests that strains that do not harbour *aacC2a* do not contain IS*1133*, further arguing for an association between these two elements in *A. baumannii.*

Outside of *A. baumannii* strains SAK and PAU, the IS element, IS*1133* has been described as part of a composite transposon, Tn*5393* in the plant pathogen *E. amylovora* (Chiou *et al*, 1993) and on plasmids in *Salmonella* spp (Pezzella *et al*, 2004). Only a portion of IS*1133* has been described linked to the *aacC2a* in *A. baumannii* strains SAK and PAU and never a full copy as described in *E. amylovora* and *Salmonella* spp. (Chiou *et al*, 1993; Pezzella *et al*, 2004). PCR assays to screen the *A. baumannii* strains containing the *aacC2a* gene linked to a portion of IS*1133* for a full copy of IS*1133* did not yield the anticipated result. Instead, a larger PCR product than expected was obtained from all 12 strains harbouring the *aacC2a* gene, as well as strains PAU and SAK, suggesting an insertion event in IS*1133* had occurred. The amplicon obtained from strain SAK was purified and sequenced directly on both strands.

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Analysis of the DNA sequence data indicated that a full copy of IS*1133* was interrupted by insertion of IS*Aba-1* at nucleotide position 1121 in the *tnpA* of IS*1133*. A 9 bp duplication (AAATGGCTT) of the target sequence had occurred at the site of IS*Aba-1* insertion (Figure 2.4.1). Subsequently, DNA-DNA hybridisation studies were carried out to determine whether the 12 strains all contain a full copy of IS*1133* disrupted by the insertion of IS*Aba-1* as in *A. baumannii* strain SAK. The same site of insertion and target duplication was identified by DNA sequence analysis in two more recent isolates, A5 and A2 from GSH and RCH, respectively.

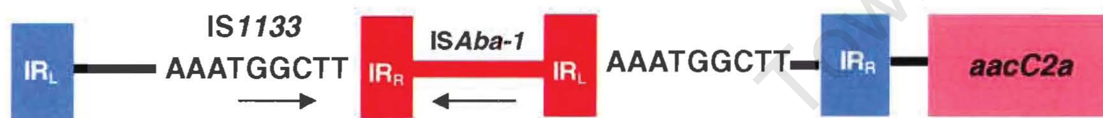

Figure 2.4.1: Genetic arrangement of *aacC2a* as determined by DNA sequence analysis in *A. baumannii* strain SAK.

It is interesting to speculate about the acquisition of IS*1133* in the *A. baumannii* genome, as IS*1133* has only been described in the plant pathogen *E. amylovora* in *Salmonella* isolates from poultry (Pezzella *et al*, 2004). It is possible *E. amylovora* is in close contact with a number of soil organisms, including the naturally competent *Acinetobacter baylii* and that IS*1133* was acquired in a genetic transfer event between these genera. However, *A. baumannii* has never been characterised outside of the hospital environment and it may be that *A. baumannii* isolates from hospitals in Cape Town originally acquired IS*1133* from another pathogenic bacterium in the hospital environment. As IS*1133* has also been identified in *Salmonella* spp isolated from poultry in Italy, it was suggested that IS*1133* was introduced into the *Salmonellae* genome following the ingestion of animal feed contaminated with *E. amylovora* (Pezzella *et al*, 2004). Furthermore, *aacC2a* has been detected in *Salmonella* serovar Typhimurium, isolated from retail meats (Chen *et al*, 2004) and the regulatory regions of plasmids isolated from both *E. amylovora* and Enterobacteriaceae have been shown to share a number of similarities (Foster *et al*, 2004). This supports the hypothesis of genetic exchange between *E. amylovora* and enteric bacteria. Therefore, it is possible to suggest that IS*1133* in *A. baumannii* was acquired from either *E. amylovora* or *Salmonella*. It would be very interesting to establish whether this IS*1133* element is present in clinical isolates of *Salmonella* and in *A. baumannii* isolates from other regions.

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The events that lead to the insertion of IS*1133* upstream of *aacC2a* are as yet unknown. To date IS*Aba-1* has only been identified in the genome of *Acinetobacter* spp (Segal *et al*, 2005) and it is likely that the insertion of IS*Aba-1* into IS*1133* occurred after the acquisition of IS*1133*. Whether, the insertion of IS*Aba-1* into IS*1133* was accidental or necessary to render IS*1133* immobile thus stabilizing the genetic organization of this region, is unknown. Nonetheless, it is interesting to postulate about the genetic arrangement between these two IS elements and their association with *aacC2a*. It may be that disruption of IS*1133 tnpA* by IS*Aba-1*, combined with the A to T transversion in the IR_R, has stabilized IS*1133* and in turn maintained the linkage with the *aacC2a* gene in these *A. baumannii* isolates. Furthermore, the necessities of this linkage may have a functional role.

IS*1133* is located immediately upstream of two phosphotransferase genes, *aph(6)-Ia* and *aph(6)-Id* (Chiou *et al*, 1993) and the expression of these two genes is dependant on promoter sequences within IS*1133* (Chiou *et al*, 1993). A number of antibiotic resistance genes in *A. baumannii* have been shown to be dependant on promoter sequences in adjacent IS elements (Corvec *et al*, 2002; Segal *et al*, 2003; Poirel *et al*, 2005; Segal *et al*, 2005; Segal *et al*, 2007). Accordingly, investigations into the expression of *aacC2a* in *A. baumannii* were carried out to determine whether the expression of *aacC2a* was derived from *aacC2a* promoters or from promoter sequences in IS*1133* or IS*Aba-1*.

Chapter Three

The expression of an aminoglycoside resistance gene, *aacC2a* in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

3.1 Introduction

Although much is known about the transcriptional control signals used in *E. coli*, there is a lack of information on the corresponding signals in *Acinetobacter*. In *E. coli* most genes are transcribed by the major RNA polymerase E δ^{70}, which can recognise a promoter that contains two conserved 6bp sequences located approximately 35bp and 10bp upstream from the transcriptional start site (Hawley & McClure, 1983). Another conserved feature of this promoter is that there is generally 17bp separating the -35 and -10 sequences (Hawley & McClure, 1983). Currently, the greater the similarity of the -35 and -10 promoter regions to that of the consensus sequences as first described for *E. coli*, the better the promoter functions *in vivo* (Dehaseth *et al*, 1998). A subclass of *E. coli* promoters have also been described that function well without a recognisable -35 region or the involvement of any acting proteins. Such promoters have what is termed an "extended -10 region" with a sequence similar to TGNTATAAT (Dehaseth *et al*, 1998). Generally, extended -10 promoters are recognised by the δ^{s} subunit of RNA polymerase (Dehaseth *et al*, 1998).

It has been suggested that an equivalent of the *E. coli* E δ^{70} is not present in all Gram-negative bacteria and studies carried out on the properties of RNA polymerases in *Acinetobacter* demonstrated that although the core RNA polymerases displays homology to RNA polymerase in *E. coli*, a corresponding *E. coli* E δ^{70} was not found in *Acinetobacter* (Kleppe & Kleppe, 1976). This suggests that either *Acinetobacter* does not have an equivalent *E. coli* E δ^{70} or that the relationship between this element and RNA polymerases is considerably weaker in *Acinetobacter* (Kleppe & Kleppe, 1976). It is therefore possible that *Acinetobacter* contains an RNA polymerase that recognises promoter sequences with similarity to *E. coli* E δ^{70} recognised promoter sequences but these sequences are not spaced the same as those in *E. coli*. Another possibility is the role of the

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δ^S subunit of RNA polymerase in *Acinetobacter* plays a more important role (Kleppe & Kleppe, 1976). This subunit is the chief regulator of general stress responses in *E. coli* and could be responsible for regulating the expression of genes in *Acinetobacter* during periods of stress (Kleppe & Kleppe, 1976).

Although a number of mechanisms can contribute to aminoglycoside resistance in *A. baumannii*, resistance to this class of antibiotics is mainly due to enzymatic modification. The expression of *aacC2a*, which encodes an enzyme responsible for the modification of the clinically important antibiotics gentamicin and tobramycin, has been described in *A. baumannii* (Shaw *et al*, 1996; Seward *et al*, 1998; Nemec *et al*, 2004). Many acetyltransferases have been identified as gene cassettes on class 1 integrons in various pathogenic bacteria, including *A. baumannii* (Nemec *et al*, 2004). The *aacC2a* gene however, has only been described previously on IncM conjugative plasmids and the transposable element, Tn*2922* (Martin *et al*, 1987).

The expression of this gene is suggested to be dependant on promoter sequences located immediately upstream in insertion sequences, IS*26*, IS*140* or IS15-Δ, or from remnants of insertion sequences (Allmansberger *et al*, 1985; Martin *et al*, 1987; Vliegenthart *et al*, 1989). Investigations into the expression of *aacC2a* when associated with the IS*140* element was shown to provide a putative -35 sequence (TTGCAA) 17 nucleotides upstream of the -10 region associated with this gene (Allmansberger *et al*, 1985). In a strain where *aacC2a* was not associated with IS*140*, and therefore was not downstream of a putative -35 region, the level of Gm resistance in this strain was half that observed in the strain where IS*140* is upstream of *aacC2a* (Allmansberger *et al*, 1985). The expression of antibiotic resistance genes due to promoter sequences supplied by insertion sequences has been well characterised in *A. baumannii*. One particular IS element, IS*Aba-1*, has been shown to be exclusively present in *Acinetobacter* spp and provides the necessary transcriptional signals required for the expression of numerous unrelated antibiotic resistance genes (Segal *et al*, 2004; Segal *et al*, 2005; Segal *et al*, 2007; Corvec *et al*, 2007).

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The observation that the ceftazidime MICs for *A. baumannii* isolates which contained the *ampC* gene linked to IS*Aba-1* were much greater (≥ 256 mg/ml) than those isolates where *ampC* was not associated with IS*Aba-1* suggested that hyperproduction of *ampC* was due to strong promoter sequences located in IS*Aba-1* upstream of this gene (Corvec *et al*, 2003). The precise transcriptional start site of *ampC* was determined as optimally spaced -35 (TTAGAA) and -10 (TATAAT) sequences located within the IS*Aba-1* element upstream of *ampC* in *A. baumannii* (Segal *et al*, 2004).

Interestingly, a number of additional -35 and -10 promoter sequences were identified in IS*Aba-1* suggesting that although only one primer extension product was obtained for *ampC* expression, transcriptional initiation could occur from more than one promoter to increase the levels of expression in some strains (Segal *et al*, 2004).

Previously, it has been shown that the IS*1133* can provide promoter sequences for expression of the aminoglycoside phosphotransferase genes, aph*(6)-Ia* and *aph(6)-Id* in *E. amylovora* (Chiou *et al*, 1993). As IS*1133* has been described upstream of another aminoglycoside resistance gene, *aacC2a* it is possible that expression of the *aacC2a* gene may originate from promoter sequences in IS*1133*.

When considering the genetic environment of the *aacC2a* gene in the 14 gentamicin resistant strains of *A. baumannii* included in this study, it is interesting to postulate whether expression of *aacC2a* (Figure 3.1.1a & 3.1.1b) occurs from its promoter sequences immediately adjacent to the gene (P1), from promoter sequences located within the portion of IS*1133* (P2) or from promoter sequences in IS*Aba-1* (P3).

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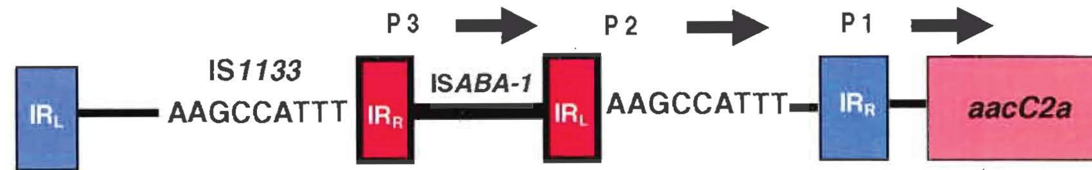

Figure 3.1.1a: Schematic representation of the genetic arrangement of the *aacC2a* gene indicating the regions where three possible sets of promoter sequences could be located for expression of the *aacC2a* gene.

HRF →
CACGAATGCAGAAGTTGATGTCTTGGTTCAATTAACCATGTAAACCACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTAC

CAAAAATGGCTATAAAGGGTTGAATCAAAGCAATGCGCTCTTTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCC

ACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTTTTCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATA

TTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTGACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCA

AGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGA

-35 (P3) **-10 (P3)**
TTTGTTTAAGATAAGATATAACTCATTGAGATGTGTCATAGTATTCGTCG**TTAGAA**AACAATTATTATGACA**TTATTT**CAATGAGTTATCTATTTTTGTC

IR$_L$ IS*Aba-1* **IS1133R →** **-35 (P2)** **-10 (P2)**
GTGTACAGAGAAATGGCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATCGCCG**GTGGCA**TTTGAGAAAAAAGCCGCT**TAAAAT**GAGAG

← IS1133F **IR$_R$ IS*1133*** **-10 (P1)**
ATAGACCGGAACACAACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGG**TAGAGT**GA

-10 (P1) **RBS** aacC2 →
TATCTCGATAGG**TATAGT**GTTTTGCAGTTTAG**AGGAGA**TATCGCG**ATG**CATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCG

← aacC2aR2
GTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTCCGGTCG —— *aacC2a*

Figure 3.1.1b: Nucleotide sequence of *aacC2a* linked by 115 bp to IS*1133* which has been interrupted by IS*Aba-1*. The primers used are blocked in yellow and the arrows indicate the 3' end of the primer from which elongation proceeds. The start codon for the *aacC2a* gene is indicated in bold type. The IR$_R$ of IS*1133* is underlined and the T to A transversion is indicated in blue. The IR$_L$ of IS*Aba-1* is underlined. The ribosomal binding site (RBS) and the two putative -10 promoter sequences (**P1**) for *aacC2a* expression are in bold type and underlined. The promoter sequences in IS*1133* (**P2**) are indicated in bold type and underlined as are the promoter sequences in IS*Aba-1*(**P3**).

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3.2 Experimental protocol

3.2.1 Preparation of *E. coli* JM109 (pRK002)

The *aacC2a* gene linked to a portion of IS*1133* interrupted by IS*Aba-1* was PCR amplified using primers aacC2aR (Table 2.2.2) designed to anneal to sequences within the structural gene of *aacC2a*, 64 bp upstream of the stop codon, and HRF [5' CACGAATGCAGAAGTTGATG '3] designed to anneal to sequences in IS*Aba-1* (Figure 3.1.1b). The PCR product obtained was cloned (2.2.8.3) into pGEM®-TEasy and transformed (2.2.8.4) into *E. coli* JM109. The resultant recombinant plasmid was designated pRK002.

The PCR reaction mix (2.2.3) containing genomic DNA from *A. baumannii* strain SAK was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 80 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour. An amplicon of the anticipated size (1638 bp) was obtained for *A. baumannii* strain SAK (Figure 3.2.1).

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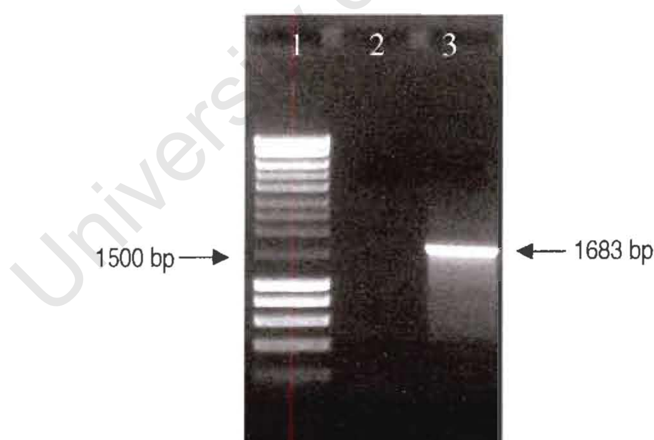

Figure 3.2.1: Agarose gel electrophoresis of amplicons following PCR amplification of the *aacC2a* linked to a portion of IS*1133* interrupted by IS*Aba-1* from *A. baumannii* strain SAK. Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: *A. baumannii* SAK

No amplification was obtained from the water control (Figure 3.2.1). A PCR product of 1683 bp was obtained from *A. baumannii* strain SAK. The PCR amplicon was excised after agarose gel electrophoresis (2.2.4) and purified (2.2.5.1). After quantification (2.2.5.3) the purified PCR product was cloned into pGEM®–TEasy [Promega Wisconsin, USA] and transformed into competent *E. coli* JM109 cells (2.2.8.4) and plated on 2xYT with appropriate selection. Blue/white screening was used, 10 white colonies were obtained, replica plated and inoculated overnight into 2xYT broth with Amplicillin selection. Small scale plasmid preparations (2.2.9) and a restriction enzyme digest with *Eco*RI was carried out to release the insert and electrophoresed (2.2.4) to confirm uptake of the correct PCR fragment. A single clone designated *E. coli* JM109 (pRK002) was obtained (Figure 3.2.2) and used during primer extension analysis.

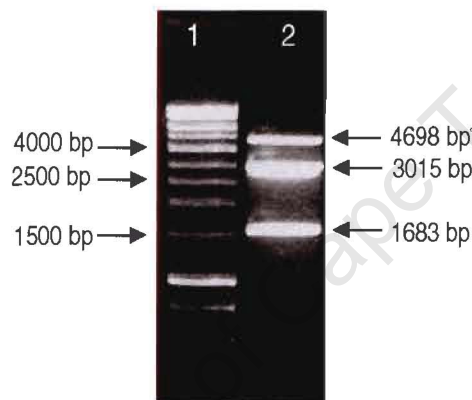

Figure 3.2.2: Agarose gel electrophoresis of the restriction enzyme digested *E. coli* JM109 (pRK002) clone. Lane 1: Hypleladder I (Bioline); Lane 2: *Eco*RI digested *E. coli* JM109 (RK002).

The 3015 bp band (Figure 3.2.2) corresponds to the pGEM®-TEasy vector and the 1683 bp band corresponds to the PCR amplicon from strain SAK, cloned into pGEM®-TEasy and cleaved from the vector by *Eco*RI digestion. The larger fragment of 4698 bp corresponds to linerized pGEM®-TEasy containing the PCR fragment.

3.2.2 The plotting of a growth curve for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

A growth curve for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) was generated to determine the optimal time point to extract RNA during mid-exponential and stationary growth phases. A 1ml aliquot taken from an overnight culture of each strain was sub-inoculated into a total volume of 50ml 2xYT broth containing gentamicin (5 µg/ml) and incubated at 37°C with shaking. Every 30 minutes a 1ml aliquot was obtained from each culture and the OD_{600} was determined

using a Beckman DU-40 at 600nm. The OD values obtained over an 8 hour period were plotted to obtain a growth curve for each of the strains as can be seen in Figure 3.2.3.

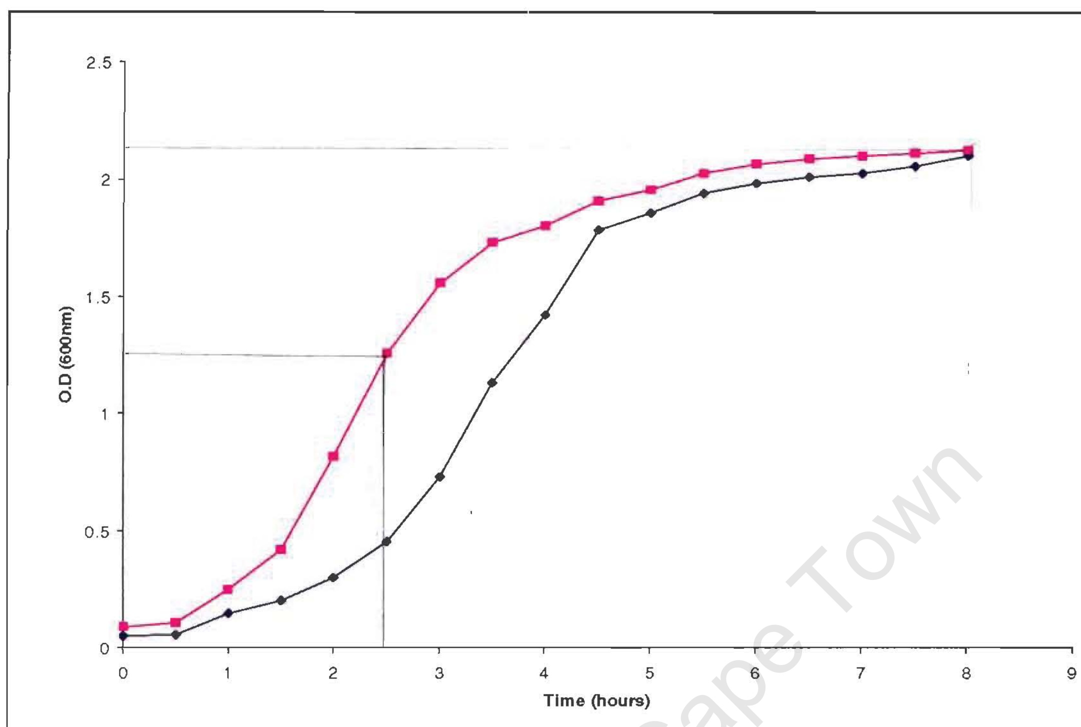

Figure 3.2.3: Growth curve for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in 2xYT with gentamicin selection (5 µg/ml). The growth curves for *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) are indicated by the pink and blue lines, respectively.

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From the growth curves obtained it was determined that two and half hours and three hours, post inoculation corresponded with the *A. baumannii* strain SAK and *E. coli* JM109 (pRK002), being in mid-exponential phase, respectively. Both cultures were in stationary phase 8 hours post inoculation.

3.2.3 Preparation of total cellular RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) using hot acid phenol

A. baumannii strain SAK and *E. coli* JM109 (pRK002) were grown in the presence of gentamicin (5µg/ml) in 50ml 2xYT broth at 37°C with aeration. Total cellular RNA was extracted from stationary and mid-exponential cultures using the hot acid phenol method (Aiba *et al*, 1981). Cells were collected from a 50ml culture by centrifugation at 7000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge.

The recovered pellet was resuspended in 1.5ml, 0.02M NaAcetate pH 5.5, 0.5% SDS and 1mM EDTA buffer. A 1.5ml aliquot of redistilled phenol (Appendix A) was added to the mixture and incubated at 60°C for 5 minutes with gentle shaking. The samples were then centrifuged at 7000rpm in order to separate out the phases. RNA in the aqueous phase was extracted and 0.5ml of redistilled phenol was added. After another incubation at 60°C for 5 minutes, with gentle shaking, the samples were centrifuged at 14 000rpm for 2 minutes at 4°C. Subsequently, the aqueous phase containing the RNA was removed and the recovered RNA precipitated by the addition of 3 volumes of 100% ethanol and chilled for 30 minutes at -70°C. The precipitated RNA was collected by centrifugation and re-suspended in 0.25ml 0.02M NaAcetate pH 5.5, 0.5% SDS and 1mM EDTA. The ethanol precipitation step was repeated twice and the recovered RNA pellet was finally resuspended in 50µl DEPC-H_2O (Appendix A) and stored at -70°C. The purity of the recovered RNA was determined using Qubit™ fluorometer [Invitrogen] (2.2.5.3) and visualised to determine its integrity by agarose gel electrophoresis (2.2.4).

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3.2.4 DNase treatment of recovered RNA

In order to eliminate any contaminating traces of DNA which may affect further analysis of the prepared RNA samples, 5µg of RNA was treated with 5U RQ_1 RNase - free DNase (1U/µg of RNA) [Promega Wisconsin, USA] in RQ1 RNase - free buffer (10x) [Promega Wisconsin, USA] and incubated at 37°C for 30 minutes. The DNase I endonuclease degrades both double and single stranded DNA and ensures that only RNA remains. The reaction was terminated by the addition of RQ1 RNase - free stop solution [Promega Wisconsin, USA] and incubation at 65°C for 10 minutes.

3.2.5 Reverse transcriptase-polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT – PCR) is a method used to confirm the expression of a particular gene of interest. The mRNA transcripts are extracted and reverse transcribed to form a cDNA product. The cDNA product can subsequently be used as a template in a PCR reaction using primers designed to anneal to sequences complementary to the gene of interest. RT- PCR was used to determine the expression levels of the *aacC2a* gene in both *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in the presence of gentamicin. 5µg of DNase treated RNA from both strains was incubated for 5 minutes at 70°C with 2µl of a 10µm 16s reverse primer [5' GCA GTG GGG AAT ATT GG '3] and 2µl of 10µm aacC2aR2 primer [5'- CGA CCG

GAC CAA TCG C - 3']. Subsequently, 5x M-MLV buffer, 10mM dNTPS, Rnase inhibitor (40U/ul) [Roche] and 400U of M-MLV RT-enzyme (200U/μl) [Promega] were added to a final volume of 25μl and incubated at 37°C for 1 hour. Reverse transcription was terminated by incubation at 85°C for 5 minutes. All remaining mRNA was removed from the cDNA product by the addition of RNase and incubated at 37°C for 1 hour. The cDNA products obtained were then analysed by PCR amplification (2.2.3) with all the appropriate controls. The PCR products obtained were visualized by agarose gel electrophoresis on a 1% agarose gel (2.2.5).

3.2.6 Primer extension studies

Primer extension analysis is used to determine the precise transcriptional start site of an mRNA transcript. This requires annealing of a primer downstream of the start codon of the gene being investigated. The primer is labeled at its 5' end with fluorescent marker and extended using reverse transcriptase.

Primer extension experiments were carried out to determine the transcriptional start of *aacC2a* gene from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002). One hundred micrograms of total RNA, extracted by using the hot acid phenol (3.2.3), was precipitated by the addition of 2.5 volumes 100% ethanol and 0.1 volumes 3M NaAcetate. The samples were incubated at -20°C for 30 minutes and subsequently, pelleted by centrifugation at 12 000rpm for 10 minutes at 4°C. The pellet was resuspended in 70% ethanol and centrifuged at 12000rpm for a further 10 minutes at 4°C. Subsequently, the pellet was resuspended in 100μl hybridisation buffer (40mM PIPES pH 6.4, 1Mm EDTA, 0.4M NaCl, 80% formamide and DEPC-H_2O). The indodicarbocyanine Cy5 labelled oligonucleotide [aacC2aR2PE; 5'- CGA CCG GAC CAA TCG C - 3'] (5 pmol) that anneals to nucleotides 87-103 downstream of the ATG initiation codon of *aacC2a* (Figure 3.1.1b), was synthesized and labeled in the Department of Molecular and Cell Biology, University of Cape Town, South Africa. The aacC2aR2PE oligonucleotide was added to the resuspended pellet and incubated at 85°C to denature the RNA. The RNA and the labeled primer were then allowed to anneal at 54°C overnight. Subsequently, the RNA plus annealed primer were precipitated by the addition of 2.5 volumes 100% ethanol and 0.1 volumes 3M NaAcetate. This mix was incubated at -20°C for 30 minutes and pelleted by centrifugation at 12 000rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol and microfuged at 12 000rpm for a further 10 minutes at 4°C.

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The pellet was then resuspended in a total volume of 20µl containing, 4µl 5x moloney murine leukaemia virus (M-MLV) reverse transcriptase buffer [Promega Wisconsin, USA], 1µl of a 10mM deoxynucleotide triphosphate mix (dNTP) [Fermentas], 1µl (40U) RNase inhibitor [Boehringer Mannheim, Germany], 2µl Actinomycin D (1mg/ml), 12µl DEPC-ddH_2O. Actinomycin D increases the production of full-length cDNA and inhibits the formation of hair pin loops when using M-MLV reverse transcriptase. M-MLV reverse transcriptase (200U) [Promega Wisconsin, USA] was added and incubated for 2 hours at 42°C. The reaction was stopped by the addition of 1µl 0.5M EDTA pH 8.0 and 1µl (0.5 mg/ml) Ribonuclease A [Boehringer Mannheim, Germany] and incubated at 37°C for 45 minutes to remove any remaining RNA in the cDNA samples. These primer extension products were then precipitated with 150µl TES buffer (10mM Tris, 1mM EDTA pH 8.0, 100mM NaCl) and 2.5 volumes of 100% ethanol. The pellet was reconstituted in 5µl DEPC-ddH_2O and stored at –20°C.

The final primer extension products were analysed alongside the corresponding sequencing reaction products (3.2.1). Analysis of the products was performed on the ALFexpress automated DNA sequencer [Amersham Biosciences] in the Department of Molecular and Cell Biology, UCT, Cape Town, South Africa. The sequencing reaction and the primer extension products were electrophoresed on a denaturing polyacrylamide gel. The appearance of a peak indicates the size of the cDNA product which corresponds to the precise transcriptional start site of the corresponding mRNA transcript. The data generated was analysed using ALFwin (Version 1.10 Pharmacia Biotech).

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3.3 Results

3.3.1 Expression of *aacC2a* in *E. coli* JM109 (pRK002)

To confirm the expression of *aacC2a* in *E. coli* JM109 (pRK002) and to compare this to the levels of aminoglycoside resistance in *E. coli* JM109 and *A. baumannii* strain SAK, MICs for gentamicin, amikacin and kanamycin were determined (2.2.1) (Table 3.3.1)

Table 3.3.1: Aminoglycoside antibiotic MICs for *E. coli* JM109, *E. coli* JM109 (pRK002) and *A. baumannii* strain SAK

Antibiotic	MIC (µg/ml)		
	A. baumannii SAK	*E. coli* JM109 (pRK002)	*E. coli* JM109
Gentamicin	≥128	6	0.190
Amikacin	4	0.750	0.750
Kanamycin	4	2	1

3.3.2 Extraction of total cellular RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

The hot phenol method (3.2.3) was used to extract total cellular RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) grown in the presence of gentamicin selection, during mid exponential and stationary growth phase. A total of 1µl of each sample was electrophoresed to check the integrity of the extracted RNA (Figure 3.3.3). Quantification of the RNA was determined using the Qubit™ fluorometer [Invitrogen] (2.2.5.3).

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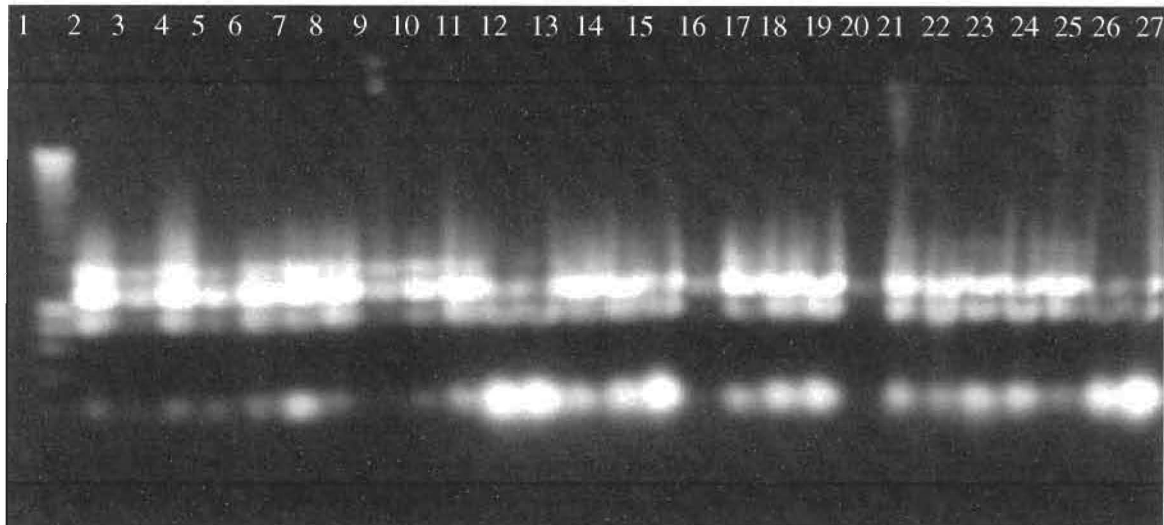

Figure 3.3.3: Electrophoresis of the extracted RNA obtained from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) during mid exponential and stationary growth phase in the presence of gentamicin selection. Lane 1 Hyperladder I (Bioline); Lanes 2 – 8: *A. baumannii* strain SAK in mid exponential growth phase; Lanes 9 - 16: *A. baumannii* strain SAK in stationary growth phase; Lanes 17 - 22: *E. coli* JM109 (pRK002) in mid exponential growth phase; Lanes 23 - 27; *E. coli* JM109 (pRK002) in stationary growth phase.

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3.3.3 Expression of the *aacC2a* gene in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

To determine *aacC2a* expression in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) RT-PCR experiments were carried out using RNA extracted during mid-exponential phase and stationary phase from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002). cDNA products corresponding to *aacC2a* were generated. A 16s RNA primer was included in the cDNA reaction as an internal control for cDNA synthesis. Subsequently, 2µl of this cDNA product was used as a template in a PCR assay.

The PCR reaction mix (2.2.3) containing cDNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) from mid exponential and stationary growth phase, respectively, was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 60 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour. Amplicons of the anticipated size corresponding to the *aacC2a* gene (786 bp) and 16s RNA gene (449 bp) were obtained (Figure 3.3.4).

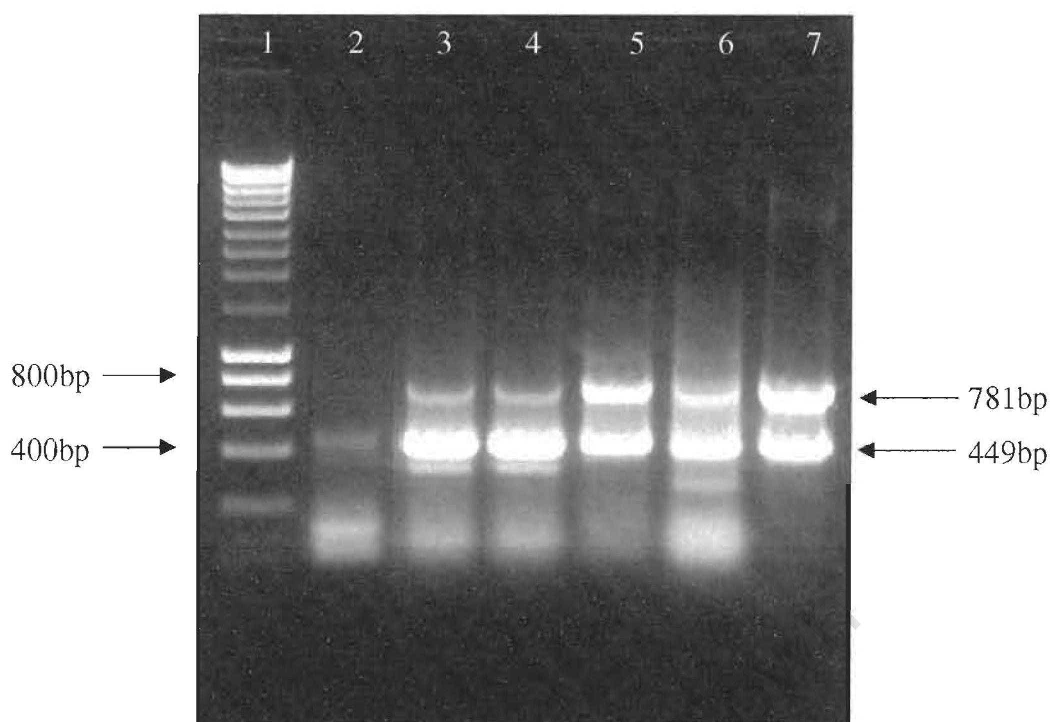

Figure 3.3.4: Agarose gel electrophoresis of amplicons following PCR amplification of *aacC2a* and 16s RNA from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002). Lane 1: Hyperladder I (Bioline); Lane 2: Water control; Lane 3: *A. baumannii* SAK in mid exponential phase; Lane 4: *A. baumannii* SAK in late stationary phase; Lane 5: *E. coli* JM109 (pRK002) in mid exponential growth phase; Lane 6: *E. coli* JM109 (pRK002) in stationary growth phase; Lane 7: *A. baumannii* SAK genomic DNA. The *A. baumannii* genomic DNA was included as a positive PCR control.

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A PCR product of 449 bp was obtained from *A. baumannii* strain SAK in mid exponential growth phase, *A. baumannii* strain SAK in stationary growth phase, *E. coli* JM109 (pRK002) in mid exponential growth phase and *E. coli* JM109 (pRK002) in stationary growth phase (Figure 3.3.4) and corresponds to the 16s RNA gene, indicating that the cDNA synthesis step worked. A band corresponding to 449 bp was also obtained in the water control. The presence of this band in the water control may be due to amplification from contaminating bacteria in the water used for PCR reaction mix. The 786 bp product corresponding to *aacC2a* was obtained from *A. baumannii* strain SAK in mid exponential growth phase, from *A. baumannii* strain SAK in stationary growth phase, *E. coli* JM109 (pRK002) in mid exponential growth phase and *E. coli* JM109 (pRK002) in stationary growth phase.

3.3.4 Investigations of the transcriptional start site of *aacC2a* in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002)

Numerous reports describe the expression of antibiotic resistance genes derived from promoter sequences located in upstream IS elements (Chiou *et al*, 1993; Segal *et al*, 2003; Segal *et al*, 2005; Segal *et al*, 2007).

When considering the genetic environment of the *aacC2a* gene in the 14 gentamicin resistant strains of *A. baumannii* included in this study, it is interesting to postulate whether expression of *aacC2a* (Figure 3.3.5) occurs from promoter sequences located immediately adjacent to the gene (P1), from promoter sequences located within the portion of IS*1133* (P2) or from promoter sequences within IS*Aba-1* (P3).

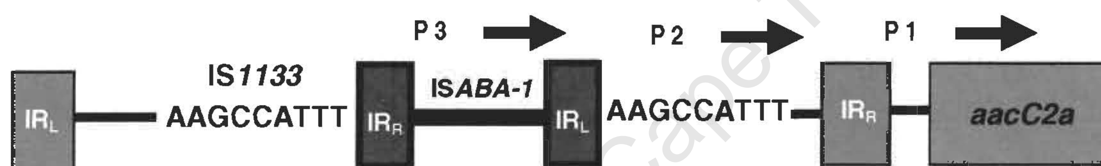

Figure 3.3.5: Schematic representation of the genetic arrangement of *aacC2a* in *A. baumannii* strain SAK. The regions where three possible sets of promoter sequences could be located and used for the expression of the *aacC2a* gene are indicated with arrows.

To determine the precise transcriptional start site, primer extension analysis was carried out using RNA extracted from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) during mid exponential and stationary growth phase (3.2.3). To check the integrity of the cDNA generated from the primer extension procedure, the cDNA products generated using the Cy5 – labelled aacC2aR2 PE primer (3.3.6) were diluted 1/10, and 2 µl was used in subsequent PCR assays. The remaining 4µl was sent to the sequencing unit at the Department of Molecular and Cell Biology, UCT, Cape Town, South Africa, for further analysis.

A PCR assay using a reverse primer designed to anneal within the structural gene of *aacC2a* (aacC2aR2) and a forward primer designed to anneal to sequences within IS*Aba-1* (HRF) (Figure 3.1.1b) was carried out to determine whether the cDNA product generated contains the regions complementary to the primers used.

The PCR reaction mix (2.2.3) containing cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) in mid exponential and stationary growth phase, was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 80 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour (Figure 3.3.6).

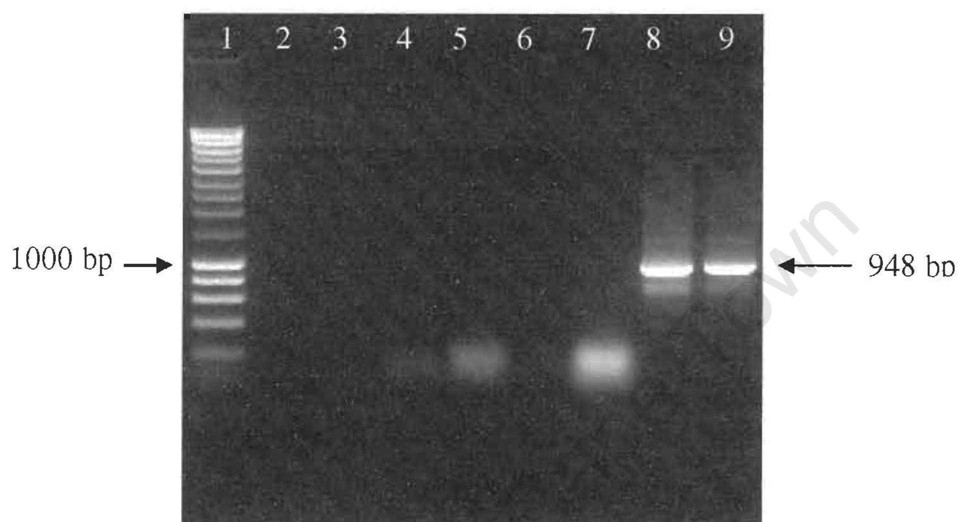

Figure 3.3.6: Agarose gel electrophoresis of PCR assays using primers HRF and aacC2aR2 and cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) as template. Lane 1: Hyperladder (Bioline); Lane 2: Water control; Lane 3: Blank; Lane 4: *A. baumannii* SAK cDNA generated from mid-exponential phase; Lane 5: *A. baumannii* SAK cDNA generated from late stationary phase; Lane 6: *E. coli* JM109 (pRK002) cDNA generated from mid-exponential phase; Lane 7: *E. coli* JM109 (pRK002) cDNA generated from stationary phase; Lane 8: *A. baumannii* SAK genomic DNA (positive control); Lane 9: *A. baumannii* MOS-1 genomic DNA. Genomic DNA from *A. baumannii* strain SAK and MOS-1 were included as PCR positive controls.

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No amplification product was obtained from the water control. A PCR product of the 948 bp, corresponding to a product of the expected size, was obtained from the genomic DNA from strains SAK and MOS-1 (Figure 3.3.6, lanes 8 and 9, respectively). Both *A. baumannii* strain SAK and MOS-1 were included as PCR positive controls as these strains are known to contain *aacC2a* linked to a portion of IS*1133* interrupted by IS*Aba-1*. No PCR product was obtained from cDNA generated from *A. baumannii* SAK in mid exponential growth phase, *A. baumannii* SAK in stationary growth phase, *E. coli* JM109 (pRK002) in mid exponential growth phase, nor *E. coli* JM109 (pRK002) in stationary growth phase (Figure 3.3.5) This result suggests that the cDNA product generated does not extend as far as the DNA sequence complementary to primer HRF, which was therefore not able to anneal. Consequently, a second PCR experiment was carried out using a reverse primer designed to anneal within the structural gene of *aacC2a* (aacC2aR2) and a

forward primer designed to anneal to complementary sequences within IS*1133* (IS1133R) (Figure 3.1.1b). The primer IS1133R anneals to sequences just downstream of the insertion of IS*Aba-1* and was used to determine whether the cDNA product generated using primer aacC2aR2 contains the region complementary to primer IS1133R.

The PCR reaction mix (2.2.3) containing cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) in both mid exponential and stationary growth phase was subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 80 seconds. A final elongation at 72°C for 5 minutes completed the reaction. PCR products were analysed by gel electrophoresis (2.2.4) on a 1% agarose gel at 90V for 1 hour (Figure 3.3.7).

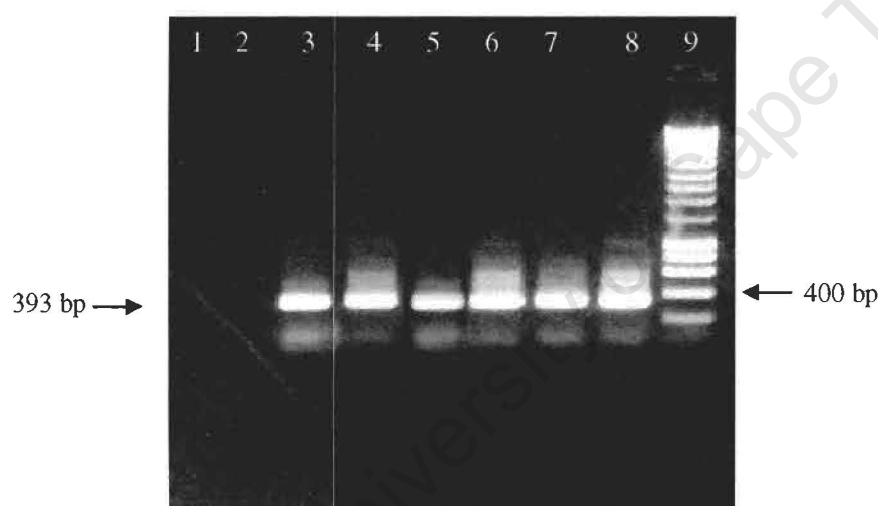

Figure 3.3.7: Agarose gel electrophoresis of PCR assays using primer IS1133R and aacC2aR and cDNA from *A. baumannii* SAK and *E. coli* JM109 (pRK002) as template. Lane 1: Water control; Lane 2: Blank; Lane 3: *A. baumannii* SAK cDNA generated from mid-exponential phase; Lane 4: *A. baumannii* SAK cDNA generated from late stationary phase; Lane 5: *E. coli* JM109 (pRK002) cDNA generated from mid-exponential phase; Lane 6: *E. coli* JM109 (pRK002) cDNA generated from stationary phase; Lane 7: *A. baumannii* SAK genomic DNA (positive control); Lane 8: *A. baumannii* strain MOS-1 genomic DNA; Lane 9: Hyperladder I. Genomic DNA from *A. baumannii* strains SAK and MOS-1 were included as PCR positive controls.

No amplification product was obtained from the water control (Figure 3.3.7). A PCR product of the expected size (393 bp) was obtained from genomic DNA from strains SAK and MOS-1(lanes 7 and 8 respectively) which contain IS*1133* linked to *aacC2a* were included as PCR positive controls. The corresponding product was obtained from cDNA generated from *A. baumannii* SAK in mid

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exponential growth phase, *A. baumannii* SAK in stationary growth phase, *E. coli* JM109 (pRK002) in mid exponential growth phase and *E. coli* JM109 (pRK002) in stationary growth phase. This result suggests that the cDNA product generated extends beyond the complementary sequences of primer IS1133R. Therefore, the 5' end of the cDNA product must correspond to a transcriptional start site upstream of primer IS1133R. Expression of *aacC2a* must therefore occur from promoter sequences upstream of this IS1133R primer and therefore more than likely from promoter sequences in IS*Aba-1*. These P(3) promoter sequences include TTAGAA (-35) and TTATTT (-10) separated by 16 bp, are promoters previously characterised in the expression of *ampC*, bla_{OXA-23} and *sulII* in *A. baumannii* (Segal *et al*, 2003; Segal *et al*, 2005; Segal *et al*, 2007).

Primer extension analysis was carried out to map the precise transcriptional start site, however the primer extension data obtained following cDNA analysis did not indicate the precise transcriptional start site. Although cDNA products were generated, as indicated following PCR analysis of the cDNA, a cDNA product could not be detected using the ALFexpress automated DNA sequencer [Amersham Biosciences]. It may be that the size of the cDNA transcript originating at the IS*Aba-1* located promoter sequences (398 bp) is too long for ALFexpress automated DNA sequencer analysis. An alternative procedure, such as 5' (Race) (Poirel & Nordmann, 2006 (a)) needs to be considered for mapping the precise transcriptional start site of the *aacC2a* gene in *A. baumannii.*

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3.4 Discussion

The genetic location of the *aacC2a* in aminoglycoside resistant bacteria is often on conjugative plasmids, transposable elements or closely associated with insertion sequences (Allmansberger *et al*, 1985; Martin *et al*, 1987; Vliegenthart *et al*, 1989; Elisha & Steyn, 1991). Elisha & Steyn (1991) identified an *aacC2a* gene in *A. baumannii* SAK on a plasmid and in the chromosome of this strain. Further analysis of *aacC2a* in strain SAK determined that the ORF is preceded by a ribosomal binding site (AGGAG) (Elisha, 1991) and upstream of the *aacC2a* coding sequence are sequences (TATAGT and TAGAGT) with homology to the -10 consensus sequence (TATAAT) of *E. coli* promoters (Hawley & McClure, 1983). However, there were no optimally spaced -35 (TTGACA) sequences relative to either of these putative -10 promoter regions (Elisha *et al*, 1991). Upstream of this region of the *aacC2a* gene are nucleotide sequences which display homology to IS*1133* (Elisha & Steyn, 1991). Other studies have suggested that the expression of *aacC2a* is dependant on promoter sequences located immediately upstream in insertion sequences IS*26*, IS*140* and IS15-Δ, or from remnants of insertion sequences (Allmansberger *et al*, 1985; Martin *et al*, 1987; Vliegenthart *et al*, 1989).

Previously, an *aacC2a* gene in *A. baumannii* strain SAK was shown to be linked to the right end of IS*1133* (Elisha, 1991). This same arrangement was later shown in *A. baumannii* strain PAU, isolated 9 years later (Segal *et al*, 2003). An identical linkage of IS*1133-aacC2a* was identified in 12 more recent Gm resistant *A. baumannii* isolates, obtained from two hospitals in Cape Town. Further investigations of the 14 strains containing the portion of IS*1133* linked to *aacC2a* revealed that a full copy of IS*1133*, linked to *aacC2a*, is disrupted by the insertion of a full copy of IS*Aba-1*.

As the expression of *aacC2a* has been previously associated with promoters provided by insertion sequences it was proposed that expression of *aacC2a*, linked to IS*1133* and IS*Aba-1* in the 14 Gm resistant *A. baumannii* strains may occur from promoter sequences from one of three possible promoter sets. It may be that the expression of *aacC2a* originates from promoter sequences immediately adjacent to the gene (P1), from promoter sequences located within the portion of IS*1133* (P2), or from promoter sequences in IS*Aba-1* (P3) (Figure 3.1.1a & 3.1.1b). Based on observations that the *aacC2a* gene has been described on a plasmid, pUO055 in a hospital isolate of *E. coli* (Terǎn *et al*, 1991) and that transcription signals recognised in *Acinetobacter* spp are different from their counterparts in *E. coli*, experiments were carried out to determine which

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promoter sequences are recognised in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) for the expression of *aacC2a* in these strains.

An important limitation on PCR and other molecular techniques is that the confirmation of the presence of a gene does not necessarily determine whether it is functionally competent and this must be established first in its host before a role in resistance can be ascribed to it. Accordingly, total cellular RNA was extracted from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in mid exponential and stationary growth phases, in the presence of Gm selection, and RT-PCR was performed to determine whether the *aacC2a* gene is expressed in these strains. Previous analysis of the *aacC2a* gene in *A. baumannii* strain SAK proposed that transcription of this gene occurs from promoter regions located within the sequences immediately upstream of the gene (Elisha, 1991). Evidence supporting this claim is that the entire *aacC2a* gene plus the 115bp region containing the two putative -10 promoter regions upstream of this gene were cloned into pUC19 and transformed into *E. coli* HB101, generating the recombinant plasmid pGSH110 (Elisha, 1991). This plasmid is able to confer gentamicin resistance on its *E. coli* host, as it is able to grow on gentamicin selection at 5 µg/ml. The initial RT-PCR results obtained (Figure 3.3.4) suggest that *aacC2a* is transcribed in *A. baumannii* strain SAK in both mid exponential growth phase and stationary growth phase, suggesting that *aacC2a* in *A. baumannii* is expressed in spite of different stages of growth. The *aacC2a* gene cloned into *E. coli* JM109 (pRK002) was also expressed in both mid exponential and stationary growth phase. Suggesting that *aacC2a* gene expression is derived from promoter sequences located upstream of this gene and expression occurs despite the growth phase in both *A. baumannii* strain SAK and *E. coli* JM109 (pRK002).

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In further studies carried out on *A. baumannii* strain SAK, high levels of kanamycin resistance were shown to be associated with the hyperproduction of the *aacC2a* enzyme and a reduced accumulation of aminoglycosides (Elisha & Steyn, 1991). The authors suggest that this may be due to tandem repeats of the gene, resulting in over expression of the gene and thus a higher MIC obtained for kanamycin, even though kanamycin is not the preferred substrate for this enzyme (Elisha & Steyn, 1991). However, in light of the recent data it could be that the hyperproduction of *aacC2a* may be due to an increased expression of the *aacC2a* gene from stronger promoters located in either IS*1133* or IS*Aba-1*, when the bacteria is exposed to a stressful environment, such as high levels of kanamycin. Accordingly, investigation of the transcriptional start site of *aacC2a* in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) was carried out.

Initially, PCR assays were carried out using primers HRF and aacC2aR2 with cDNA generated by using the primer aacC2aR2, as template. Using primers HRF and aacC2aR2 no PCR product was obtained, suggesting that the cDNA product does not extend to the regions containing sequence complementary to the primer HRF, which is therefore not able to anneal.

A second PCR assay was carried out using primer IS1133R, located in the IS*1133* region, 393 bp upstream of the *aacC2a* start codon. A product of 393 bp was obtained from cDNA from both *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in mid exponential and stationary growth phase, suggesting that the cDNA products generated using primer aacC2aR2 extends beyond the region complementary to IS1133R, which is therefore able to anneal. This suggests that the mRNA transcript for *aacC2a* expression originates at a nucleotide position upstream of the IS1133R binding site. This region of sequence corresponds to *ISAba-1*, which contains a number of putative promoter sequences including TTGACA (-35) (N_{16}) TATAAT (-10) which were suggested as the putative promoters for *ampC* in *A. baumannii* strain RAN (Segal *et al*, 2004) and *sulII* in *A. baumannii* strain RAM (Segal *et al*, 2005). The sequence immediately upstream of the -10 hexamer (TTATTT) generates an extended -10 hexamer (TGACATTATTT) which shows similarity to sequences recognized by $E\delta^S$ (Hengge-Aronis, 2002). The promoter sequences necessary for the expression of *aacC2a* are therefore most likely in IS*Aba-1* (Figure 3.1.1b). Many IS elements contain promoters that play a role in the expression of downstream antibiotic resistance genes and previous studies have shown that IS*Aba-1* provides promoter sequences necessary for the expression of adjacent antibiotic resistance genes, *ampC*, *bla*$_{OXA-23}$ and *sulII* in *A. baumannii* (Segal *et al*, 2003; Segal *et al*, 2005 and Segal *et al*, 2007). In a recent study, primer extension analysis indicated that *bla*$_{OXA-23}$ is transcribed from different promoters from IS*Aba-1* in *A. baumannii* and *E. coli* (Segal *et al*, 2007) and that in *A. baumannii* strain RAM, an extended -10 promoter located in IS*Aba-1* is used to initiate expression of *bla*$_{OXA-23}$ (Segal *et al*, 2007). This result suggests that different transcriptional factors may be involved in the expression of the *aacC2a* in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002). A difference in the transcription of genes from *A. baumannii* and *E. coli* has been described previously (Schembri *et al*, 1995). Analysis of the expression of the polyhydroxyalkanoic acid biosynthetic genes (*pha*A$_{AC}$, *pha*B$_{AC}$, *pha*C$_{AC}$) in *A. baumannii* and *E. coli* was carried out using primer extension analysis. It was shown that the expression of this operon in *A. baumannii* compared to in *E. coli* occurs from several different promoter regions in *E. coli* compared to *A. baumannii* (Schembri *et al*, 1995). Another study, which compared the expression of an aminoglycoside resistance gene in *A. baumannii* to

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that in *E. coli,* suggested that the transcriptional control signals recognised in *A. baumannii* are similar but not identical to those utilised by *E. coli* (Segal & Elisha, 1999).

The results obtained from cDNA generated from RNA extracted from *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) suggest the transcription of *aacC2a* may originate from promoter sequences within IS*Aba-1*. However, as *aacC2a* gene expression can originate from promoter sequences immediately upstream of the gene as described by Elisha & Steyn (1991) it may be that the *aacC2a* gene is expressed at low levels from promoter sequences immediately upstream of the gene, however as growth conditions become more stressful expression of *aacC2a* is derived from stronger promoter sequences within IS*Aba-1*.

Interestingly, the same genetic arrangement of *aacC2a* has been maintained in 14 *A. baumannii* strains isolated over a 23 year period from two different hospitals. Since the genetic relatedness of these strains is highly probable, PFGE analysis was carried out.

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Chapter four

Investigation into the clonal relatedness amongst *A. baumannii* isolates from GSH and RCH

4.1 Introduction

Increasingly, noscomial infections caused by *A. baumannii* are being reported worldwide. Recent trends in microbiology have moved towards establishing the genetic origin of these bacterial strains and it has been shown that *A. baumannii* infections can occur either as sporadic cases associated with a single genotype species or as outbreaks caused by genotypically related organisms. A clone describes a group of isolates that are highly similar although not necessarily genetically related to one another, which still remain distinct from other strains and need not have an epidemiological link (Dijkshoorn *et al*, 1996; Turton *et al*, 2005).

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A number of clonal lineages of *A. baumannii* have been reported including the European clones I, II and III, which are widespread in Europe (Dijkshoorn *et al*, 1996). There have also been reports of another two *A. baumannii* clonal lineages, one containing an OXA-40 carbapenemase, prevalent at various hospitals in Spain and Portugal (Da Silva *et al*, 2004) and the other containing a VEB-1 carbapenemase that has spread throughout parts of France and Belgium (Poirel *et al*, 2003). However, these are by no means the only reports of clonal lineages of *A. baumannii* in a clinical setting. The United Kingdom alone has published numerous reports on the increasing prevalence of clonally related *A. baumannii* isolates. This was demonstrated in the emergence of a southeast (SE) clone which has been prevalent in southern England since 2000 (Turton *et al*, 2004) and the recent discovery of two carbapenem resistant *A. baumannii* lineages, designated OXA-23 clone 1 and 2 (Coelho *et al*, 2006).

Numerous typing tools have been established for the identification of epidemic strains and the clonally related strains, and all methods described to date have merits and weaknesses. No single typing tool has gained acceptance for typing *Acinetobacter* spp and this area is still the subject of much research.

Ribotyping involves the purification and digestion of chromosomal DNA which is subsequently electrophoresed, blotted and then hybridised with a labelled cDNA probe is derived from *E. coli* rRNA (Bergogne-Bérézin & Towner, 1996). Hybridisation patterns generated are compared and excellent results have been obtained using this method, particularly when used in combination with another typing method, such as PFGE (Brisse *et al*, 2000).

Amplified fragment length polymorphism (AFLP) is a more recent PCR-based DNA fingerprinting method that was first described in 1993. It can be used for the characterization and comparison of DNA and has been used to genotype a number of different bacterial species including *Acinetobacter* (Bergogne-Bérézin & Towner, 1996). AFLP fingerprinting with a radioactively labeled primer or a fluorescently labeled primer has been shown to be highly discriminatory in the identification of *Acinetobacter* spp (Koeleman *et al*, 1998). In a study carried out by Koeleman *et al* (1998), they clearly identified 18 *Acinetobacter* genomic species and typed 13 clinical *Acinetobacter* isolates using fluorescent AFLP. They concluded that this technique is technically very fast, simple to perform and seemed particularly well suited for investigating the epidemiology of nosocomial infections and outbreaks caused by *Acinetobacter* species (Koelman *et al*, 1998).

Analysis of *Acinetobacter* spp by pulsed-field gel electrophoresis (PFGE) is often considered the "gold standard" (Tenover et al, 1995) and aims to compare the restriction fragment length polymorphisms obtained from intact genomic DNA (Bergogne-Bérézin & Towner, 1996). This method has indicated discrimination between DNA polymorphism obtained from clinically important *A. baumannii* isolates and is an extremely efficient epidemiological tool. PFGE involves the in situ lysis of bacterial cells and subsequent, digestion of genomic DNA with an enzyme that cuts the genome infrequently. The application of an alternating pulsing electric field allows the larger genomic fragments to be separated, producing unique banding patterns for each strain. However, the disadvantages of this method are that the equipment required is costly and the preparation of intact chromosomal DNA digestion and gel electrophoresis is a lengthy process, whilst the interpretation of the data obtained requires individual and computer based analysis often with the comparison of results with those of another laboratory extremely difficult. To overcome these difficulties Tenover *et al* (1995) described guidelines that can be followed for the standardisation of PFGE analysis. A study aimed at the standardisation and interlaboratory reproducibility of PFGE fingerprints obtained from the same *A. baumannii* isolates was carried out in three separate laboratories (Seifert *et al*, 2005). In conclusion it was

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found that there was an 87% matching of the strains if they were processed at different laboratories and 95% if they were processed at each local laboratory (Seifert *et al*, 2005).

To determine whether 44 aminoglycoside resistant *A. baumannii* strains considered in this study are genetically related, pulsed-field gel electrophoresis (PFGE) was carried out.

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4.2 Experimental protocol

4.2.1 Pulsed-field gel electrophoresis

The PFGE protocol used in this study has been adapted from that described by Ribot *et al* (2001).

4.2.1.1 Plug preparation

A. baumannii strains were streaked out onto 2xYT plates and incubated overnight at 37°C. Cells obtained were suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA pH 8.0) to an OD_{540nm} of 1.8 - 2.0 (BioMate 5, ThermoElectron Corporation, Cambridge, UK), and incubated at 55°C for 10 minutes to disrupt the bacterial cell walls. Subsequently, 25 µl of proteinase K (20 mg/ml) [Lyophilized, Novagen] and 5µl of lysozyme (100 mg/ml) [Roche] were added to the cell suspension buffer. A 1% low melting point Seakem® gold agarose [Cambrex] with 1% SDS was used in the preparation of the plugs. The agarose mixture was briefly heated and a 500 µl aliquot of this mixture was added to the cell suspension buffer, pipetted up and down to mix, before removing 500 µl and adding it to the plug moulds. Plugs were incubated at 4°C for fifteen minutes to set.

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4.2.1.2 Lysis of plugs

Plugs were removed from the casting apparatus, resuspended in cell lysis buffer (50 mM Tris, 50 mM EDTA pH 8, 1% sarcosine, proteinase K [20mg/ml] and lysozyme [100mg/ml]) and incubated at 55°C with constant shaking. After a 2 hour incubation, cells were washed twice for 15 minutes with ddH_2O at 55°C and washed a further three times for 15 minutes at 55°C with TE buffer (10 mM Tris, 1 mM EDTA pH 8.0).

4.2.1.3 Restriction digestion

A 2 mm slice of each plug was incubated in buffer A (Roche) with 100 µg/ml BSA [Roche] at 30°C for 15 minutes. The plug slices were incubated at 30°C with 30U *ApaI* (Roche) in a fresh aliquot of buffer A for 2 hours. Plugs were then washed with 200µl 0.5 x TBE (Appendix A) to

remove all remaining buffer and enzyme and incubated at 4°C for 10 minutes. The plug slices were then loaded into the wells alongside a PFGE marker [Ammersham Bio Sciences] in a 1.5 % agarose gel prepared using pulsed-field gel electrophoresis agarose (ultra pure DNA grade) [BIO-RAD Laboratories] in 1x TBE buffer. The gel was electophoresed from 5 seconds to 35 seconds for 23 hours at 200v using a gene navigator [Amersham Bio Sciences]. Subsequently, the gel was stained in 0.5 μg/ml EtBr (10 mg/ml) for 45 minutes and then destained in dH2O for several hours before visualization was achieved using a fotodyne Inc. UV light box and the results were captured using a Kodak EDAS 290 Camera (New York).

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4.3 Results

PFGE analysis was carried out to on the 44 Gm resistant *A. baumannii* strains as well as strain TOM, which was included as a susceptible control. The PFGE data obtained was interpreted according to the Tenover criteria (Tenover *et al*, 1995). A well characterised control strain is necessary when processing unknown isolates. Accordingly, a European control strain, RUH, was included. A molecular size standard marker [Ammersham Bio Sciences] was included on every gel to enable sizing of the fragments.

The PFGE profiles obtained (Figure 4.3.1a - f) were analysed and all the strains were assigned profiles (Table 4.3.2) according to their genetic relatedness based on the Tenover criteria (Tenover *et al*, 1995). According to this criterion, strains are considered indistinguishable if there are no visible fragment differences. However, if two or three fragments differences are visible when comparing the profiles obtained to the outbreak strain, these isolates are considered subtypes. If there are 7 or more visible fragment differences between strains then these strains are said to be not related.

Accordingly, strains RAM, MOS-2, TOM, A3, A7 and A27 were all assigned to profile A, suggesting that these strains are genetically related. Similarly strains SAK, PAU, REI, RAN, MOS-1, A2, A5, A6, A22, A23, A26, A31, A34 and A36 were all designated profile B. Strains A1, A4 and A39 were all assigned to profile C, whilst strain A8 was unique to profile D. Strains A35, A40, A42, A46, A59, A60, A64, A67 and A68 were all isolated from GSH in 2006 and were assigned to profile E, suggesting that these strains are all clonally related. Strain A20 isolated from GSH was unique to profile F. Profile G was assigned to strains APL, A12, A19, and A28, as these strains all seem to have a common ancestor. Profile H was assigned to strains A14 and A25, whilst profile I was assigned to A9, A10 and A11. Both strain A13 and A17 were assigned to profile J.

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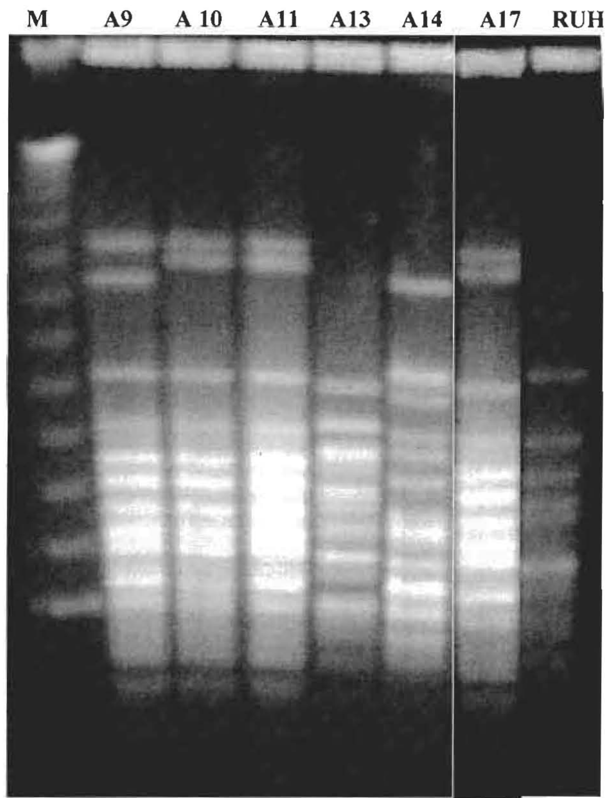

Figure 4.3.a: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: Marker; Lane 2: A9; Lane 3: A10; Lane 4: A11; Lane 5: A13; Lane 6: A14; Lane 7: A17; Lane 8: European type strain RUH.

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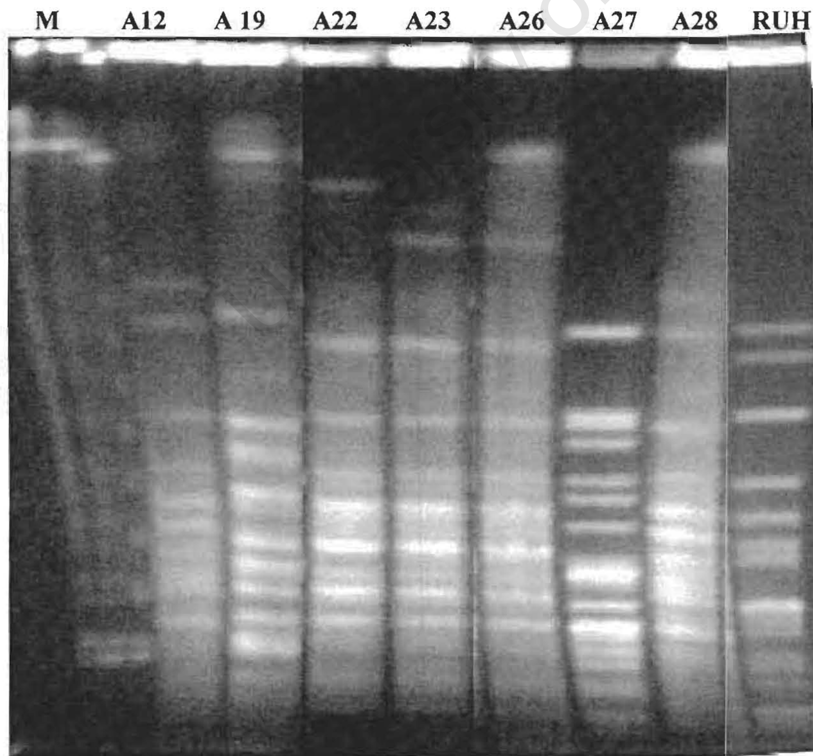

Figure 4.3.b: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: Marker; Lane 2: A12; Lane 3: A19; Lane 4: A22; Lane 5: A23; Lane 6: A26; Lane 7: A27; Lane 8: A28; Lane 9: European type strain RUH.

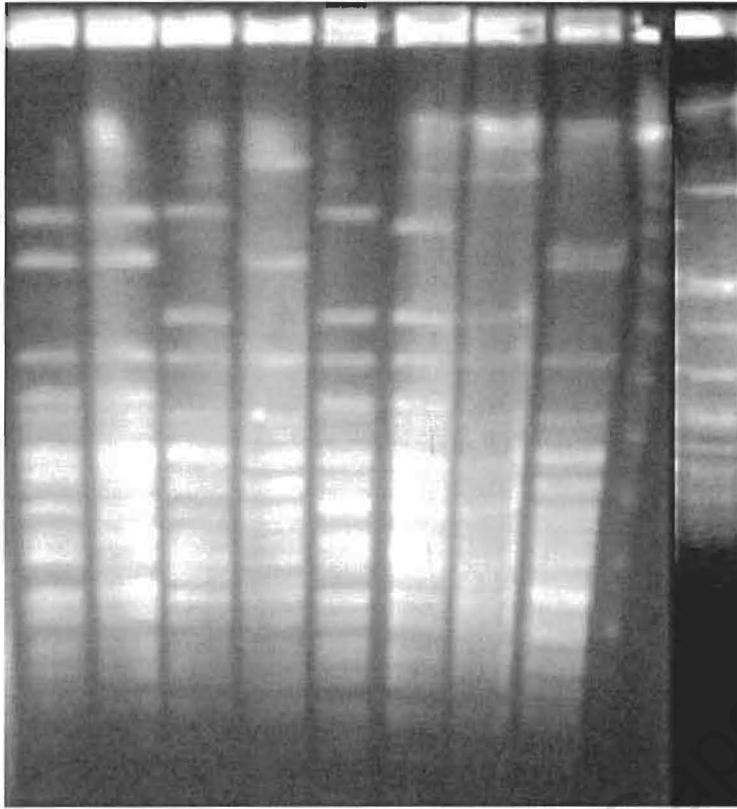

Figure 4.3.c: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: REI; Lane 2: RAN; Lane 3: RAM; Lane 4: TOM; Lane 5: SAK; Lane 6: PAU; Lane 7: MOS-1; Lane 8: AMOS-2; Lane 9: Marker; Lane 10; APL.

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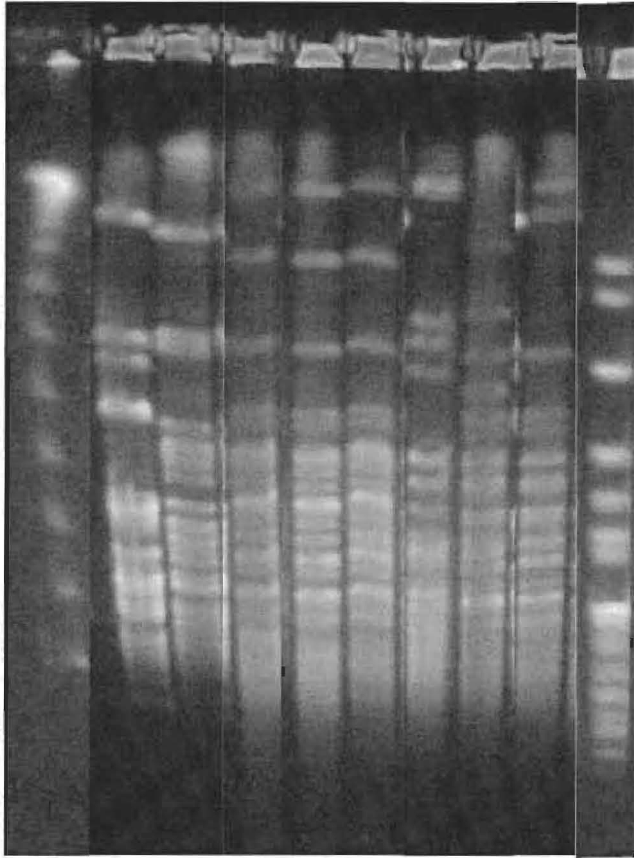

Figure 4.3.d: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: Marker; Lane 2: A20; Lane 3: A25; Lane 4: A31; Lane 5: A34; Lane 6: A36; Lane 7: A39; Lane 8:A40; Lane 9: A42; Lane 10: European type strain.

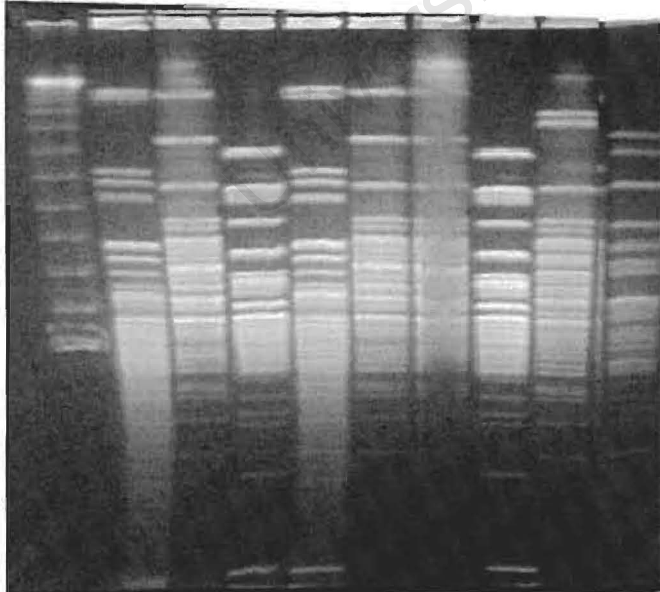

Figure 4.3.e: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: Marker; Lane 2: A1; Lane 3: A2; Lane 4: A3; Lane 5: A4; Lane 6: A5; Lane 7: A6; Lane 8:A7; Lane 9: A8; Lane 10: European type strain

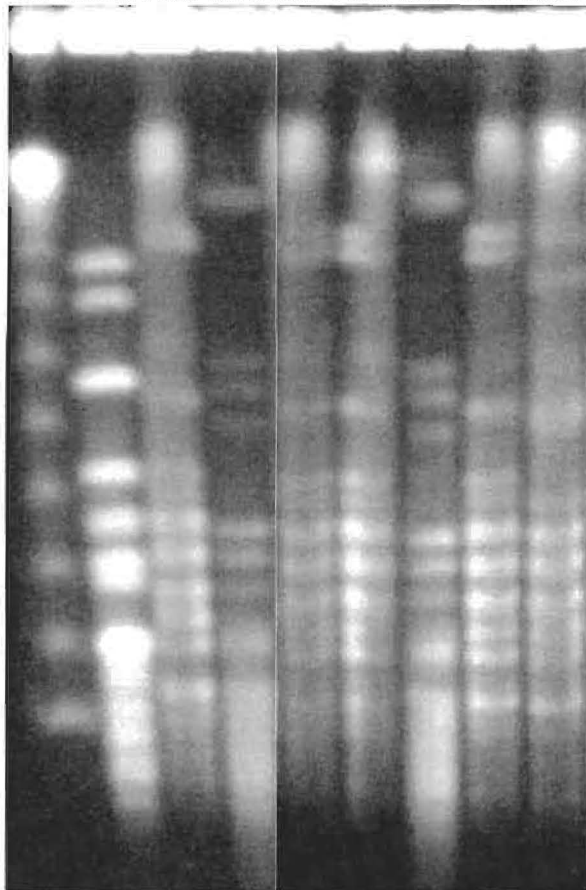

Figure 4.3.f: Agarose gel electrophoresis of restriction enzyme fragments obtained during PFGE from *A. baumannii* strains. Lane 1: Marker; Lane 2: RUH; Lane 3: A35; Lane 4: A46; Lane 5: A59; Lane 6:A60; Lane 7: A64; Lane 8:A67; Lane 9: A68.

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Table 4.3.2: PFGE profiles of the 45 *A. baumannii* strains included in this study.

Strain	Year	Hospital	[a] *aacC2a* linkage	PFGE Profile	Strain	Year	Hospital	[a] *aacC2a* linkage	PFGE profile
SAK	1983	GSH	+	B	**A9**	2006	GSH	-	I
PAU	1992	GSH	+	B	**A10**	2006	GSH	-	I
REI	1996	GSH	+	B	**A11**	2006	RCH	-	I
RAN	2001	GSH	+	B	**A12**	2006	GSH	-	G
MOS-1	2005	GSH	+	B	**A13**	2006	GSH	-	J
A2	2006	RCH	+	B	**A14**	2006	RCH	-	H
A5	2006	GSH	+	B	**A17**	2006	GSH	-	J
A6	2006	RCH	+	B	**A19**	2006	GSH	-	G
A22	2006	RCH	+	B	**A20**	2006	GSH	-	F
A23	2006	RCH	+	B	**A25**	2006	RCH	-	H
A26	2006	GSH	+	B	**A27**	2006	GSH	-	A
A31	2006	RCH	+	B	**A28**	2006	GSH	-	G
A34	2006	RCH	+	B	**A35**	2006	GSH	-	E
A36	2006	GSH	+	B	**A39**	2006	GSH	-	C
APL	1996	GSH	-	G	**A40**	2006	GSH	-	E
RAM	2003	GSH	-	A	**A42**	2006	GSH	-	E
MOS-2	2005	GSH	-	A	**A46**	2006	GSH	-	E
TOM	2005	GSH	-	A	**A59**	2006	GSH	-	E
A1	2005	GSH	-	C	**A60**	2006	GSH	-	E
A3	2006	GSH	-	A	**A64**	2006	GSH	-	E
A4	2006	GSH	-	C	**A67**	2006	GSH	-	E
A7	2006	GSH	-	A	**A68**	2006	GSH	-	E
A8	2006	RCH	-	D					

a *aacC2a* linked to IS*1133* interrupted by IS*Aba-1*

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4.4 Discussion

Of the 44 Gm resistant *A. baumannii* strains included in this study, 12 contained the aminoglycoside resistance gene *aacC2a*. PCR assays, hybridisation experiments and DNA sequence analysis revealed that in these same 14 strains, the *aacC2a* gene is associated with a full copy of the insertion element IS*1133* which has been interrupted by a full copy of IS*Aba-1*. The genetic arrangement of *aacC2a* has therefore been maintained in *A. baumannii* isolates obtained from two separate hospitals from 1983 to 2006, suggesting a common genetic link between these strains.

Accordingly, PFGE analysis was carried out on all *A. baumannii* isolates included in his study. Following PFGE analysis, profiles were assigned to banding patterns obtained from all the strains. Analyses of the PFGE patterns generated for all 44 gentamicin resistant *A. baumannii* strains revealed that the 14 *aacC2a* positive *A. baumannii* strains all had similar PFGE patterns and are therefore considered to be genetically related. These 14 strains have been clustered into the clone B lineage. The remaining strains 30 strains all had distinct profiles which included profiles A, C, D, E, F, G, H, I and J. Not surprisingly, the PFGE patterns obtained from the remaining 30 isolates shown to be negative by PCR assays for the presence of *aacC2a* gene and its genetic location show no similarity to the PFGE profiles obtained for the 14 strains which do *aacC2a*.

Interestingly, although these 14 *A. baumannii* strains clustered into the B profile are genetically identical they had been isolated from two separate hospitals in the Western Cape. These hospitals are located more than ten kilometres apart and the chance of the cross transfer of infected patients is highly unlikely. RCH is a children's hospital whilst in comparison GSH is a large academic hospital. As a result the transfer of *A. baumannii* strains from one hospital to the other would have to either occur via the transfer of an infected individual or via a medical practitioner. It is of course not unreasonable to suggest that the original ancestor of these clones may have been acquired from a source outside of the Western Cape, overseas perhaps. Whether these strains belonging to the clone B profile are confined to these two hospitals is unknown and it would be valuable to know whether this clone is prevalent in any other hospitals around South Africa.

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It is interesting to note that the 14 strains carrying *aacC2a* linked to IS*1133* and IS*Aba-1* have the same PFGE profile (A) and are therefore genetically related. It is also interesting that 9 of the 34 isolates obtained from GSH belong to profile E, which does not seem to be present in strains obtained from RCH.

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Chapter five

Final discussion and concluding remarks

Acinetobacter spp are becoming increasingly important as nosocomial pathogens. The ability of *A. baumannii* to rapidly acquire antibiotic resistance combined with its simple growth requirements and extreme hardiness are perhaps the secret to its successful spread in the hospital environment (Bergogne-Bérézin & Towner, 1996).

In a previous study, DNA sequence analysis revealed that the 5' end of an aminoglycoside resistance gene, *aacC2a*, from *A. baumannii* strain SAK isolated in 1983, is linked to the right end of an IS element, IS*1133* (Elisha, 1991). This same arrangement was later identified in another *A. baumannii* isolate, strain PAU, isolated 9 years after strain SAK (Segal *et al*, 2003). DNA sequence analysis identified a T to A substitution in IR_R of this IS*1133* element from both strains. This led to the suggestion that this substitution event may have prevented the excision of IS*1133* from *A. baumannii* and thus inhibited further insertion events. It may be that the substitution and subsequent inhibition of excision of IS*1133* has stabilized *aacC2a* in the genome of these two strains.

A total of 42 Gm resistant *A. baumannii* isolates and one Gm susceptible isolate (TOM) collected from two hospitals in Cape Town from 1996-2006, as well as *A. baumannii* strain SAK and strain PAU, were included in this study. An *aacC2a* gene, encoding resistance to the clinically important aminoglycosides, gentamicin and tobramycin, was identified in 12 of the more recent *A. baumannii* isolates. The mechanism of aminoglycoside resistance in the remaining 30 isolates may be due to an alteration of the antibiotic target site, a diminished uptake of the antibiotic or enzymatic modification of the aminoglycoside or an interplay of these mechanisms. However, this was not further investigated.

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An identical linkage of IS*1133* - *aacC2a* was observed in the 12 A. *baumannii* isolates shown to harbor *aacC2a*. DNA sequence analysis of PCR products obtained from two strains, RAN and MOS-1, isolated in 2001 and 2005, respectively, confirmed the same T to A substitution in the IR_R of IS*1133* in these strains. It is noteworthy that the remaining 30 *A. baumannii* strains, in which *aacC2a* was not detected were similarly negative when screening for IS*1133* sequence, suggesting perhaps an absolute association between IS*1133* and *aacC2a* in *A. baumannii* in our strains. It would be interesting to screen strains from other regions for *aacC2a* to determine whether in these strains the *aacC2a* gene is similarly associated with IS*1133*.

In *A. baumannii* strain SAK and strain PAU, IS*1133* is truncated following the insertion of IS*Aba-1* (Segal *et al*, 2003). However, it was not confirmed whether *A. baumannii* strain SAK or PAU contained a full copy of IS*1133*. Accordingly, PCR assays were carried out to screen for the presence of a full copy of IS*1133* using primers directed against the left and right ends of this element. However, larger amplicons than anticipated were obtained, suggesting an insertion event. Sequence analysis of the amplicons from strain SAK showed that IS*Aba-1* has inserted in the *tnpA* gene (nucleotide 1121) of IS*1133*, thereby disrupting the gene and rendering the IS element inactive. The products obtained from one GSH strain (MOS-1) and one RCH strain (A2) isolated in 2005 and 2006, respectively, were sequenced. The DNA sequences across the IS*1133*-IS*Aba-1* junctions were identical to the corresponding sequences in strain SAK. Furthermore, DNA-DNA hybridization studies confirmed that in the remaining 10 strains carrying IS*1133-aacC2a*, the IS*1133* element has been disrupted by the insertion of IS*Aba-1*.

Until recently, IS*1133* had been described only in the plant pathogen *E. amylovora* where it is associated with Tn*5393* and supports the expression of two streptomycin resistance genes (Chiou *et al*, 1993). Increased transcription of these two genes in *E. amylovora* is driven by promoter sequences located in IS*1133* (Chiou *et al*, 1993). These promoter sequences are present in the left end of the IS*1133* gene sequence identified in the 14 *A. baumannii* isolates and were not disrupted by the insertion of IS*Aba-1*. However, it is assumed that the transcription of *aacC2a* is not dependant on IS*1133* promoters and that previously identified sequences immediately upstream of the *aacC2a* start codon are recognized in the transcription of this gene (Elisha & Steyn, 1991).

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This was further confirmed by plating the recombinant plasmid *E. coli* HB101 pGSH110, which contains the entire *aacC2a* gene plus the 115bp region containing the two putative -10 promoter regions upstream of this gene, on gentamicin selection at 5 μg/ml. This plasmid is able to confer gentamicin resistance on its *E. coli* host, which is able to grow on gentamicin selection at 5 μg/ml.

Latterly, IS*1133* has been found on plasmids and in the chromosome of *Salmonella* isolates from poultry (Pezzella *et al*, 2004). The authors suggest that this IS*1133* element was introduced into the *Salmonellae* genome following the ingestion of animal feed contaminated with *E. amylovora* (Pezzella *et al*, 2004). It is interesting to consider the events that may have led to the insertion of IS*1133* upstream of *aacC2a* in *A. baumannii*. To date, IS*Aba-1* has only been found in the genome of *Acinetobacter* spp (Segal *et al*, 2005) and it is likely that the insertion of IS*Aba-1* into IS*1133* occurred after the acquisition of IS*1133*. Whether the insertion of IS*Aba-1* into IS*1133* was accidental or necessary to render IS*1133* immobile, stabilizing the genetic organization of this region is unknown. The invariability of this arrangement in 14 *A. baumannii* strains spanning 23 years suggests that the inability of IS*1133* to be excised due to the insertion of IS*Aba-1* may also assist in the stabilization of the *aacC2a* gene by contributing to the expression of this gene in *A. baumannii*.

The expression of antibiotic resistance genes from promoter sequences located within IS elements immediately upstream of these genes have been described in *A. baumannii* (Corvec *et al*, 2002; Segal *et al*, 2003; Poirel *et al*, 2005; Segal *et al*, 2005; Segal *et al*, 2007). Using RT-PCR, the expression of *aacC2a* was confirmed in *A. baumannii* strain SAK and *E. coli* JM109 (pRK002) in both mid exponential and stationary phase cultures grown in the presence of 5 μg/ml of gentamicin. Analysis of the cDNA transcripts generated suggested that promoter sequences necessary for the expression of *aacC2a* in *A. baumannii* strain SAK are located in IS*Aba-1*. Analysis of the cDNA products generated for the expression of *aacC2a* in *E. coli* JM109 (pRK002) suggest that expression originates either from sequences upstream of *aacC2a* or from promoter regions in IS*Aba-1*.

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The genetic arrangement of *aacC2a* in association with IS*1133* and IS*Aba-1* has been maintained in *A. baumannii* isolates obtained from two separate hospitals over a 23 year period, which suggests that there may be a common ancestral link between these strains. Accordingly, PFGE analysis was carried out on all *A. baumannii* isolates included in this study. Analyses of the PFGE patterns generated for all 45 Gm resistant *A. baumannii* strains indicated that the 14 *aacC2a* positive *A. baumannii* strains were assigned the same PFGE profile (clone B) and therefore considered genetically related. Not surprisingly, none of the strains that did not contain *aacC2a* nor IS*1133* were clone B strains. This evidence clearly indicates that the 14 strains containing the *aacC2a* genetic arrangement have all originated from a common ancestor.

The original ancestor of clone B is at present unknown but it is possible that the original isolate acquired *aacC2a* from another pathogenic bacterium in the hospital environment. *Acinetobacter* spp have the tendency to rapidly acquire antibiotic resistance and this is thought to be as a consequence of its long term evolutionary exposure to antibiotic-producing organisms in the soil environment (Bergogne-Bérézin & Towner, 1996). There is evidence to suggest that the increased use of particular antibiotics, in this case aminoglycosides, may have had a favorable effect on emergence and spread of *Acinetobacter* spp, by selecting for those that are able to survive. Subsequently, the insertion of IS*1133* upstream of *aacC2a* provided stabilization of this gene which in turn was later followed by the interruption of IS*1133* by IS*Aba-1*, perhaps enabling increased expression of *aacC2a* under certain stress conditions, as described for the expression of bla_{OXA-23} in *A. baumannii* (Segal *et al*, 2007).

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Appendix A

Media, Buffers and Solutions

A.1 Media

2xYT

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

Make up to 1 litre with dH_2O and autoclave.

Agar:	2xYT broth	100ml
	Agarose	1.5g

Autoclave.

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A.2 Buffers and solutions

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

NaCl	4.1g
CTAB	10g

Dissolve the NaCl in 80ml dH_2O, and then slowly add the CTAB. Once all the CTAB is added heat to 65°C to dissolve and then adjust the volume to 100ml with dH_2O.

Denaturation buffer

NaCl	43.83g
NaOH	10g

Make up to a final volume of 1 litre with dH_2O.

ECL hybridisation solution

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

Mix a room temperature for 1 hour with shaking, then heat to 42°C for 1 hour with occasional stirring.

Gel tracking dye (6X)

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

Make up to 10ml with dH_2O. Separate into aliquots of 1ml and store at 4°C.

0.25M HCL

Concentrated HCL	10.8ml

Make up to 500ml with dH_2O.

Lysis buffer

30% lauroyl sarcosine	1.17ml
1M EDTA	17.5ml

100mg/ml Proteinase K 175ul

Make up to 35ml with dH_2O.

Neutralisation solution

Tris	30.29g
NaCl	43.83g

Adjust pH to 7.5 and make up to a final volume of 500ml with dH_2O.

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Phenol

Commercial crystallized phenol	500g
8-hydroxyquinolone	0.6g
2M NaOH	7.4ml
dH_2O	130ml

Buffered in 10mM Tris, pH7.6, liquefy at 40°C, separate into aliquots and store at -20°C.

Primary wash buffer without urea

SDS	4g
20X SSC	25ml

Make up to 1 litre with dH_2O and store at 4°C for up to 3 months.

20X SSC

NaCl	175.32g
Tri-sodium citrate	88.23g

Adjust the pH to 7.0 and make up to 1litre with dH_2O, autoclave.

X-gal

X-gal	0.4g

Dissolve in 1ml DMF and store at -20°C.

Tris-HCl (1M)

Tris	121.g

Adjust the pH as required with concentrated HCl and make up to a final volume of 1 litre with dH_2O, autoclave.

Tris-EDTA (TE) buffer

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

Adjust pH to 8.0 and make up to a final volume of 500ml with dH_2O, autoclave.

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10X Tris-Borate-EDTA (TBE) buffer

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

Make to a final volume of 500ml with dH_2O and autoclave.

50X Tris-Acetate-EDTA (TAE) buffer

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

Make up to 1 litre with dH_2O and autoclave

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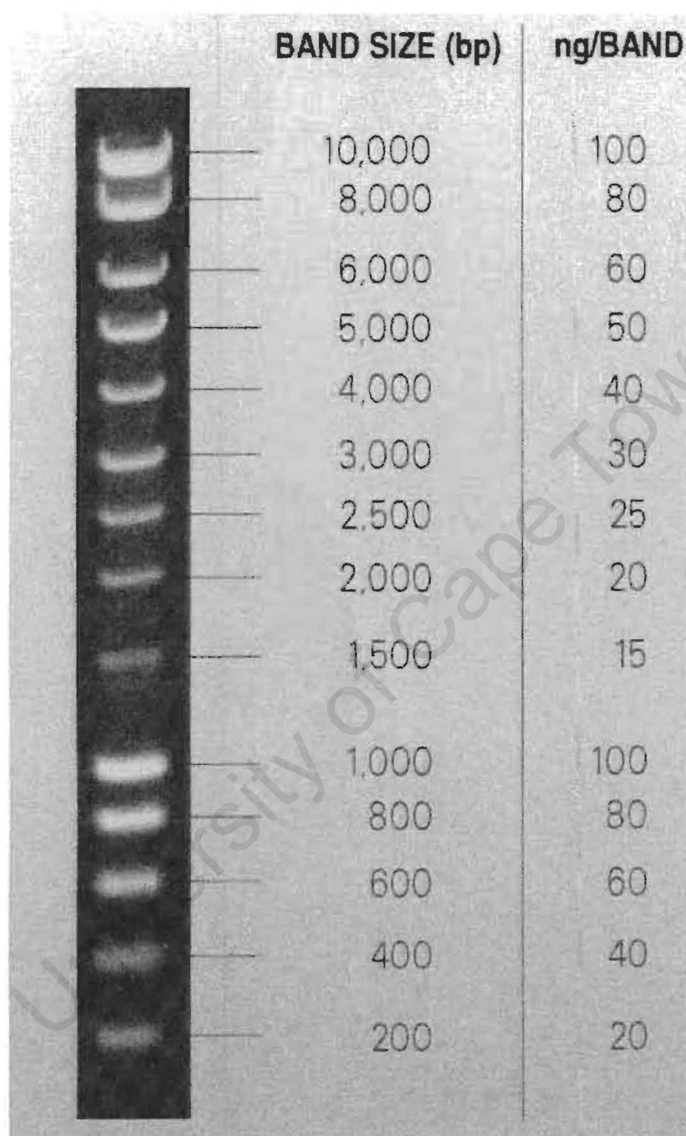
Appendix B

B.1 **Hyperladder I (Bioline)**



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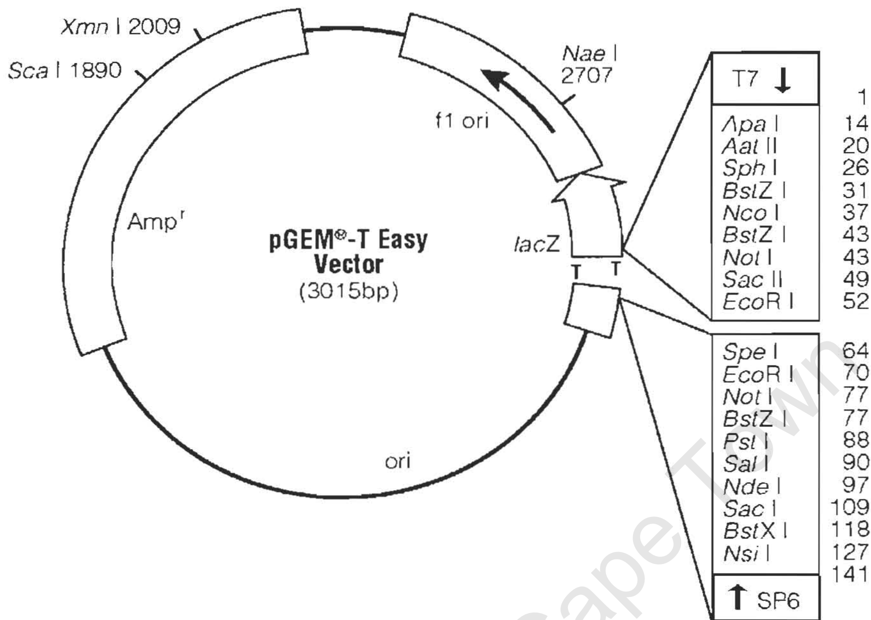
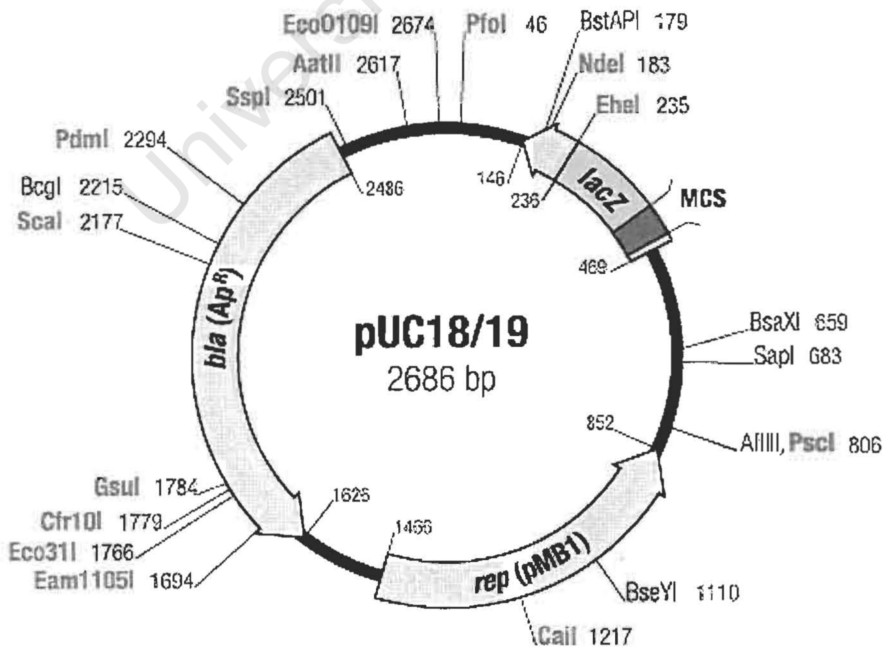
B.2 pGEM®-TEasy vector



B.3 pUC19 vector



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