Distribution, frequency and contribution to the expression of antibiotic resistance gene of an IS element in *Acinetobacter baumannii*

Seike Garny

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With love, this thesis is dedicated to my parents

DECLARATION

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

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ABSTRACT

A novel insertion sequence, IS_{Aba-1}, which was recently identified in *Acinetobacter baumannii* was investigated. With hybridization studies, this element was identified in 8/14 *A. baumannii* stains and 2/3 *A. lwoffii* strains, but no signals were detected in *A. calcoaceticus* (1 strain), *Escherichia coli* (13 strains), *Klebsiella pneumoniae* (7 strains), *Klebsiella oxytoca* (2 strains), *Enterobacter cloacae* (1 strain) and *Pseudomonas aeruginosa* (4 strains). With similar experiments, the copy number of IS_{Aba-1} in *A. baumannii* ranged from 6 to 19 copies and *A. lwoffii* strains harboured up to 13 copies of this IS in their genomes.

To determine the linkage of IS_{Aba-1} with antibiotic resistance genes, a partial genomic DNA library from one *A. baumannii* strain (RAM), which contained up to 14 copies of IS_{Aba-1}, was constructed. Ten recombinant plasmids were obtained which contained a portion of IS_{Aba-1}, but only one recombinant plasmid harboured a sulphonamide resistance gene (*sul2*) downstream of this IS element. This recombinant plasmid, pSUL100, conferred sulphonamide resistance (100µg disk) to its *E. coli* host.

Sequence analysis of 2.29kb insert in pSUL100 revealed four open reading frames encoding the transposases of IS_{Aba-1}, sulphonamide resistant DHPS, and phosphoglucose mutase GlmM. Several putative promoter sequences including one that shows similarity to sigma S, located in IS_{Aba-1} were identified and their contribution to the expression of the downstream sulphonamide resistance gene was determined using primer extension studies. The expression of *sul2* in *A. baumannii* strain RAM was driven from one promoter which contains an extended -10 hexamer, suggesting recognition by both σ^{70} and σ^{S}. *sul2* in *E. coli* (pSUL100) was expressed from multiple promoters, including the hexamers recognized in strain RAM.

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ABBREVIATIONS

°C	degrees Celsius
2YT broth/ agar	2x Yeast-Tryptone broth/ agar
Amik	amikacin
AAC	aminoglycoside acetyltransferase
AAD	aminoglycoside adenyltransferase
APH	aminoglycoside phosphotransferase
AGE	agarose gel electrophoresis
AME	aminoglycoside-modifying enzyme
bp	base pairs
cDNA	complimentary DNA
CAP	catabolite activator protein
CTAB	hexadecyltrimethylammonium bromide
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthetase
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphospahte
DR	direct repeat
DSMO	dimethyl sulfoxide
EDTA	disodium ethylenediaminetetra acetate
ESBL	extended-spectrum β-lactamase
EtBr	ethidium bromide
γ	gamma
g	gram
Gm	gentamicin
GSH	Groote Schuur Hospital
HRR/HRF	homologous region reverse/forward primer
IPTG	isopropylthio-β-thio-galactoside

IR	inverted repeat
IR_R	right inverted repeat
IS	insertion sequence
kb	kilobase pairs
Km	kanamycin
l	litre
m	milli
M	Molar
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute
M-MVL RT buffer	M-MVL reverse transcription buffer
n	nano
NaCl	sodium chloride
NCCLS	National Committee for Clinical Laboratory Standards
Neo	neomycin
nm	nano meter
OD	optical density
OMP	outer membrane protein
ORF	open reading frame
p	pico
PBP	penicillin binding protein
PCR	polymerase chain reaction
Pipes	1, 4-piperazinedicthanesulfonic acid
R	resistance
RCF	relative centrifugal force
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	room temperature

RTase	reverse transcriptase
SDS	sodium dodecyl sulfate
spp.	species
SSC	standard sodium citrate
Sm	streptomycin
TAE	Tris-acetate/EDTA electrophoresis buffer
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TE buffer	Tris- EDTA Buffer
TH	Tygerberg Hospital
Tob	tobramycin
Tn	transposon
TU	Tjernberg and Ursing
U	units
UH	Universitas Hospital
UK	United Kingdom
UV	ultra-violet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
λ	lamda
μ	micro
YT	yeast tryptone
xg	centrifugal force

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Chapter 1

Literature Review

1.1. The genus *Acinetobacter*

*Acinetobacter*s are γ-proteobacteria, superfamily Pseudomonadales, classified into *Moraxellaceace* (Prescott *et. al.*, 1999; Bergogne-Bérézin & Towner, 1996) and the genus *Acinetobacter* consisting of several species: *calcoaceticus, lwoffii, baumannii, haemolyticus, junii, johnsonii, radioresistens* as well as some unnamed genomic species. The '*A. baumannii* complex' comprises four different species (*A. baumannii*, *A. calcoaceticus*, genomic species 3 and 13TU) and should be regarded as different from remaining *Acinetobacter* spp. (Joly-Guillou, 2005). They are gram negative coccobacilli, strict aerobes, oxidase negative and catalase positive with a G+C content of 40-43% (Bouvet & Grimont, 1986; Barbe *et al.*, 2004). *Acinetobacter*s do not form spores and grow at temperatures ranging from 15 to 30°C (Bergogne-Bérézin & Towner, 1996). Bouvet & Grimont (1986) have identified up to 60 different carbon and energy sources, which makes *Acinetobacter*s versatile chemoheterotrophes (Barbe *et al.*, 2004). Most strains utilize acetate, lactate and pyruvate as carbon and energy source (Bergogne-Bérézin & Towner, 1996). The colonies on solid media are yellow to white in colour and have a smooth circular appearance (Bergogne-Bérézin & Towner, 1996; Bouvet & Grimont, 1986). The size of diplococcoids on solid media is 1.0μm by 0.7μm and the diameter of short rods range from 1-2.5μm by 0.9-1.6μm (Bouvet & Grimont, 1986). *Acinetobacter*s strains are widely distributed in nature and are increasingly located in the hospital environment. As ubiquitous organisms, *Acinetobacter*s have been isolated from soil, water, sewage and skin (Bergogne-Bérézin & Joly-Guillou, 1991; Bergogne-Bérézin & Towner, 1996).

1.2. Clinical significance of the genus *Acinetobacter*

More than two decades ago, *Acinetobacter* spp. were one of the relatively unknown gram-negative bacilli, which have since emerged as nosocomial pathogens and contributed increasingly to outbreaks of hospital infections and colonization (Bergogne-Bérézin & Joly-Guillou, 1991). Paradoxically, it is the widespread use of antibiotics in the treatment of bacterial infections that has enabled the more resistant *Acinetobacter*s to take-over a niche previously colonized by other bacteria (van Looveren *et al.*, 2004). Our understanding of the epidemiology of *Acinetobacter* has been hampered by the chequered history of the taxonomy of this genus. As recently as 1984 only one species, *A. calcoaceticus*, was described in Bergy's Manual of Systemic Bacteriology (Juni, 1984). However, medical microbiologists did not always report the single-species definition with the result that two variants of the type species (var *anitratus* and var *lwoffii*) are frequently described in the old literature. Perhaps simplistically, for at least some medical microbiologists, var *anitratus* equates to *A. baumannii*, the most frequently reported genomic species involved in nosocomial infections and hospital outbreaks (Bergogne-Bérézin & Towner, 1996; Gales *et al.*, 2001; Marais *et al.*, 2004; Wang & Chen, 2005). *Acinetobacter*s were reported as the most common gram-negative nosocomial pathogens causing secondary infections in patients in Groote Schuur Hospital, South Africa (Hammond & Potgieter, 1995). And in China, the Nosocomial Pathogen Resistance Surveillance (NPRS) program identified *Acinetobacter* spp. as the fourth most common nosocomial pathogen isolated from patients in intensive care units (Wang & Chen, 2005). Although *Acinetobacter*s are considered to be low-virulent organisms, a mortality rate ranging from 20% to 60% has been reported in various studies (Joly-Guillou, 2005).

*Acinetobacter*s are opportunistic pathogens and children under 10 years of age, elderly and patients with severe underlining diseases are most at risk (Gales *et al.*, 2001; Urban *et al.*, 2003; Coehlo *et al.*, 2004; Turton *et al.*, 2004). Having previously received anti-microbial therapy is another predisposing risk factor for infections of nosocomial pathogens, such as *Acinetobacter*s (Coehlo *et al.*, 2004). *Acinetobacter* spp. are most frequently isolated from the respiratory tract; however this organism has been isolated

from wounds, blood and other body fluids (Bergogne-Bérézin & Towner, 1996; Gales *et al.*, 2001; Turton *et al.*, 2004; Wang & Chen, 2005). This organism has also been isolated from inanimate material in the hospital environment, such as catheter tips, respiratory equipment, bed linen and curtains (Marais *et al.*, 2004). In some cases, apparatus, such as ventilator monitor boards and continuous positive airway pressure (CPAP) masks have been implicated in the transmission of *Acinetobacter* spp. resulting in hospital outbreaks (Pimentel *et al.*, 2005). The stress tolerance of *Acinetobacter*s complicates the containment of hospital outbreaks of this organism (Jawad *et al.*, 1998). A study done by Pimentel *et al.* (2005) has shown that improved cleaning methods and hand-hygiene of medical staff facilitated the control of an outbreak of multi-drug resistant *A. baumannii*.

The treatment of *Acinetobacter* infections can be problematic for clinicians. *A. baumannii* often exhibits resistance to multiple antibiotics. By 2000 most of the antibiotics, including extended-spectrum penicillins, cephalosporins, and aminoglycosides, previously used to treat *Acinetobacter* infections, were ineffective (Abbo *et al.*, 2005). More recently, the emergence of carbapenems resistance in *Acinetobacter* has resulted in pan-resistant strains and it has become necessary to restore colistin and rifampicin to treatment regimens (Urban *et al.*, 2003; Falagas *et al.*, 2004; Montero *et al.*, 2004).

A surveillance study (1997-1999) done by Gales *et al.* (2001) showed that while most *Acinetobacter* clinical isolates from Canada, United States and Latin America were sensitive to carbapenems and amikacin, almost 11% of the nosocomial isolates were resistant to carbapenems. Similarly, Hsueh *et al.* (2002) reported a sharp increase (from 5.88% in 1993 to 21.5% in 2000) in imipenem resistant *Acinetobacter*s in Taiwan. Southeast England also reported that most of the *A. baumannii* isolates investigated, were carbapenem resistant (Turton *et al.*, 2004). In 2000, carbapenem resistant *Acinetobacter* strains were identified in an intensive care unit in Johannesburg, South Africa (Marais *et al.*, 2004). During the same year less than 1% of the *Acinetobacter* isolates from Groote Schuur Hospital (Cape Town, South Africa) showed resistance to meropenem but within four years 30% of *Acinetobacter* isolates demonstrated resistance to this antibiotic (C. Bamford; personal communication).

1.3. Antibiotic resistance and mechanisms of resistance in *Acinetobacter* spp.

Acinetobacters are able to survive in the hostile environment of hospitals (Jaward *et al.*, 1998) in part, because they are intrinsically resistant to many antimicrobials, but mostly because of their ability to rapidly acquire a plethora of different antibiotic resistance determinants (van Looveren *et al.*, 2004). Genes, encoding mechanisms to counteract the lethal actions of antimicrobials, have been identified in the chromosome and on mobile elements, such as R-plasmids, transposons and integrons (Koeleman *et al.*, 2001; Miranda *et al.*, 2003; van Looveren *et al.*, 2004). An overview of mechanisms of resistance in *Acinetobacter* is presented.

1.3.1. Resistance to β-lactams antibiotics

1.3.1.1. β-Lactamases

Enzymatic inactivation is the most common defence strategy, not only to β-lactams, but also to other antibiotics (Jacoby & Munoz-Price, 2005). A number of schemes have been described to classify β-lactamases (Livermore, 1995). In this review, the sequence-based classification system derived by Ambler (Bradford, 2001) will be used to discuss the β-lactamases in *Acinetobacter*.

Many different types of β-lactamases have been described in the genus *Acinetobacter* but most often in *A. baumannii* (Table 1.1.). The Ambler class A extended spectrum beta lactamases (ESBL) are rare resistance determinants in *Acinetobacter* spp. compared to other cephalosporinases, oxacillinases and carbapenemases (Ambler class C, D or B enzymes). Of the Temoniera (TEM)-variants, only TEM-1-type plasmid-mediated enzyme has been identified in *A. baumannii* (Bou *et al.*, 2000[b]). Sulfhydryl (SHV) β-lactamase appears to have been derived from *Klebsiella* spp. (Stürenburg & Mack, 2003), and Huang *et al.* (2004) were the first research group to describe a plasmid-mediated SHV-12 in a multi-drug resistant *A. baumannii*. Another ESBL, VEB-1 which has

activity against cefotaxime and ceftazidime was first identified in *Pseudomonas aeruginosa* and subsequently in *A. baumannii* (Poirel *et al.*, 2003). The genes encoding this enzyme were identified on integrons in the chromosome of both organisms (Poirel *et al.*, 2003). Contrary to $bla_{\text{VEB-1}}$, $bla_{\text{PER-1}}$ has been located in the chromosomes of nosocomial isolates of *A. baumannii* strains from Turkey, France and Korea (Vahaboglu *et al.*, 1997; Poirel *et al.*, 1999; Jeong *et al.*, 2005). CTX-M-2 and CTX-M-5, members of the recently described class A CTX-M-family, which have high activity against cefotaxime, have been described in *A. baumannii* (Nagano *et al.*, 2004). $bla_{\text{CTX-M-2}}$ was carried on a plasmid in *A. baumannii* while no details of CTX-M-5 have been published yet (Nagano *et al.*, 2004).

Chromosomally encoded cephalosporinase, AmpC (Ambler class C) is intrinsic to *Acinetobacter* spp. and a gene encoding this enzyme from *A. baumannii* was described for the first time by Bou *et al.* (2000[a]). Another five bla_{AmpC} genes have been identified in *A. baumannii*, of which the most recent allelic variant was called ADC-7 β-lactamase (Bou *et al.*, 2000[b]; Hujer *et al.*, 2005). High-level resistance to ceftazidime, which was only inhibited by cloxacillin, was accounted to the over-expression of a non-inducible AmpC in *A. baumannii* (Corvec *et al.*, 2003).

Ambler class D β-lactamases, also known as oxacillinases, have similar antimicrobial hydrolysing spectrum as the CTX-M enzymes (Jacoby & Munoz-Price, 2005). Like the CTX-M enzymes, not all Ambler class D enzymes confer resistance to ceftazidime; for example, OXA-10 (Poirel *et al.*, 2003), OXA-20 (Ploy *et al.*, 2000), OXA-21 (Vila *et al.*, 1997[b]; Zarrilli *et al.*, 2004) and OXA-37 (Navia *et al.*, 2002) do not hydrolyse ceftazidime but have activity against many broad-spectrum cephalosporins (van Looveren *et al.*, 2004; Jacoby & Munoz-Price, 2005). Other members of the OXA- family identified in *A. baumannii* are of concern, as these confer resistance to carbapenems. These include OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, OXA-51 and OXA-58 (Donald *et al.*, 2000; Bou *et al.*, 2000; Afzal-Shah *et al.*, 2001; Héritier *et al.*, 2003; Héritier *et al.*, 2005; Poirel *et al.*, 2005). Plasmid encoded OXA-23 and OXA-58 and chromosome located OXA-40 notwithstanding, the genetic location of most bla_{OXA}

genes in *A. baumannii* is unknown. In other genera bla_{OXA} genes are frequently associated with type 1 integrons, which is not the case in *A. baumannii*: to date, none of the bla_{OXA} gene from carbapenem resistant *A. baumannii* have been integron associated (Bown & Amyes, 2006).

Besides the class D carbapenemases, class B metallo-β-lactamases, the IMP and VIM family members, confer resistance to carbapenems in *Acinetobacter*. Metallo-β-lactamases hydrolyse most β-lactams and are resistant to all β-lactamase inhibitors, except EDTA, which chelates the zinc ions required for the activity of these enzymes (Poirel & Nordmann, 2002). Of bla_{IMP} and bla_{VIM} alleles, IMP-1, IMP-2, IMP-4, IMP-5, VIM-1 and VIM-2 have been found in the variable regions of class 1 integrons identified thus far in *A. baumannii* (Yum *et al.*, 2002; Poirel & Nordmann, 2002; Weldhagen, 2004).

1.3.1.2. Altered outer membrane porins (OMP) and penicillin binding proteins (PBPs)

There is a paucity of data on the role outer membrane protein (OMP) and penicillin binding protein (PBP) in β-lactam resistance in *A. baumannii*. Most is known about their role in carbapenem resistance in this organism (Fernández-Cuenca *et al.*, 2003; Mussi *et al.*, 2005). The presence of a carbapenemase, and the loss of an outer membrane porin, Omp7 (22.5kDa OMP) as well as the reduced expression of penicillin binding protein 2 (PBP2) contributed to carbapenem resistance in a Spanish *A. baumannii* strain as well as in an endemic *A. baumannii* strain in New York (Fernández-Cuenca *et al.*, 2003; Quale *et al.*, 2003). Just recently, Mussi *et al.* (2005) identified a β-barrel outer membrane protein, CarO, in a multi-drug resistant *A. baumannii* strain. Carbapenem resistance observed in this clinical isolate was accounted to the disruption of *carO* allele by novel insertion elements, IS_{Aba825} and IS_{Aba125} (Mussi *et al.*, 2005).

Table 1.1. Summary of β-lactam resistance mechanisms in *Acinetobacter baumannii*

β-lactamase	Genetic location	Substrate	Molecular Class	Reference
TEM-1	plasmid	penicillins	A	Bergone-Bérézin & Towner, 1996
SHV	plasmid	penicillins	A	Bergone-Bérézin & Towner, 1996; Huang *et al.*, 2004
PER	plasmid; chromosome	penicillins; narrow spectrum cephalosporins	A	Urban *et al.*, 2003; Jeong *et al.*, 2005
VEB	chromosome; integron	extended-spectrum cephalosporins	A	Poirel *et al.*, 2003
CTX-M	chromosome; plasmid; integron	narrow spectrum cephalosporins	A	Nagano *et al.*, 2004; Bonnet, 2004
CARB	plasmid	penicillins; cephalosporins	A	Bergone-Bérézin & Towner, 1996
AmpC	chromosome	penicillins; 3rd generation cephalosporin	C	Bou *et al.*, 2000; Urban *et al.*, 2003; Corvec *et al.*, 2003
OXA	chromosome; plasmid; integron	penicillins; cephalosporins; carbapenems	D	Donald *et al.*, 2000; Bou *et al.*, 2000; Afzal-Shah *et al.*, 2001; Urban *et al.*, 2003; Da Silva *et al.*, 2004; Zarrilli *et al.*, 2004; Poirel *et al.*, 2005
IMP	plasmid; integron	penicillins; cephalosporin; carbapenems	B	Poirel & Nordmann, 2002; Urban *et al.*, 2003; Weldhagen, 2004
VIM	plasmid; integron	penicillins; cephalosporins; carbapenems	B	Yum *et al.*, 2002; Poirel & Nordmann, 2002; Urban *et al.*, 2003
Non enzymatic resistance: PBP; OMP	chromosome	carbapenems		Urban *et al.*, 2003; Quale *et al.*, 2003; Fernández-Cuenca *et al.*, 2003; Mussi *et al.*, 2005

PBP-penicillin binding proteins; OMP- outer membrane proteins

1.3.2. Aminoglycosides

Aminoglycoside-modifying enzymes (AMEs) alter the antibiotic at the hydroxyl or amino group either by acetylation, adenylation or phosphorylation (Dever & Dermody, 1991; Van Looveren *et al.*, 2004). Modified aminoglycosides bind poorly to their target, the ribosome, and thus do not interfere with protein synthesis (Vakulenko & Mobashery, 2003). Aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside andenylyltransferase (AAD) are the three aminoglycoside-modifying enzymes families, which hydrolyse various aminoglycosides. These three enzymes families are categorized based on the site of modification and substrate profile (Vakulenko & Mobashery, 2003). Many of these enzymes types from different families have been identified in *Acinetobacter* spp. (Table 1.2.). The phosphotransferase, APH(3')VI is most frequently associated with this genus and is responsible for amikacin resistance in *A. baumannii* (Bergogne-Bérézin & Towner, 1996; Vakulenko & Mobashery, 2003; Van Looveren *et al.*, 2004).

The genes encoding for AMEs are often located on plasmids, transposons or integrons (Bergogne-Bérézin & Towner, 1996; Lambert *et al.*, 1994[a]; Lambert *et al.*, 1994[b]; Ploy *et al.*, 2000). For example, the aminoglycoside modifying enzyme genes, *aph(3')*, *aac(6')-Ih* and *aadB*, have been located on plasmids (Lambert *et al.*, 1988; Lambert *et al.*, 1994[b]; Segal & Elisha, 1997). The novel aminoglycoside resistance gene, *aac(6')-Iad*, has been suggested to be located in transposons on a plasmid (Doi *et al.*, 2004). Most of the aminoglycoside resistance genes are located in gene cassettes of integrons (Ploy *et al.*, 2000; Wu *et al.*, 2004), whereas others are associated with insertion elements (Rudant *et al.*, 1997: Rudant *et al.*, 1998). Not all genes for aminoglycoside modifying enzymes are mobile; the gene for AAC(6') is in the chromosome of *A. haemolyticus* and *Acinetobacter* sp. 13 (Lambert *et al.*, 1993; Lambert *et al.*, 1994[b]; Rudant *et al.*, 1993; Van Looveren *et al.*, 2004).

Table 1.2. Aminoglycoside modifying enzymes in *Acinetobacter* spp.

Enzyme	Substrate	Reference
Acetyltransferases		
AAC(6')	Amik, Km, Tob	
AAC(2')I	Gm, Neo, Tob	Bergogne-Bérézin
AAC(3)I	Gm	& Towner, 1996;
AAC(3)II	Gm, Tob	Seward *et al.*, 1998
AAC(3)IV	Gm, Tob	
AAC(3)V	Gm, Tob	
Adenylylases		
AAD(3")I	Sm	Bergogne-Bérézin
AAD(3")(9)		& Towner, 1996;
AAD(2")I	Gm, Km, Tob	Seward *et al.*, 1998
AAD(2")		
Phosphotransferases		
APH(3')I	Km, Neo	Bergogne-Bérézin
APH(3')II	Km, Neo	& Towner, 1996;
APH(3')III	Amik, Km, Neo	Seward *et al.*, 1998;
APH(3')IV	Km, Neo	Vakulenko &
APH(3")I	Sm	Mobashery, 2003
APH(3')VI	Amik, Km, Neo	

Modification of table taken from Bergogne-Bérézin & Towner , 1996; Amik-amikacin; Gm-gentamycin; Km-kanamycin; Neo-neomycin; Sm-streptomycin; Tob-tobramycin

1.3.3. Quinolones

The mechanism of resistance to quinolones is mainly mediated by target modification, such as mutations in the chromosome located in the *gyrA* or *parC* genes, resulting in structural changes of DNA gyrase and topoisomerase IV, respectively (Dever & Dermody, 1991). The single mutation in *gyrA* contributes to low-level ciprofloxacin resistance in *Acinetobacter* spp., whereas high resistance is associated with a double mutation, in other words, a mutation in *gyrA* and *parC* (Vila *et al.*, 1997[a]; Spence & Towner, 2003). Mutational changes at Ser-83 and Gly-81 in GyrA and mutations at Ser-80 and Glu-84 in ParC are most common amino acid changes identified in *A. baumannii* (Hamouda & Amyes, 2003; Spence & Towner, 2003). Besides mutational changes in the target protein for antibiotic action, reduction in drug influx (reduced expression of outer membrane porins) and the up-regulation of a chromosome encoded efflux-pump could also be accounted for reduced susceptibility to quinolones (Bergogne-Bérézin & Towner, 1996; Vila *et al.*, 1997[a]; Higgins *et al.*, 2004; van Looveren *et al.*, 2004).

1.3.4. Resistance to other antibiotics: tetracycline, trimethoprim and sulphonamide

Tetracycline is bacteria static as it binds to the 30S ribosomal subunit and thus inhibits protein synthesis (Roberts, 1996; van Looveren, 2004). To date, three mechanisms mediating resistance to tetracycline have been identified: energy depended efflux and ribosomal protection or enzymatic inactivation of tetracycline (Roberts, 1996). Sixteen tetracycline resistance determinants have been identified in gram-negative bacteria of which *tet*(A), *tet*(B), *tet*(H) and *tet*39 (genes encoding active efflux pumps) and *tet*(M) (gene encoding tetracycline resistance by ribosomal protection) confer resistance to tetracycline in *Acinetobacter* spp. (Roberts, 1996; Agersø & Guardabassi, 2005). The presence of a non-specific AdeB efflux pump has contributed to tetracycline resistance in *A. baumannii* strains isolated from European hospitals (Huys *et al.*, 2005). Tetracycline resistant determinants are located in transposons and on a diverse group of plasmids, which facilitate their distribution amongst gram-negative genera (Roberts, 1996). For example, Miranda *et al.* (2003) identified a *tet*(H) with 100% identity to a Tn*5706*-associated *tet*(H) gene found in *Pasteurella multocida* on a carrying plasmid in an environmental *A. radioresistens* isolate. The *tet*(A) gene identified in *A. baumannii* was suggested to be part of Tn*1721* (Ribera *et al.*, 2003).

Trimethoprim and sulphonamides are metabolic antagonists as they inhibit two enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS), involved in bacterial folic acid synthesis (Jenkins, 1996; Huovinen, 2001). The combination of trimethoprim and sulfamethoxasole (TMP-SMX) has a synergistic bacteria static effect and cover a wide bacterial spectrum (Huovinen, 2001). Alterations in cell permeability, antimicrobials insensitive enzymes as well as the overproduction of these metabolic enzymes (Jenkins, 1996) can compromise the antimicrobial effect of TMP-SMX. Trimethoprim resistance genes (*dfr1* and *dfrA7*) were identified as gene cassettes of class 1 integrons harboured by *Acinetobacter* spp. (Petersen *et al.*, 2000; Segal *et al.*, 2003), and a similar resistance determinant, *dfrA1*, has been described on a hybrid class 2 integron (Ploy *et al.*, 2000). Of the three, sulphonamide resistance determinants (*sul1*, *sul2* and *sul3*) described thus far; *sul1* is part of one of the 3'-conserved region of class 1

integrons (Abbott *et al.*, 2005), whereas *sul2*, is mostly associated with IncQ plasmids (Rådström & Swedberg, 1988; Rawlings & Tietze, 2001). Resistance to sulphonamides is described in more detail in chapter 3.

Similar to resistance to sulphonamide, resistance to chloramphenicol is common in *Acinetobacter*s, but little is known about its genetic basis (van Looveren *et al.*, 2004). Thus far, a gene encoding an enzyme inactivating the antibiotic, chloramphenicol acetyltransferase I (CATI), has been described in chromosomes and on plasmids in the genus of *Acinetobacter* (Elisha & Steyn, 1991; Bergogne-Bérézin & Towner, 1996; van Looveren, 2004). Some of the chloramphenicol resistance determinants; for example, *catB8* (Wu *et al.*, 2004; Turton *et al.*, 2005) and *catB3* (Lee *et al.*, 2005) have been observed as integron gene cassettes

1.4. Dissemination of antibiotic resistance: "Russian doll effect"

The spread of antibiotic resistance determinants is mostly due to the dynamic movement of bacteria to different environments (Figure 1.1.A.), the inter-relationships of bacteria in the same environment (Figure 1.1.B.), and the interactions of the mobile elements with each other (Figure 1.1.C. and Figure 1.1.D.).

Various mobile genetic elements are "piggi backs" for the genetic information from one location to the next (Salyers & Amádile-Cuevas, 1997). Just as information is exchanged between cells, genetic material moves around within the cell (Figure 1.1.C.). The genetic fluidity of mobile genetic elements and their association with other mobile elements (Figure 1.1.D.) enables bacteria to accumulate resistance determinants (Rice, 2002) creating large multi-resistant genetic elements in a manner which was figuratively described by Amábile-Cuevas & Chicurel (1992) as the "Russian doll effect".

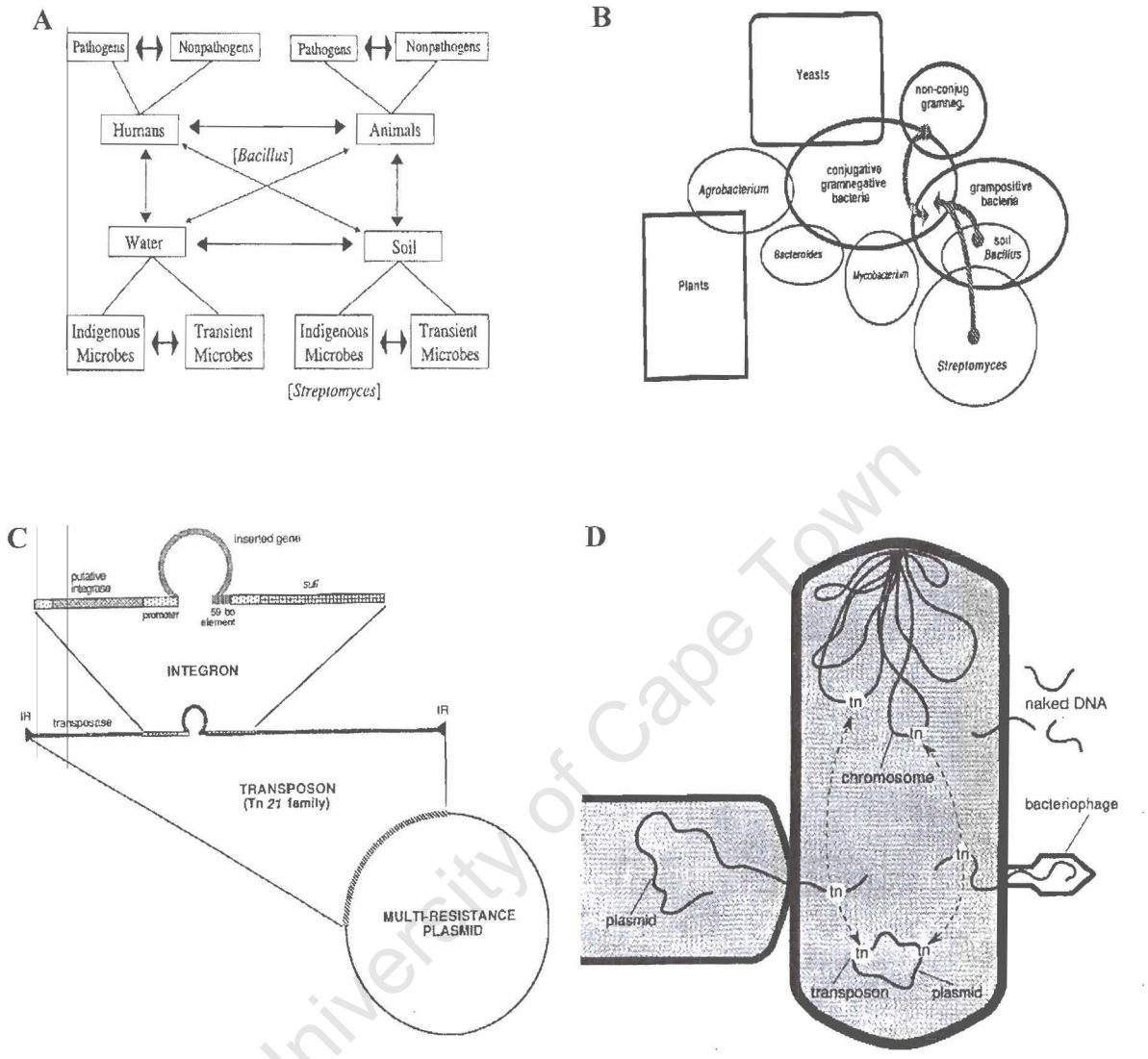


Figure 1.1. Spread of antibiotic resistance determinants A) Schematic representation of the relationship of microorganism within the four major ecosystems. Movement of bacteria within soil, water, animals, and humans outlines the possible spread of antibiotic resistance determinants throughout these ecosystems. (Figure taken from Nwosu, 2001). **B) Genetic flux amongst genetically related microorganisms.** Most genetic exchange occurs on plasmids and other conjugative elements. The arrows indicate the origin and the direction of dissemination of various genetic elements within the micro organism (Figure taken from Amábile-Cuevas & Chicurel, 1992). **C) Mechanism of inter- and intracellular exchange of genetic information amongst microorganisms**. Conjugation and transduction as well as transformation involve exchange of DNA molecules with other microorganisms and the environment. Intracellular movement of DNA includes transposition of various genetic elements within the microorganism. (Figure taken from Levy, 1997). **D) A schematic diagram of the Russian doll effect.** Antibiotic resistance genes are captured in gene cassettes of integrons, which reside in transposons, and these are carried on plasmids. The integration of small mobile elements into larger conjugative elements results in accumulation of several resistance genes on one mobile element. (Figure taken from Amábile-Cuevas & Chicurel, 1992).

The dissemination of antibiotic resistance genes is amplified as the cycle of antibiotic resistance (Figure 1.2.) acquisition is repeated, powered by antibiotic selective pressure (Davies, 1995).

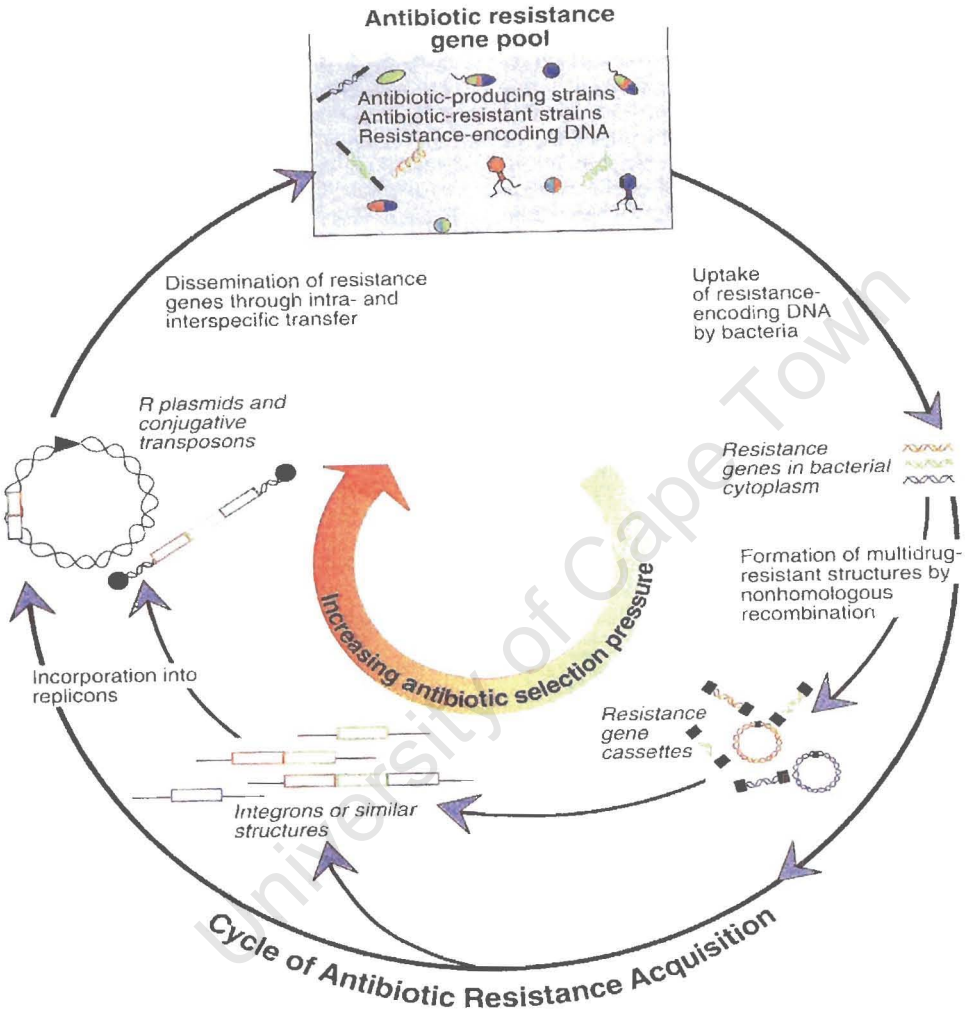


Figure 1.2. Cycle of antibiotic resistance acquisition amplifies the dissemination of antibiotic resistance determinants. Three mechanisms of genetic exchange (conjugation, transformation and transduction) disseminate antibiotic traits into the ecosystems. Various vehicles (plasmids, ICE, integrons and IS elements) transpose antibiotic resistance traits within the organism and other organisms. Recombination of these mobile elements facilitates stacking of antibiotic traits (emergence of multidrug resistance). Inter and intra species exchange of genetic material and the formation of recombinant genetic mobile elements create an enormous diverse pool of antibiotic resistance determinants. Figure taken from Davies, 1994)

1.5. Dissemination of resistance genes from and to *A. baumannii*

Resistance determinants observed in *Acinetobacters* have been identified in *Pseudomonas*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and other members of *Enterobacteriaceae* (Lévesque *et al.*, 1995; White *et al.*, 2001; van Looveren, 2004), indicating dissemination. On the other hand, some genes appear to be unique to *Acinetobacter* spp.: aminoglycoside resistance (*aac(6')-Ik*; Rudant *et al.*, 1994; Doi *et al.*, 2004) and an oxallinase (OXA-40) have only been identified in this genus.

Early studies indicated that *Acinetobacter* harbour many naturally occurring plasmids (Towner, 1991), but plasmid transfer from *Acinetobacter* to other organisms does not appear to occur frequently (Goldstein *et al.*, 1983; Towner, 1991). Transfer of small plasmids conferring resistance to tetracycline was observed amongst *Acinetobacter* spp. within the same ecological niche, but experimental procedures failed to demonstrate in-*vitro* transfer of tetracycline resistance from clinical *Acinetobacter* isolates to aquatic isolates of the same genus, or to *E. coli* K-12 (Guardabassi *et al.*, 2000). Similarly, Lambert *et al.* (1988) observed the transfer of an amikacin resistance gene, *aph*(3') located on a self-transferable plasmid in *A. baumannii* to other *Acinetobacter* spp., but not to *E. coli*. In 1994, the same amikcin gene, *aph*(3'), was identified within Tn*1528* located on a plasmid in *Providencia stuartii* (Lambert *et al.*, 1994[a]) indicating that dissemination of resistance genes is just a matter of time. Seward *et al.*, 1998 also observed the transfer of aminoglycoside resistance genes from clinical *Acinetobacter* isolates via conjugation to a sensitive *A. baumannii* strain. The genetic environment of the chromosome located oxacillinase gene, bla_{OXA-23}, from *Proteus mirabilis* was identical to the corresponding plasmid located gene, bla_{OXA-23}, of *A. baumannii* (Donald *et al.*, 2000), suggesting horizontal gene transfer from *A. baumannii* to *P. mirabilis* (Bonnet *et al.*, 2002). Further evidence that gene transfer occurs between *Acinetobacter* and different genera comes from the observation that the sequence of the chromosomally located bla_{ABA-1} from *Oligella urethralis* is almost identical to the chromosome *ampC* of *A. baumannii*, which suggests a "chromosome-to-chromosome" transfer of a β-lactamase resistance gene (Mammeri *et al.*, 2003). In addition, Joshi *et al.* (2003) demonstrated the

transfer of a plasmid borne ESBL gene from *A. baumannii* via conjugation to *E. coli*, indicating that ESBLs could spread to other organisms from *A. baumannii*.

Although transfer from *Acinetobacter* to other genera may not be efficient, *Acinetobacter*s have the ability to acquire resistance rapidly through horizontal gene transfer (Bergogne-Bérézin, 2001). Chopade *et al.* (1985) have shown that *A. calcoaceticus* readily accepted self-transferable R plasmids from *E. coli* and the transfer of multiple drug resistance to *A. calcoaceticus* from plasmid pIP1031, which is commonly found amongst the *Enterobacteriaceae* family, has been demonstrated (Goldstein *et al.*, 1983). A plasmid encoding cefotaxime resistance isolated from three *A. baumannii* strains has been shown to have an identical restriction enzyme digestion profile to a $bla_{\text{CTX-M-2}}$-carrying plasmid which was previously identified in *Proteus mirabilis* (Nagano *et al.*, 2004) suggesting that *A. baumannii* has acquired the plasmid. Devaud *et al.* (1982) have observed transposable multiple drug resistance in chromosomes of *Acinetobacter* strains and suggest that it was acquired by this genus from the hospital flora. Wu *et al.* (2004) suggested that $bla_{\text{IMP-1}}$ gene was acquired by *Acinetobacter* spp. on a conjugative transferable plasmid and Ribera *et al.* (2003) suggest that tetracycline resistance resulted from a transposition event involving Tn*1712*-like transposon. The abundance of integrons identified in *Acinetobacter* spp., which often contain more than one resistance determinant, is indirect evidences of antibiotic resistance determinant acquisition by *Acinetobacter*s (Ploy *et al.*, 2000; Gombac *et al.*, 2002; van Looveren, 2004; Wu *et al.*, 2004).

1.6. Transcription of resistance genes in *Acinetobacter*

As part of my research project involved studying the transcription of a resistance gene in *A. baumannii*, I shall provide an outline of what is known about transcription of resistance gene in *Acinetobacter* and further details are presented in chapter 4.

Transcription of the majority of prokaryotic genes involves the recruitment of the bacterial core RNA polymerase complex (five subunits: ββ'αω) by the sigma subunit (σ^{70}) to DNA sequences upstream of the transcription initiation signals (Paget & Helman, 2003), which recognizes the consensus sequence, TTGACA (-35) N_{17} TATAAT (-10) in *E. coli* (Harley & Reynolds, 1987). Kleppe & Kleppe (1976) observed similar holoenzyme components in *A. calcoaceticus* when compared to *E. coli*, but the β subunits were not separated by gel electrophoresis and no sigma 70 unit was apparent in the preparations from *A. calcoaceticus*. This led to suggestion that σ^{70} may not be the major σ factor in *Acinetobacter*, or that association between σ^{70} and the core RNA polymerase (RNAP) may be weak (Kleppe & Kleppe, 1976). Further, gene expression in *Acinetobacter* spp. has been observed to proceed from non-conventional sequences (Segal & Elisha, 1999).

For example, atypical promoter sequences were identified upstream of chromosomally located aminoglycoside 6'-N-acetyltransferase type I gene, *aac(6')-Ig* (Lambert *et al.*, 1993). The putative transcription signals, TTGAAA (-35) and TAAGTT (10), are separated by 21 nucleotides (Table 1.3.) and the -10 hexamer is positioned within one of the two 11bp invert repeat sequences upstream the start codon. Lambert *et al.* (1993) have speculated that this feature could correspond to an operator region and function as a regulator for this gene. Promoter sequences with atypical spacer sequences (19 nucleotides) have been described also in the transcription of *trpFB* (Kishan & Hillen, 1990).

The upstream region of an aminoglycoside resistance gene, *aacC2*, contains two putative -10 hexamers (Table 1.3.) and no -35 hexamer (Elisha, 1991). In that study, the conserved sequence of a CAP/cAMP complex was identified but its contribution to expression of this gene can only be speculated (Elisha, 1991). Similarly, only a -10 hexamer (Table 1.3.) was identified upstream of an *aadB* gene in *A. baumannii* (Segal & Elisha, 1999). Interestingly, this sequence is upstream of a region containing four putative promoters, typical of those recognized by σ^{70} which were not recognized in the transcription of *aadB* (Segal & Elsiha, 1997). However, one of these promoters (TTGACA (-35) N_{17} CAGAAT (-10)) was recognized when the *aadB* gene was cloned and expressed in *E. coli* (Segal & Elisha, 1999), suggesting that transcription signals may be similar but different in the two organisms. Although not related to the transcription of resistance genes, studies done by Schembri *et al.* (1995) support this notion. While studying the expression of *Acinetobacter* derived $phaB_{AC}$ genes in the host organism and *E. coli*, primer extension products were observed in the latter species that were not apparent in *Acinetobacter* (Schembri *et al.*, 1995).

Putative promoters, typical of those recognised by σ^{70} have been identified in *Acinetobacter*. Optimally spaced (17 to 18 nucleotides) -35 and -10 hexamers were proposed for the expression of a plasmid located *aac(6')-Ih* in *A. baumannii* and for a chromosome-located *aac(6')-Ij* gene in *Acinetobacter* species 13 (Lambert *et al.*, 1994[b]). These sequences (Table 1.3.) showed good homology to the consensus sequence of *E. coli* σ^{70} (Harley & Reynolds, 1978). The transcription of an *aac(6')-Ij* gene identified in *Acinetobacter* sp. 13 is possibly driven from a hybrid promoter which shows similarity to sequences recognised by σ^{70} (Rudant *et al.*, 1998). The putative -10 hexamer is just downstream of one of the direct repeat of IS*18*, whereas an optimally spaced -35 hexamer is situated within the inverted repeats (Table 1.3.). It was speculated that the transposition of IS*18*, and hence the formation of this putative transcription control signal could have been involved in the activation of the previously silent aminoglycoside gene (Rudant *et al.*, 1998).

Table 1.3. Promoter sequences of antibiotic resistance genes identified in *Acinetobacter* spp.

		-35 hexamer	Inter–region spacing	-10 hexamer	Reference
CONSENSUS σ^{70}		**TTGACA**	**17**	**TATAAT**	Harley & Reynolds, 1987
Organism	**Gene**				
A. baumannii	*aacC2*	None		TATAGT TAGAGT	Elisha, 1991
A. baumannii	*aadB*	None		TATTCA*	Segal & Elisha, 1999
A. heamolyticus	*aac(6')-Ig*	TTGAAA	21	TAAGTT	Lambert *et al.*, 1993
A. species 13	*aac(6')-Ij*	TTGAGT	#	TATGAT	Lambert *et al.*, 1994[b]
A. baumannii	*aac(6')-Ih*	TTGATT	17	TTTAAT	Lambert *et al.*, 1994[b]
A. species 13	*aac(6')-Ij*	TTGCCG	17	TAAAAT	Rudant *et al.*, 1998
A. baumannii	bla_{OXA27}	TTGTCG	17	TATTTT	Segal *et al.*, 2003
A. baumannii	*ampC*	TTGTTC	17	TATGAT	Corvec *et al.*, 2003
A. baumannii	*ampC*	TTAGAA*	16	TTATTT*	Segal *et al.*, 2004

*Promoters identified by primer extension analysis. The non-annotated sequences are putative promoter sequences. # not given.

Recently, primer extension studies showed that transcription of *ampC* in *A. baumannii* was dependent on promoters (Table 1.3.) within an IS element (Segal *et al.*, 2004) designated $IS_{Aba\text{-}1}$ by Corvec *et al.* (2003). Interestingly, Corvec *et al.* (2003) proposed different putative promoter sequences within $IS_{Aba\text{-}1}$ for the transcription of *ampC* in *A. baumannii* strains (Table 1.3.). In an earlier study, Segal *et al.* (2003) observed $IS_{Aba\text{-}1}$ related sequences immediately upstream of oxacillinase genes ($bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}27}$) and they proposed that transcription of these genes was similarly dependent on promoters within $IS_{Aba\text{-}1}$. These findings raised a number of questions, including:

1. What is the frequency and distribution of $IS_{Aba\text{-}1}$ in *Acinetobacter* spp., *Enterobacteriaceae* and *P. aeruginosae*?
2. What is the frequency of $IS_{Aba\text{-}1}$ associated with antibiotic resistance genes in *A. baumannii*, and is the expression of associated genes dependent on $IS_{Aba\text{-}1}$ located promoters.

The aim of this project was to answer these questions.

Chapter 2

Distribution and Frequency of IS_{Aba-1} in *Acinetobacter* spp., *Pseudomonas aeruginosa* and *Enterobactericeae*

2.1. Introduction

Many insertion sequences (IS) are widely distributed amongst several bacterial genera (Table 3 in Mahillon & Chandler, 1998). For some IS families, for example IS*21*, the host range includes both gram-positive and gram-negative genera (Yeo *et al.*, 1998). IS*15*-related sequences have been detected on plasmids isolated from phylogenetically distant bacteria (Labigne-Roussel & Corvalin, 1983), and IS elements from IS*3* family are widely spread amongst different genera (Steibl & Lewecke, 1995; Mahillon & Chandler, 1998). On the other hand, some IS elements are limited to genera or host. For example, IS*1* was identified in *Escherichia coli*, but not in *Salmonella*, *Citrobacter*, *Edwardsiella* and *Erwinia* (Nymann *et al.*, 1983). Similar findings were observed with respect to IS*1222*: hybridization studies identified this IS only amongst strains closely related to *Enterobacter agglomerans* 339, indicating the limited distribution of some IS elements (Steibl & Lewecke, 1995). Other elements, such as IS*1491*, are widely distributed amongst Pseudomonads (Yeo *et al.*, 1998) and IS*2000* has been shown to be unique to *Klebsiella pneumoniae* (Aubert *et al.*, 2003). In some instances, IS elements, including IS*1542*, IS*1251* and IS*1476*, have been shown to be confined to a geographical area (Huh *et al.*, 2004).

The copy number of a particular IS may vary between disparate genera. IS*1* is abundant in *E. coli* (one to twenty copies amongst different isolates); on the other hand, *Klebsiella aerogenes* and *Serratia marcescens* contain one and two copies of the element, respectively (Nyman *et al.*, 1983). One explanation for these differences is that the transposition frequency of an IS varies greatly amongst different genera (Mahillon & Chandler, 1998). Secondly, the repetitive introduction of the IS element into the same strain might contribute to the variation of copy number (Nyman *et al.*, 1983) and, thirdly,

the genomic plasticity of the strains could have facilitated these events (Schneider & Lenski, 2004).

Insertion sequences, which are widely distributed amongst several genera were also identified in *Acinetobacter* spp., including IS*15* (Labigne-Roussel & Courvalin, 1983), IS*26* (Kim & Aoki, 1994; Sundström *et al.*, 1995; Segal *et al.*, 2003), IS*1* (Elisha & Steyn, 1991), IS*1597* (Miranda *et al.*, 2003) and IS*1999* (Poirel *et al.*, 2003; www-is.biotoul.fr). IS-elements, which have their origin in *Acinetobacter* spp., have been shown to be either widely distributed amongst this genus or have been shown to be exclusive to a species. For example, IS*17* was detected in *A. heamolyticus* and *Acinetobacter* genomic species 6, 13, 14, 16 and 17 (Rudant *et al.*, 1997); in contrast, IS*1236* and IS*18* has been detected only in *A. calcoaceticus* and *Acinetobacter* sp. 13, respectively (Gerischer *et al.*, 1996; Rudant *et al.*, 1998). Recent studies identified novel insertion sequence elements, IS_{Aba3}, IS_{Aba825} and IS_{Aba125} in *A. baumannii* (Poirel *et al.*, 2005[b]; Mussi *et al.*, 2005). No studies were carried out to investigate the distribution of these novel IS elements amongst other bacteria. Nevertheless, it has been shown that these *A. baumannii* strains contain more than one copy of IS_{Aba3} and IS_{Aba825} (Poirel *et al.*, 2005[b]; Mussi *et al.*, 2005).

Characterization of a 6.080-kb *Hin*dIII-fragment cloned from *A. baumannii* strain PAU in pUC19, generating pSK1F100 (Segal *et al.*, 2003), identified a 946bp sequence which was previously found adjacent to $phaB_{Ac}$ gene (Schembri *et al.*, 1995) and antibiotic resistance genes bla_{OXA-23} and bla_{OXA-27} (Donald *et al.*, 2000; Afzal-Shah *et al.*, 2001). Segal *et al.* (2003) proposed that a site specific recombination system could account for the occurrence of several different genes downstream of this sequence (Figure 2.1.). Further analysis of this region identified two open reading frames, ORF1 (543bp) and ORF2 (230bp), which show similarities to transposases of *Deinococcus radiodurans* (Segal *et al.*, 2003), suggesting a putative insertion sequence. Based on homology searches the transposases are similar to Tn*10*-like transposases, which have been assigned to the Tn*11* family of these enzymes. In the absence of the complete IS Segal *et al.* (2003) termed the sequence homologous region. Subsequently, this same sequence (homologous region) was cloned upstream of a β-lactamase gene, *ampC*, from *A.*

baumannii strain RAN (Segal *et al.*, 2004) and at the same time, Corvec *et al.*, 2003 identified an entire copy of the IS element, which they called IS_{Aba-1}, upstream of an *ampC* gene from *A. baumannii*. Characterization of IS_{Aba-1} (1198bp) showed that the element has 11bp inverted repeat sequences (5'-CTCTGTACACG-3') and that integration was accompanied by duplication of 9bp of the target DNA

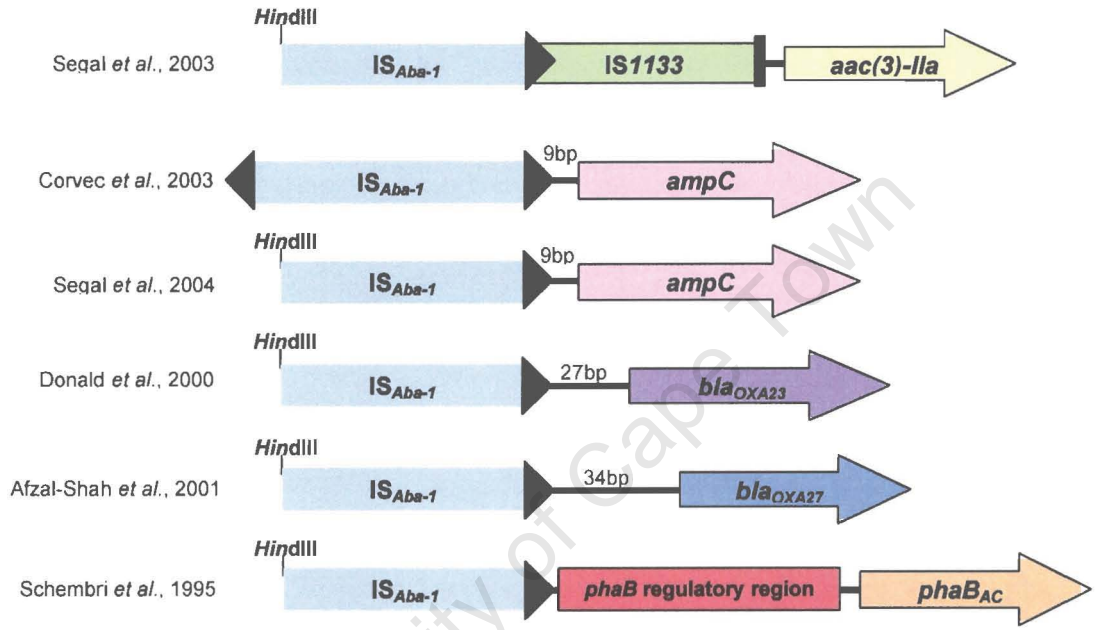


Figure 2.1. Genetic environment of IS_{Aba-1}. IS_{Aba-1} is represented by a blue block. Inverted repeats of IS_{Aba-1} are represented in black triangles. Downstream of IS_{Aba-1} are the genes (block arrows), which are separated by non-coding sequence (black horizontal line). The vertical black box indicates the inverted repeat of IS*1133* (green box) and the dark pink box the regulatory region of $phaB_{AC}$. The source of the data is indicated on the left.

To date, the IS_{Aba-1} has been identified only in an unspeciated *Acinetobacter* (Schembri *et al.*, 1995) and *A. baumannii* strains. PCR assays done by Segal *et al.* (2004) indicate that *A. baumannii* strain PAU contains at least two copies of this insertion sequence. These data raised two questions: (1) what is the frequency and distribution of IS_{Aba-1} in clinical isolates of *Acinetobacter* spp. and (2) is IS_{Aba-1} present in similar isolates of *Enterobacteriaceae* and *P. aeruginosae*?

2.2. Experimental protocol

2.2.1. Bacterial isolates

The bacterial strains included in this study are presented in Table 2.1. Fourteen *A. baumannii*, 3 *A. lowffii* and one *A. calcoaceticus*, BD413 (Segal, 1998) subsequently called *Acinetobacter baylyi* BD413, were investigated. Of the 14 *A. baumannii* isolates, 2 strains were from Universitas Hospital (UH), Bloemfontain, Free State and 3 strains were from Tygerberg Hospital (TH) Cape Town, Western Cape. The balance (9) were obtained from patients at Groote Schuur Hospital (GSH), Cape Town, Western Cape. *A. baumannii* strains, PAU and RAN were also from GSH and were previously shown to contain $IS_{Aba\text{-}1}$ (Segal *et al.*, 2003; Segal *et al.*, 2004). Clinical isolates of *Escherichia coli* (13), *Klebsiella pneumoniae* (7) and *Klebsiella oxytoca* (2), *Enterobacter cloacae* (1) and *Pseudomonas aeruginosa* (4) were also investigated in this study. All of these isolates were obtained from GSH. The identification of the clinical isolates was performed in the clinical medical laboratories of the hospitals mentioned above.

Table 2.1. Clinical isolates

Bacterial strains	Number of isolates	Hospital	Isolated
Acinetobacter baumannii	9#	GSH	1993-2003
Acinetobacter baumannii	3	TH	2002
Acinetobacter baumannii	2	UH	1994
Acinetobacter calcoaceticus	1*		
Acinetobacter lwoffii	3	GSH	2004
Escherichia coli	12+1*	GSH	1997-2002
Klebsiella pneumonia	7	GSH	2000-2002
Klebsiella oxytoca	2	GSH	1999
Pseudomonas aeruginosa	4	GSH	2000
Enterobacter cloacae	1	GSH	2003

#includes $IS_{Aba\text{-}1}$ positive strains, PAU and RAN (Segal *et al.*, 2003 and Segal *et al.*, 2004); *non-clinical isolate

2.2.2. Genomic DNA preparation

A single colony was inoculated into 50ml 2 times Yeast-Trytone (2YT) broth (Appendix 1) and incubated at 37°C for 18 hours. Bacterial cells were harvested by centrifugation in a GS-6 rotor at 3000 rpm for 20 minutes. The cells were gently re-suspended in Tris-

EDTA buffer (Appendix 1) and lysed by 10% SDS (Sigma). Proteinase K (0.1mg/ml, Sigma) was added to the solution to hydrolyse cellular proteins. Genomic DNA was extracted by the CTAB/NaCl-method as described in Current Protocols in Molecular Biology Vol.1 (Ausubel *et al.*, 1987). RNase A (0.1µg/ml, Southern Cross Biotechnology, QIAGEN, Germany) was added to the cell lysate. The DNA was precipitated with isopropanol, washed with 70% ethanol and air dried for 5 minutes. The DNA was resuspended in 100µl TE buffer. To estimate the DNA concentration, a 1/10 dilution was prepared and an aliquot was subjected to agarose gel electrophoresis [2.2.4.]. The level of the fluorescence was compared to the fluorescence emitted by a known standard (50µg to 5µg λ-DNA, Roche).

2.2.3. Isolation of pMERL100

pMERL100, containing a portion of IS_{Aba-1} (Segal *et al.*, 2004), was used as a positive control and as a source of the probe in hybridization experiments. *E. coli* JM109 (pMERL100) was cultured in 2YT broth containing the appropriate selection (50µg/ml kanamycin (Novo Nordisk, South Africa)). Bacterial cells were harvested from an overnight culture and recombinant plasmids were isolated according to the protocol of QIAGEN plasmid midi kit (Germany). The DNA was precipitated with isopropanol and washed with 70% ethanol, air-dried and re-dissolved in 20µl ddH_2O. The emission of fluorescence of a molecular weight marker VI (Roche) was used to estimate DNA concentration [2.2.4.].

2.2.4. Agarose gel electrophoresis (AGE)

DNA was separated on horizontal gels consisting of 0.8-1% (w/v) agarose (Hispangar, Spain), which was dissolved in 1x Tris-acetate EDTA buffer (TAE; Appendix 1). The running buffer was 1x TAE buffer and the agarose gel was stained with 100µg/ml ethidium bromide (Boehringer Mannheim). Aliquots of DNA were loaded with gel tracking dye (Appendix 1) into the wells of the agarose gel. The DNA was visualized with an ultra violet light source (302nm) and the fluorescence was photographed with a Kodak DC290 digital camera (New York).

2.2.5. DNA-DNA hybridization studies

2.2.5.1. Preparation of DNA blots for hybridization

2.2.5.1. A) Slot blot

Genomic DNA (5µg) was heated at 95°C for 10 minutes, snap-cooled on ice for 10 minutes, subsequently, an equal volume of 20x SSC (Appendix 1) was added to facilitate denaturing. The Hybond N+ membrane (Amersham Bioscience, UK) was wetted in 10x SSC before the single stranded DNA was loaded into Minifold II slot blot apparatus (Schleicher & Schuell Inc. Keene NH, Germany). A vacuum (60mbar, VacuGene Pump, Pharmacia Biotech, Sweden) was applied to the apparatus to transfer the DNA to the Hybond N+ membrane. After transfer, the membrane was soaked in denaturing solution (Appendix 1) for 5 minutes at room temperature and subsequently in neutralization solution for 1 minute at room temperature. The DNA was fixed to the membrane with UV radiation (UV cross linker, Hoefer, Scientific Instruments, San Francisco) for 1200 counts at 254nm.

2.2.5.1. B) Southern transfer

Genomic DNA was digested overnight at 37°C in a total reaction volume of 30µl, containing 1µg genomic DNA, 20U of *Pst*I (Roche) and 1x restriction enzyme buffer (Roche). The DNA fragments were separated overnight in a 0.8% agarose gel [2.2.4.].

The agarose gel was depurinated in 250mM HCl for 10-15 minutes, followed by a denaturing step of 25 minutes in 1.5M NaCl and 0.5 NaOH. Neutralization was achieved by incubating the gel in 1.5M NaCl and 0.5M Tris-Cl for 30 minutes. All steps were carried out with agitation (The Belly Dancer, Stovall Life Science, USA) at room temperature (RT) and between each step the gel was rinsed with ddH_2O. The DNA was transferred from the gel to a Hybond N+ membrane (Amersham Bioscience, UK) via capillary blotting with 20x SSC buffer for 18 hours at RT. The blot was rinsed in 6x SSC for 1 minute at RT and the DNA was fixed to the blot with an ultra violet cross linker (254nm) for 1200 counts.

2.2.5.2. Preparation of the probe for the detection of IS_{Aba-1}-related sequences

pMERL100 (Segal *et al.*, 2004) was used as a source of IS_{Aba-1} probe. Primers, HRR and HRF, designed by Heidi Segal, as indicated in Figure 2.2. were synthesized in the Department of Molecular and Cellular Biology, University of Cape Town, South Africa. The primers, HRF (5'-CACGAATGCAGAAGTTG-3') and HRR (5'-CGACGAATACTATGACAC-3'), were annealed to DNA of pMERL100 and were subjected to PCR amplification to generate a 549bp product. The PCR was carried out in a thermocycler (Perkin Elmer GeneAmp PCR System 2400). The reaction mix contained of 3mM $MgCl_2$, 1x PCR Buffer, 10mM dNTPs, 2.5U of Taq (all reagents from TaKaRa, Japan) and 20pmoles primers in a total reaction volume of 50µl. The DNA was initially denatured at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 54°C for 45 seconds and elongation at 72°C for 3 minutes. The PCR product was separated by AGE [2.2.4.]. A fragment corresponding to the expected size was excised and purified by the Min Elute Gel extraction kit (50) QIAGEN, Germany. The purified 549bp fragment (400ng) was labelled using ECL Direct Nucleic Acid Labelling Detection System (Amersham Bioscience, UK) according to the manufactures instructions.

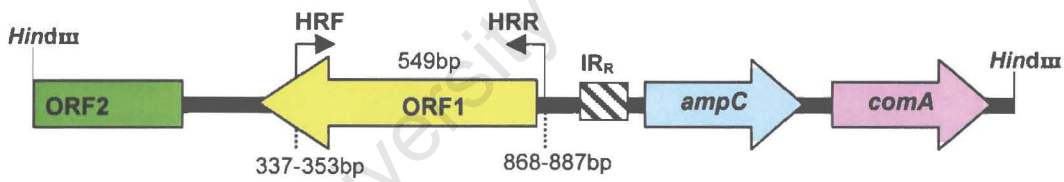


Figure 2.2. The genetic organization of 3.123kb *Hin*dIII-insert of pMERL100. For the detection of IS_{Aba-1}-related sequences, a probe was generated from pMERL100 with primers, HRF and HRR. These primers anneal within the structural gene of ORF1 and a PCR product of 549bp was obtained. The primers are indicated by the arrows and the nucleotides corresponding to the sequencing data of pMERL100, is indicated by the dotted line. Two open reading frames (ORF1 and ORF2) encode the transposases of IS_{Aba-1}. The blue and pink block arrow downstream of the right inverted repeats (IR_R: diagonally striped box), represent the genes encoding a cephalosporinase (*ampC*) and ComA (*comA*), respectively. The direction of gene transcription is indicated by the block arrow. Non-coding sequence is indicated by a black bar. (pMERL100 Accession no AY325306)

2.2.5.3. Hybridization and post-hybridization washes

The DNA containing membrane was made wet with 6x SSC prior to the hybridization procedures. The hybridization buffer (Appendix 1) was prepared as directed by the manufacturer in the ECL Direct Nucleic Acid Labelling Detection System kit. The pre-hybridization and the hybridization were carried out in a hybridization tube and oven (Techne Hybridiser HB 1D, England). Pre-hybridization was carried out in 50ml hybridization buffer for 1 hour at 42°C. Subsequently, the probe was added to the hybridization buffer and hybridization was allowed to proceed over night at 42°C. The hybridization buffer was discarded and replaced with 5x SSC and the membrane was washed for 10 minutes at 55°C. Thereafter, the membrane was washed in primary washing buffer, consisting of 0.4% SDS and 0.5x SSC for 10 minutes at 55°C. This step was repeated twice, for 5 minutes and 3 minutes, respectively; after which, the membrane was removed from the hybridization tube, placed in plastic container and rinsed with 2x SSC for 5 minutes at room temperature with gentle agitation. The solution was discarded and replaced with 2x SSC. The membrane was washed for another 5 minutes at room temperature (RT) with gentle agitation.

2.2.5.4. Signal generation and detection

The wet membrane was transferred to a sheet of cling wrap and covered with previously mixed equal volumes of detection reagents (provided by ECL Direct Nucleic Acid Labelling Detection System) for one minute at RT. The blot was exposed to X-ray film (AGFA, CP-BU, medical X-ray film, 100NIF, 180 x 210mm) for 1 minute, 1 hour or overnight. To visualize the signals, the X-ray film was incubated in G128 Developer (AGFA, Belgium) for three minutes, followed by one minute in 2% acetic acid (Merck), two minutes in G333c rapid fixer (AGFA, Belgium) and rinsed in water for 5 minutes. The autoradiograph was air dried and photographed with a digital camera (Kodak DC290).

2.3. Results

2.3.1. Hybridization studies

2.3.1.1. Distribution of IS_{Aba-1} in *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella*, *Enterobacter* and *E. coli*

When genomic DNA was transferred to Hybond N+ membrane usig the slot blot apparatus, the probe hybridized to pMERL100, the source of the probe, and to *A. baumannii* strains PAU and RAN, previously shown to contain IS_{Aba-1} (Segal *et al.*, 2003; Segal *et al.*, 2004). In addition, signals were obtained from 6/14 *A. baumannii* strains and 2/3 *A. lwoffii* strains (Figure 2.3.). No signal was obtained with the DNA from the *A. calcoaceticus*. Similarly, signals were not obtained with DNA from *Escherichia coli* (13), *Klebsiella pneumniae* (7) and *Klebsiella oxytoca* (2), *Enterobacter cloacae* (1) and *Pseudomonas aeruginosa* (4) (Figure 2.3.).

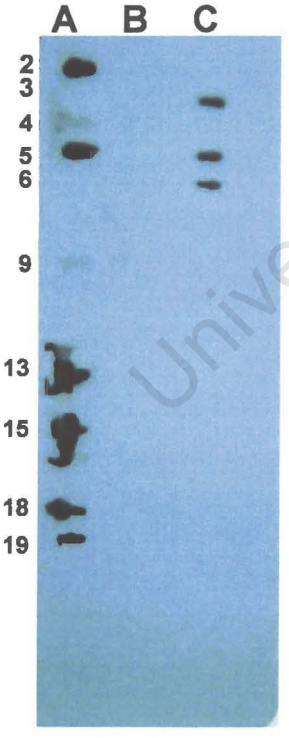


Figure 2.3. Autoradiograph of genomic DNA hybridized to the IS_{Aba-1} -probe.
2A, pMERL100;
4A, RAN;
5A, PAU;
6A, *A. calcoaceticus* BD413;
A8-A15, *A. baumannii* strains;
A17-A19, *A. lwoffii* strains;
2B-7B, *E. coli* strains;
9B-13B, *Klebsiella* spp.;
15B-18B, *P. aeruginosa* strains;
2C-7C, *A. baumannii* strains;
9C-15C, *E. coli* strains;
16C, *E. cloacae*;
18C-21C, *K. pneumonia* strains.

2.3.2.2. Frequency of IS_{Aba-1} in *A. baumannii* and *A. lwoffii*

To determine the copy number of the IS element, genomic DNA from IS_{Aba-1} positive *Acinetobacter* strains was digested with *Pst*I (this restriction site is absent in IS_{Aba-1}), separated by AGE [2.2.4.], transferred to a nylon membrane (Hybond N+) and hybridized to a portion of IS_{Aba-1} [2.2.5.1.B.]. The probe hybridized to itself (lane12 in Figure 2.4.) and to pMERL100 (lane 2 in Figure 2.4), which was the source of the probe. In addition, the probe hybridized to multiple bands in PAU and RAN (lane 8 and 9, Figure 2.4). With respect to the remaining isolates, the probe hybridized to between 1 and 13*Pst*I fragments in the *A. baumannii* and *A. lwoffii* strains (Figure 2.4.).

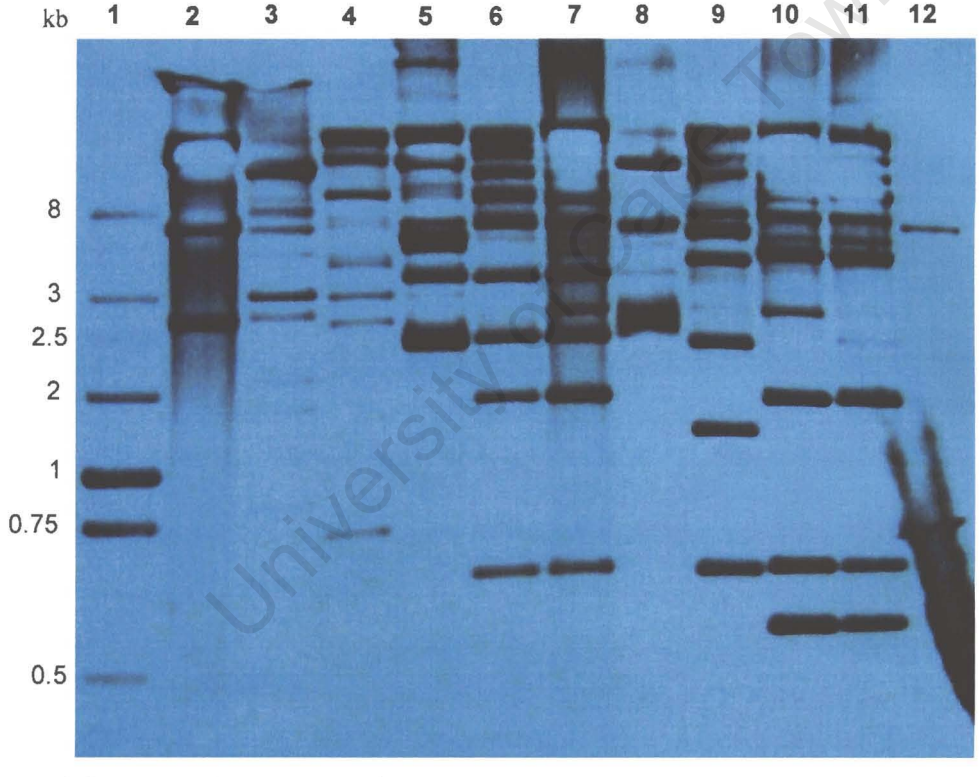


Figure 2.4. Autoradiograph of IS_{Aba-1} -probe hybridized to *Pst*I-digested genomic DNA of *Acinetobacter* strains. Lane 1, 1kb DNA ladder (fragment size (kb) is indicated by the numbers on the left); lane 2, pMERL100; lane 3, *A. lwoffii* strain 2; lane 4, *A. lowffii* strain 4; lane 5, *A. baumannii* strain TYG01; lane 6, *A. baumannii* strain TYG02; lane 7, *A. baumannii* strain TYG03; lane 8, PAU; lane 9, RAN; lane 10, *A. baumannii* strain RAM; lane 11, *A. baumannii* strain GA13; lane 12, *A. baumannii* strain GA5; lane 13, no DNA; lane 14, probe.

2.4. Discussion

A portion of IS_{Aba-1} was first identified in *A. baumannii* in 2003 (Segal *et al.*, 2003), and an entire IS_{Aba-1} from *A. baumannii* was characterized in the same year (Corvec *et al.*, 2003). To study the distribution of IS_{Aba-1}, hybridization studies were carried out. The IS was detected in 8 of the 14 *A. baumannii* strains and in two of the three *A. lwoffii* isolates (Figure 2.3.). That the IS was present in *A. baumannii* strains from geographically separate hospitals, indicates that it is not confined to isolates from a specific hospital. Further, the data suggest that IS_{Aba-1} is not present in all strains of a particular *Acinetobacter* species (Figure 2.3.). In this respect IS_{Aba-1} is similar to IS*18*, which was identified in 6 of 29 *Acinetobacter* sp. 13 strains, but was not detected in 10 *A. baumannii* or in 10 *A. heamolyticus* strains (Rudant *et al.*, 1998).

Sequences related to IS_{Aba-1} were not identified in *E. coli* (13 strains), *Klebsiella* spp. (9), *P. aeruginosa* (4) and one *E. cloacae* (Figure 2.3.) from GSH; albeit that they were isolated from Groote Schuur Hospital during the same time period as some *A. baumannii* and *A. lwoffii* strains. Since it has been suggested that some plasmids may be specific to *Acinetobacter* (Towner, 1991), transfer of IS_{Aba-1} from this genus to other genera may not have been efficient. Although the number of strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* is small, the data suggest that IS_{Aba-1} may be unique to *Acinetobacter* spp. Interestingly, IS_{Aba-1} putative transposase shares similarities to its counterpart in *D. radiodurans*, a common soil organism. *Acinetobacter* spp. are also found in soil and this might reflect the source of the insertion sequence (Segal *et al.*, 2003).

The abundance of IS_{Aba-1} in *Acinetobacter*s reflects its mobility and indicates that transposition had occurred frequently. This frequency of insertion suggests plasticity of the *Acinetobacter* genome as transposition of IS element can cause a variety of genome rearrangements. Interestingly, with respect to the *A. baumannii* strains from GSH, the copy number of IS_{Aba-1} is higher in the more recent isolates. Further, as the *A. lwoffii* strains were susceptible to most of the clinically useful antimicrobials, IS_{Aba-1} is not associated with only a resistance phenotype. Nosocomial isolates are constantly under

selective pressure; it has been proposed that IS expansion might occur during evolutionary bottlenecks, which favours only the survival of the fittest (Nagy & Chandler, 2004). Therefore, IS_{Aba-1} might enable *Acinetobacter*s to survive in a hostile environment.

Chapter 3

The association of IS_{Aba-1} with antibiotic resistance genes in *Acinetobacter baumannii*

3.1 Introduction

It is well established that IS elements contribute to chromosomal rearrangements (Mahillion & Chandler, 1998). They facilitate the mobilization of antibiotic resistance determinants and often contribute to the expression of genes downstream by providing promoter sequences (Mahillion & Chandler, 1998).

Insertion sequences, IS*1*, IS*15*, IS*1597* and IS*4321*, have been associated with antibiotic resistance determinants forming composite transposons. Therefore, it is possible that these IS elements might have been involved in the acquisition of a chloramphenicol resistance gene (*catI*), aminoglycoside resistance gene (*aph(3')VIa*) and tetracycline resistance determinants (*tet*(A) and *tet*(H)), respectively, in *Acinetobacter* spp. (Elisha & Steyn, 1991; Lambert *et al.*, 1994; Miranda *et al.*, 2003; Ribera *et al.*, 2003). Similarly, IS*1999* and IS*Pa12* could have contributed to the dissemination of β-lactamase resistance genes, $bla_{VEB-1}$and bla_{PER-1}, respectively, to various bacteria, including *A. baumannii* (Poirel *et al.*, 2003; Aubert *et al.*, 2003; Poirel *et al.*, 2005[a]). Recently, a plasmid-mediated bla_{OXA-58} was associated with a novel IS element, IS_{Aba3} in *A. baumannii*, which has the potential to facilitate the dissemination of this gene to other bacteria (Poirel *et al.*, 2005[b]).

IS elements not only have the potential to facilitate mobilization of antibiotic resistance genes but also provide promoters to the genes located at the 3'-end of the IS element. One of the best examples of the contribution of IS-located promoters to the expression of antibiotic resistance has been observed in *Bacteriodes fragilis*. Up to date, ten insertion elements have been shown to activate a silent imipenem resistance gene, *cfiA*, of *B. fragilis* (Kato *et al.*, 2003). Another example is IS*Ecp1*, which has been identified

upstream of several different bla_{CTX-M} genes and plasmid-mediated *ampC* genes in *Enterobacteriaceae* (Bonnet, 2004). Putative promoter sequences were identified within this element (Bonnet, 2004). Less is known about the role of IS-located promoters in the transcription of antibiotic resistance genes in *Acinetobacter*. IS*Pa12* was identified upstream of bla_{PER-1} in *Salmonella enterica* serovar Typhimuriumm, *P. aeruginosa* and *A. baumannii* and transcription of bla_{PER-1} is dependent on IS*Pa12*-located promoters in *Salmonella enterica* serovar Typhimuriumm and *P. aeruginosa* (Poirel *et al.*, 2005[a]). It is not known whether transcription of this gene in *A. baumannii* is similarly dependent on IS*Pa12*-located promoters (Poirel *et al.*, 2005[a]). Similarly, IS*1999*, which has been shown to increase the expression of bla_{VEB-1} in *P. aeruginosa* (Aubert *et al.*, 2003) could also be responsible for the expression of this gene in *A. baumannii* (Poirel *et al.*, 2003). The insertion of IS*18* immediately upstream of a silent amikacin resitance gene (*aac(6')-Ij*) in *Acinetobacter* sp. 13 was thought to facilitate transcription of this gene by providing the -35 component of the promoter (Rudant *et al.*, 1998).

With respect to IS_{Aba-1}, previous studies have identified β-lactamase resistance genes encoding oxacillinase (Donald *et al.*, 2000) and cephalosporinase (Corvec *et al.*, 2003; Segal *et al.*, 2004) downstream of this element. Segal *et al.* (2004) have identified promoter sequences in IS_{Aba-1} which drive the expression of *ampC* in strain RAN and Corvec *et al.* (2003) have accounted putative promoter sequences in IS_{Aba-1} to the overexpression of *ampC*. This raised the question of the frequency of association of IS_{Aba-1} with antibiotic resistance genes in *A. baumannii*. This chapter describes the undertaking of identifying antibiotic resistance genes downstream of IS_{Aba-1}.

3.2. Experimental protocol

3.2.1. Preparation of partial DNA library from strain RAM

A) Preparation of genomic DNA

To determine the frequency of association of IS_{Aba-1} with antibiotic resistance genes *A. baumannii* strain RAM was chosen, as it contains multiple copies of IS_{Aba-1} (Figure 2.4.) and was resistant to most useful antibiotics. Genomic DNA (80ng) from strain RAM [2.2.2.] was digested with 10U of each restriction enzyme, *Hin*dIII and *Bam*HI (Roche), and 1x restriction enzyme buffer (Roche) in a final reaction volume of 20µl. The reaction was incubated over night at 37°C. An aliquot of the digested DNA was separated on AGE [2.2.4.] to ensure complete digestion of the genomic DNA. Subsequently, the DNA fragments generated by the restriction enzyme digestion were purified with phenol/choloform/isoamylalcohol (Appendix 1) and precipitated with 100% ethanol according to the protocol described by Ausubel *et al.* (1987). The precipitated DNA fragments were washed with 70% ethanol and resuspended in ddH_2O.

B) Preparation of pUC19 vector

The vector, pUC19 (Appendix 2), was used in the cloning studies. For the plasmid isolation, *E. coli* JM109 (pUC19) was cultured in 2YT broth, containing the appropriate selection (100µg/ml ampicillin (Ranbaxy, South Africa)), at 37°C and cells were harvested the next morning by centrifugation. QIAGEN plasmid midi kit (Germany) was used to lyse the cells and to extract plasmid DNA. One microgram of pUC19 was linearized with 10U of each restriction enzyme, *Hin*dIII and *Bam*HI (Roche), and the appropriate restriction enzyme buffer (1x) in a final reaction volume of 20µl at 37°C for 3 hours. Thereafter, the reaction mix was subjected to AGE [2.2.4.]; the linearized plasmid band was excised and purified using Min Elute Gel extraction kit (50) QIAGEN, Germany.

C) Ligation and transformation

The *Bam*HI-*Hin*dIII digested genomic DNA (80ng) was ligated to similarly digested pUC19 (1µg) in a final ligation reaction volume of 20µl, containing 10U T4 ligase

(Roche) and 1x ligation buffer (Roche). Following incubation over night at 16°C, the ligation mix (5µl) was introduced into 50µl One Shot Top10 competent cells (Invitrogen Life Technologies) using heat shock as described in the manufacture's instructions. To facilitate gene expression, the transformation mix was incubated for one hour at 37°C. After which 100µl aliquots were plated onto 2YT agar containing the appropriate selection (Appendix 1) and incubated at 37°C overnight. White colonies were subcultured onto ampicillin (100µg/ml) and X-gal (100µg/ml X-gal) containing 2YT agar, which were incubated over night at 37°C.

D) Screening for IS_{Aba-1} containing clones

PCR with IS_{Aba-1}-specific primers (HRF & HRR, 2.2.5.2.) was used to screen the white clones for IS_{Aba-1}. For DNA extraction, ten colonies were suspended in 20µl ddH_2O and were boiled for 10 minutes. The cell debris was collected by brief centrifugation. The supernatant contained the DNA for the PCR reaction. All assays included a negative control (no DNA) and positive control, pMERL100 [2.2.3.]. The same PCR conditions used in this experiment were as in section 2.2.5.2., except that the annealing temperature was raised to 56°C. Following amplification, the PCR products were subjected to AGE [2.2.4.]. When necessary, individual IS_{Aba-1}- containing clones were identified by screening the 10 colonies in separate reactions. Colonies producing a PCR-product of the excepted size were cultured overnight in 2YT broth with the appropriate selection and recombinant plasmids were extracted with QIAGEN plasmid midi kit (Germany). The sizes of the inserts were determined following digestion with *Bam*HI and *Hin*dIII [3.2.1.1.] and the DNA fragments were separated on 1% agarose gel and the fluorescence of ethidium bromide was photographed [2.2.4.].

3.2.2. Antibiotic susceptibility testing

When necessary, disk diffusion tests were performed by Iva Shankland (National Health Laboratory Services, Groote Schuur Hospital, Cape Town, South Africa) according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS).

3.2.3. DNA sequencing

DNA sequencing was performed on recombinant plasmids with an ABI PRISM 3100 Genetic Analyser supplied by Applied Biosystems and analyzed with DNA Sequencing Analysis Software Version 3.7 at the Central DNA sequencing Unit in the Department of Genetics, University of Stellenbosch, Stellenbosch, South Africa. Initial sequencing data was generated with universal primers, M13-40F ('5-GTTTTCCCAGTCACGAC-3') and M13R ('5-CAGGAAACAGCTATGAC-3') and thereafter primers were designed to complete the entire sequence. All the primers were synthesized in the Department of Molecular and Cellular Biology, University of Cape Town, South Africa

3.2.4. DNA sequencing analysis

DNA sequence data was edited with software programs such as DNAMAN (version 4.7) and ChromasPro (version 1.21) and analysed with the Basic Local Alignment Search Tool, BLAST (Altschul *et al.*, 1997; http://www.ncbi.nlm.nih.gov/BLAST/Blast) and ORF finder (http://www.ncbi.nih.gov/gorf/gorf.html).

3.2. Results

3.2.1. Antibiotic susceptibility profile and preparation of partial DNA library from strain RAM

A. baumannii strain RAM harbours multiple copies of IS_{Aba-1} (Figure 2.4.) and was resistant to amikacin, cefepine, ceftazidine, chloramphenicol, ciproflex, cotrimoxazole, gentamycin, imipenem, meropenem, pipercillin and tazobactum. Genomic DNA from this strain was digested and ligated to pUC19 and introduced into *E. coli* (One Shot Top10 competent cells, Invitrogen Life Technologies). Approximately 1000 white clones were obtained of which 820 were screened for the presence of IS_{Aba-1} using PCR [2.2.5.2.]. Twelve recombinant plasmids containing IS_{Aba-1} related sequences were identified. Following digestion of the recombinants and AGE [2.2.4.], the sizes of the inserts were estimated to be between 0.75kb and 5.5kb. To determine the genetic environment of the IS_{Aba-1} sequences, the inserts of 10 recombinants were sequenced. Two recombinant plasmids were excluded as they contained inserts that are too small (0.75kb to 0.8kb) to carry both a resistance gene and IS_{Aba-1}. These recombinants were not characterized further.

3.2.2. Analysis of DNA sequencing data

Sequencing data showed that only one of the recombinant plasmids (designated pSUL100) contains IS_{Aba-1} adjacent to an antibiotic resistance gene. Three recombinants contain IS_{Aba-1} adjacent to a variety of genes (transcriptional regulator, putative signal peptide and acintobactin biosynthesis), but these were not investigated further. The remaining 6 recombinant plasmids did not contain IS_{Aba-1} related sequences and an explanation for the positive PCR was not established.

3.2.3. Analysis of the nucleotide sequence of the insert of pSUL100

Based on restriction enzyme digestion with *Bam*HI and *Hin*dIII, pSUL100 contains an insert of 2.29kb which was sequenced on both strands and deposited onto the EMBL database, (http://www.ncbi.nlm.nih.gov/BLAST/Blast), with a GenBank Accession No. AY823412. The nucleotide sequence of 2.29kb *Bam*HI/*Hin*dIII insert is shown in Figure

3.3. The first 230bp showed 100% sequence homology with a portion of ORF2 of IS_{Aba-1} previously described (Segal *et al.*, 2003; Corvec *et al.*, 2003). The region between nucleotides 231 and 945, including ORF1 and the right inverted repeat of IS_{Aba-1}, shows 100% sequence homology to the corresponding region described by Corvec *et al.*, 2003. The sequence derived from strain RAM contains an A instead a G at nucleotides 902 and 933 when aligned with the sequences described by Segal *et al.*, 2004 (Figure 3.3.). Twenty one base pairs separate the right inverted repeat from an open reading frame (816 nucleotides) which contains a *sul2* structural gene that encodes a sulphonamide resistance enzyme, dihydropteroate synthase (DHPS). Downstream of *sul2* (86bp) is another open reading frame (304bp), encoding phosphoglucosamine mutase (GlmM).

Analysis of the sequence (21bp) between the IR_R of IS_{Aba-1} and the ATG start codon of *sul2* showed that this region has 100% homology with the corresponding regions upstream of *sul2* on of several plasmids, including IncQ plasmid, RSF1010 (Rådström & Swedberg, 1988). A putative ribosome binding site (AGGACC) but none of the previously described putative promoters were located within this region (Rådström & Swedberg, 1988; Rådström *et al.*, 1991), suggesting that transcription of *sul2* is dependent on promoter sequences located in IS_{Aba-1}.

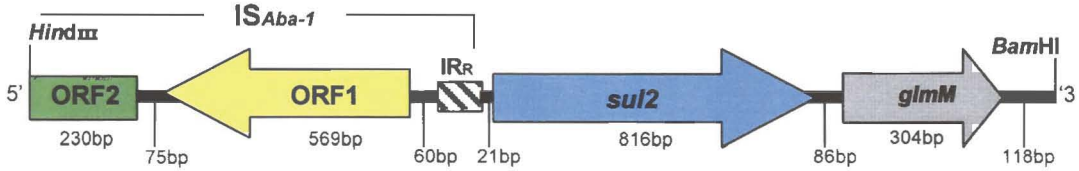


Figure 3.1. The genetic organization of 2.29kb *Hin*dIII-*Bam*HI insert of pSUL100. A) Schematic outline of the insert pSUL100. IS_{Aba-1} sequence on pSUL100 consists of a portion of ORF2 and the complete ORF1 as well as the right inverted repeat (IR_R). The *sul2* is followed by *glmM* gene and the *Bam*HI site marks the 3'-end. The block arrows indicate the direction of transcription of the demarked open reading frames. The thick black line represents the non-coding sequence. Numbers of nucleotide of the ORF's are indicated below the ORF's and the base pairs in-between the non coding area are indicated by the numbers attached to a thin black line. ORF-open reading frame; *sul2*-sulphonamide resistance gene; *glmM*- phosphoglucosamine mutase.

*Hin*dIII

AAGCTTATAGCTAACACCGCAATCAATTTTTTCACTCGTCTAGGGTCTGTCAAGCGCGTATTTTCAAGATTAAACCCGCGTCCTTTGAGACAACTGAATA 100

AGGTTTCAATTTCCCAGCGTAATGCATAATCCTGAATAGCATTGGCATTAAACTGAGGAGAAACGACGAGTAAAAGCTCTCCATTTTCTAACTGTAGTGC 200

ACTTATATATAGTTTCACCCGACCAACCAAAATCCGTCGTTTACGACATTCAATTTGACCAACTTTAAGATGGCGAAATAAATCACTAATTTTATGATTC 300

TTTCCTAAATGATTGGTGACAATGAAGTTTTTTTAACACGAATGCAGAAGTTGATGTCTTGTTCAATTAACCATGTAAACCACTGCTCACCGATAAACTC 400

TCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCAAAGCAATACGCTCTTTCGTATCTGAATTTCCACGTTTA 500

TTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTTTTCCCCATTTCCAATTGGTTCTAT 600

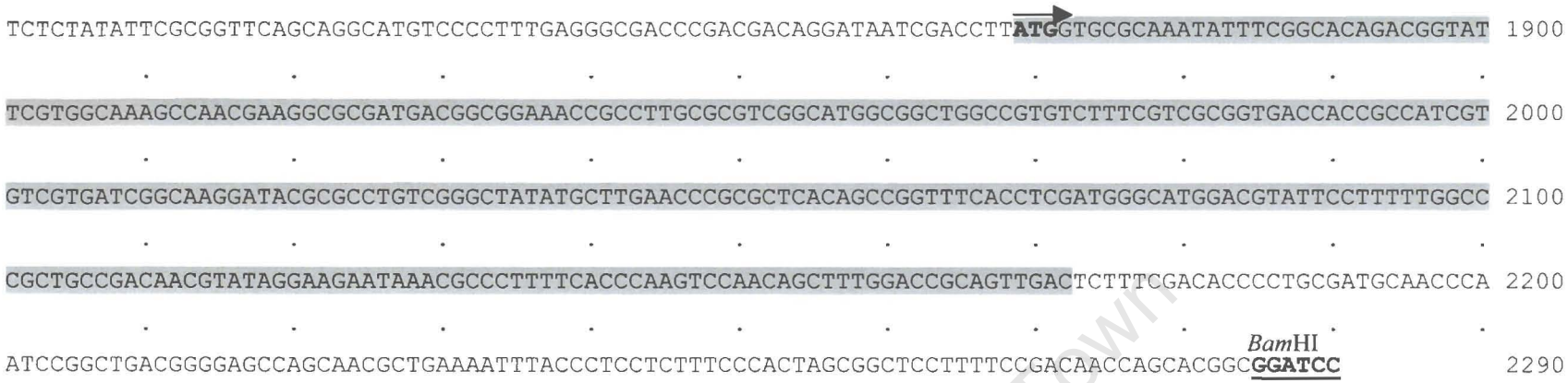


Figure 3.1.B) Nucleotide sequence of the insert of pSUL100. Open reading frames (ORFs) 1 and 2 are the putative transposases OFR 1 and ORF 2 previously identified by Corvec *et al.*, 2003; Segal *et al.*, 2003. Right inverted repeat and putative ribosome binding site (RBS) are underlined with bold line and a thin line, respectively. The remaining 1345bp containing two open reading frame of *sul2* gene and *glmM* gene (highlighted in turquoise and grey, respectively) are identical to the corresponding genes in the complete genome sequence of *Shigella flexneri* serotype 2a strain 2457T (Accession No. AOE 16986.1). The pink blocked sequence highlights the 21bp upstream of the *sul2* gene, which is identified on many plasmids, such as pGS05. The start codons are bold features with an arrow on top indicating the direction of transcription. The amino acid sequence of the sulphonamide resistant DHPS is presented under the nucleotide sequence of *sul2* open reading frame and the stop codon (***) demarks the end of the translation. Nucleotide differences and deletions (-) are indicated on top of the respective nucleotide sequence. The numbers on the right hand side indicate nucleotide bases. Enzyme restriction sites are bold and underlined. The sequence has been deposited onto the EMBL database, (http://www.ncbi.nlm.nih.gov/BLAST/Blast), with a GenBank Accession No. AY823412.

3.4. Discussion

IS_{Aba-1} has been identified adjacent to *ampC*, bla_{OXA-23} and bla_{OXA-27} in *A. baumannii*. Transcription of *ampC* proceeds from promoters within IS_{Aba-1} (Segal *et al.*, 2004) and it is likely that transcription of bla_{OXA-23} and bla_{OXA-27} is similarly dependent (Segal *et al.*, 2003). To better understand the frequency of association of this element with antibiotic resistance genes, strain RAM, which was resistant to many antibiotics [3.2.1.] and contains multiple copies of IS_{Aba-1}, was studied. Only one antibiotic resistance gene (*sul2*) adjacent to IS_{Aba-1} was identified in a partial DNA library prepared from stain RAM and the recombinant plasmid, pSUL100, conferred resistance to sulphamethoxasole (100µg disk, Mastdiscs, Merseyside, UK) on its *E. coli* host. However, this is not an accurate reflection of the association of antibiotic resistance genes with IS_{Aba-1} in this strain, since further studies have identified bla_{OXA-23} and *ampC* adjacent to IS_{Aba-1} in strain RAM (unpublished observations).

The *sul2* gene encodes a sulphonamide resistant dihydropteroate synthetase (DHPS) which is involved in the synthesis of dihydropteroic acid (Sköld, 2000). This gene is one of three plasmid-mediated sulphonamide resistance determinants, which are widely spread amongst gram-negative bacteria (Antunes *et al.*, 2005). The three sulphonamide resistant DHPSs, share 57% amino acid identities which makes them distinct enzymes (Rådström & Swedberg, 1988; Peterren & Boerlin, 2003). The *sul1* gene is most frequently found in class 1 integrons on several plasmids (R1, R100, R6 and R388) and is linked to other resistance genes (Swedberg, 1987), whereas *sul2* is most frequently found on IncQ plasmids (RSF1010) and on other small plasmids (pBP1) as well as on conjugative plasmids such as pGS05 (Huovinen *et al.*, 1995). The third sulphonamide resistant DHPS encoding gene, *sul3*, was identified on a plasmid found in several *E. coli* clonal linages (Perreten & Boerlin, 2003). In 1991, an equal distribution of *sul1* and *sul2* amongst 339 gram-negative bacilli was reported (Rådström *et al.*, 1991); however, in 2002/2003 *sul1* was identified in 76% of gram-negative bacilli, whereas only 37% of the strains contained *sul2* (Antunes *et al.*, 2005). Only 7% of strains contained *sul3* (Antunes *et al.*, 2005). That *sul1* is associated with integrons, which have been responsible for the acquisition and spread

of resistance genes in recent decades (Antunes *et al.*, 2005), probably accounts for the increase in prevalence of this gene.

The genetic environment of *sul2* has been shown to be diverse (Figure 3.2.). The nucleotide sequence (5'-ATTATTATTATAGAAGCCCCC-3') on pSUL100 between the right inverted repeat of IS_{Aba-1} and the start codon of *sul2* gene seems to be conserved. This sequence is the last 21bp of the non coding sequence between *repC* and *sul2* gene on RSF1010 and other *sul2*-carrying plasmids (EMBL database; http://www.ncbi.nlm.nih.gov/BLAST/Blast). The *sul2* gene on pSUL100 is linked to *glmM*, which encodes phosphoglucose mutase (Mengin-Lecreulx & van Heijenoort, 1996). As shown in Figure 3.2.B., a similar combination (*sul2-glmM*) has been identified on various IncQ-like plasmids such as pRSV1 (AJ289135) from *Vibrio salmonicida*, pGS05 (M36657) and a R-plasmid from *Pasteurella pisticidia* (D37825). In addition, the combination has been identified in chromosomal DNA of *Shigella flexneri* 2a str 2457T (AEO016986) as well as on a conjugative transposon SXT^{MO10} (AY055428) from *Vibrio cholerae* (Hochhut *et al.*, 2001).

On some IncQ-like plasmids *sul2* is linked to either a streptomycin resistance determinant (*strAB*) encoding an aminoglycoside phosphotransferase APH-(3") (van Treeck *et al.*, 1981; Rådström & Swedberg, 1988) or to a chloramphenicol resistance gene (*catAIII)-strA* cluster (Kehrenberg & Schwarz, 2001; Ito *et al.*, 2004). On pVM111 a tetracycline resistance gene (*tetR-tet*(H)) was inserted between *sul2* and *strA* genes (Kehrenberg *et al.*, 2003). The identification of these resistance genes suggests that the intergenic region downstream of *sul2* might be recombinationally active and potential recombination sites have been identified (Ito *et al.*, 2004). One of the potential sites (5'-CGCGGTTCAGCAGGCAT-3') is present on pSUL100 at the nucleotides 1811 to 1827 (Figure 3.2.C.). A recently identified trimethoprim resistance gene (*drfA20*) at the sequence ATACGT (inverse complementary: ACGTAT on pSUL100) within *glmM* (Kehrenberg *et al.*, 2005) suggests that this may be an additional recombination site for the acquisition of resistance genes.

It is interesting to speculate on how strain RAM acquired *sul2*. Although IncQ-like plasmids can be transferred to *Acinetobacter*s, they are, by and large, unstable in this

organism (Chopade *et al.*, 1985; Smalla *et al.*, 2000). Therefore, it is possible that IS_{Aba-1} in conjunction with a second copy of this element transferred *sul2* from an IncQ-like plasmid acquired by RAM to the genome of this strain.

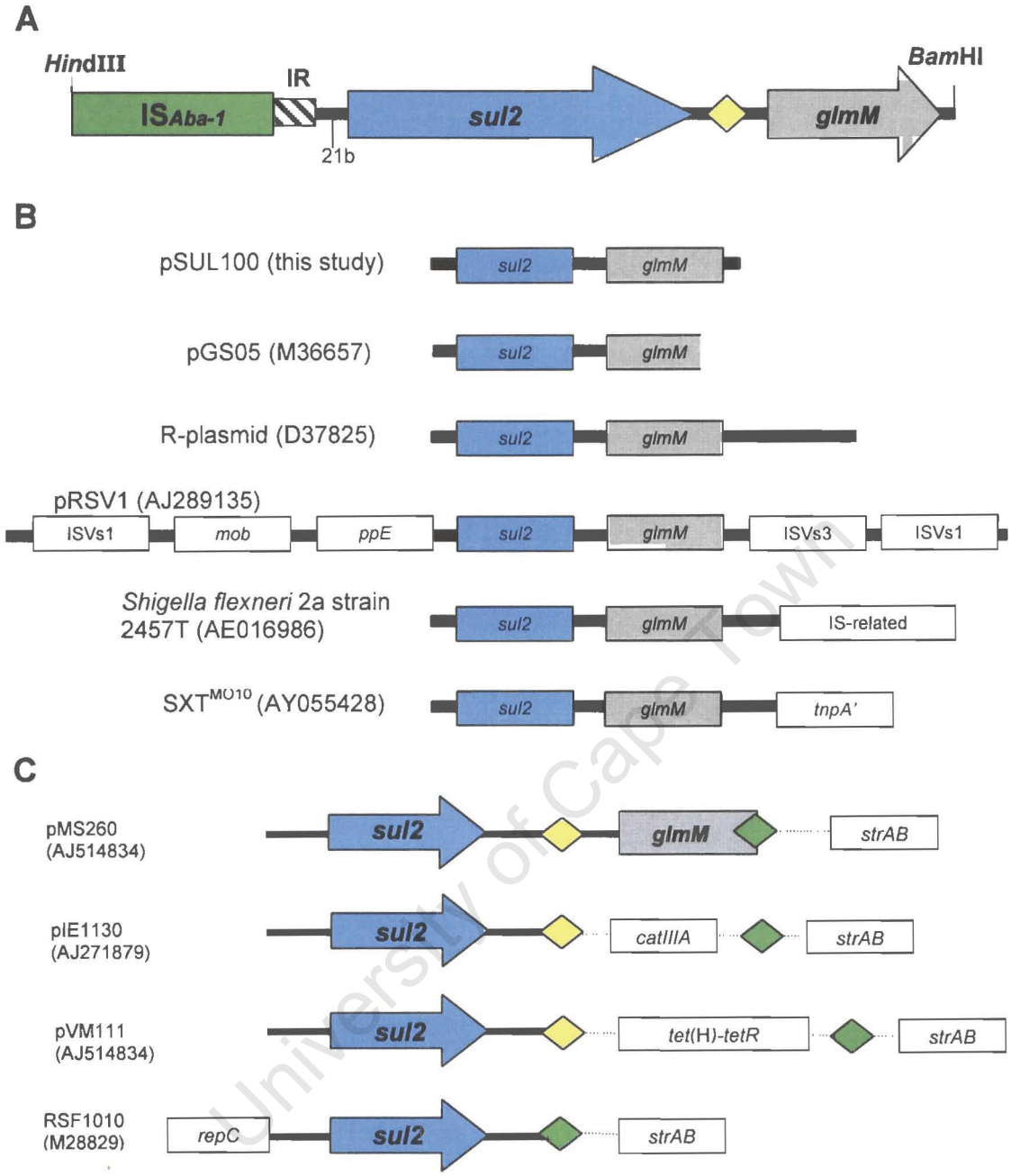


Figure 3.2. Genetic environment of *sul2*.
A) The genetic organization of *sul2* on pSUL100. A portion of IS_{Aba-1} is depicted by a green rectangle and the inverted repeat (IR) by a striped square. Conserved 21bp between IR and *sul2* is depicted by "21bp". ORFs are represented by block arrows and the noncoding sequence by a black bar. The yellow diamond represents a putative recombination site. *Hin*dIII & *Bam*HI-restriction enzyme sites, IS-insertion sequence; *sul2*- sulphonamide resistance gene; *glmM*- phosophoglucose mutase. **B) *sul2-glmM* clusters.** ORFs are indicated in coloured boxes. Non-coding sequences are represented in black bars. Accession numbers are shown in parentheses. *mobC*- mobilization protein gene C, *ppE*- putative protein E, *tnpA'*- truncated transposase. **C) Genetic environment *sul2* on various IncQ-like plasmids**. Putative recombination sites are indicated by yellow and green diamonds. ORF of *sul2* is represented by turquoise arrow and the other ORFs are indicated by rectangles. The dark black bar depicts the noncoding sequence identical to the corresponding sequence on pSUL100 and the dotted line represents nucleotides diverting from the pSUL100 sequence. *repC*- repressor protein C; *strAB*- aminoglycoside resistance gene; *catIIIA*- chloramphenicol resistance gene; *tet*(H)-*tetR*– tetracycline resistance genes.

Chapter 4

The expression of sulphonamide resistance gene, *sul2*, in strain RAM and *E. coli* (pSUL100)

4.1. Introduction

Primer extension studies showed that IS_{Aba-1} provides the transcription signal TTAGAA (-35) N_{16} TTATTT (-10) for the expression of *ampC* in strain RAN (Segal *et al.*, 2004). This is the only study to identify promoter sequences within IS_{Aba-1}. Corvec *et al.* (2003) have suggested that the hyperproduction of AmpC cephalosporinase could be accounted to a strong promoter located within IS_{Aba-1}. This was based on the observation that the ceftazidime MIC for *A. baumannii* containing *ampC* linked to IS_{Aba-1} was higher (≥256mg/L) than the corresponding MIC (≤8mg/L) for strains carrying non IS_{Aba-1} associated *ampC*. Interestingly, the ceftazidime MIC for strain RAN was 16mg/L. Since a number of promoter sequences were identified in IS_{Aba-1} (Segal *et al.*, 2003; Segal *et al.*, 2004) it is possible to speculate that transcription from more than one promoter, or a different promoter, could increase AmpC and MICs of appropriate antibiotics. In this context, hyperproduction of TEM-1 is, at least in part, associated with transcription from more than one promoter (Nelson & Elisha, 2005).

Further, it has been suggested that transcription control signals recognized in *Acinetobacter* are similar but not identical to their counterparts in *E. coli* (Segal & Elisha, 1999). In one study (Segal & Elisha, 1999), the promoter recognized in the expression of an aminoglycoside resistance gene (*aadB*) was different from the corresponding sequences recognized in *E. coli*. Relevant to my study are the experiments carried out by Schembri *et al.* (1995). During the course of their studies on polyhydoxyalkanoic acid biosynthetic genes in *Acinetobacter*, Schembri *et al.* (1995) identified $phaB_{Ac}$ downstream of what was later recognized as IS_{Aba-1} (Segal *et al.*, 2003). That primer extension products were obtained in *E. coli* were not identified in *Acinetobacter* supports the notion that transcription control signals may be different in the two organisms.

This chapter describes experiments carried out to determine the transcription signals recognized in the expression of *sul2* in strain RAM and *E. coli* (pSUL100).

4.2. Experimental protocol

4.2.1. Determination of growth phases of strain RAM and *E. coli* (pSUL100)

An overnight culture was used to inoculate 50ml 2YT broth for the determination of growth phases of each bacterium in the absence of sulfadimidine. The growth of the bacterium was monitored using spectrophotometric analysis (BioMate 5, ThermoElectron Corporation, Cambridge, UK) by recording the optical density (600mn) at 20 minutes intervals. The same procedure was repeated to investigate the growth phases of the two organisms in the presence of 200µg/ml sulfadimidine (Jiangsu Qiaoji Biochem Co., Ltd, China). Mid exponential phase was defined at ½ of OD at stationary phase.

4.2.2. Preparation of RNA

Strain RAM and *E. coli* (pSUL100) were sub-cultured in 2YT broth from an overnight culture (containing 100µg/ml ampicillin for *E. coli* (pSUL100)) and incubated at 37°C. TRIzol Reagent (Invitrogen Life Technologies) was used to extract total RNA from mid exponential phase (3 hours for RAM and 4 hours for *E. coli* (pSUL100) and stationary phase (18 hours) cultures. Bacterial cells were harvested by centrifugation and resuspended in 1ml TRIzol reagent. The lysation reaction was allowed to proceed for 5 minutes at room temperature (RT) and to optimise denaturing of cellular proteins 200µl chloroform was added. To mix the reagents thoroughly the Eppendorfs were shaken vigorously for 15 seconds and were subsequently incubated at RT for 3 minutes. Phase separation was achieved by centrifugation at 12000xg at 4°C for 10 minutes. The supernatant containing RNA was transferred to a fresh Eppendorf and 500µl isopropanol was used to precipitate the RNA. The ribonucleic acids were taken out of solution by centrifugal force at 12000xg at 4°C for 10 minutes. The RNA pellet was washed with 75% ethanol, mixed, briefly vortexed and collected again by centrifugation at 7500xg at 4°C for 5 minutes. To re-dissolve RNA, the pellet was briefly air-dried, resuspended in 20µl DEPC-treated ddH_2O and incubated at 55°C for 10 minutes. RNA was quantified by using spectrophotometric analysis (BioMate 5, ThermoElectron Corporation, Cambridge, UK) with an ultraviolet source at 280nm considering 1 unit OD equal to 40µg/ml RNA. The RNA was stored at -70°C until further investigations.

4.2.3. Determination of transcription start site: primer extension analysis

A primer (5'-CGAGGTCGATCACATCTG-3') that annealed 124bp downstream of *sul2* start codon was designed to generate cDNA transcripts. This primer was synthesized and labeled with indodicarbocyanine, Cy5, in the Department of Molecular and Cellular Biology, University of Cape Town, South Africa. RNA (100µg) and 5pmol Cy5-labeled primer was precipitated at -20°C for 30 minutes in the presence of 0.3M sodium acetate and 100% ethanol (Sambrook *et al.*, 1989). RNA-primer precipitate was collected by centrifugation at 12000xg for 10 minutes at 4°C and washed with 70% ethanol. The pellet was recollected as before and air-dried briefly before it was re-suspended in 30µl hybridization buffer (40mM Pipes, 1mM EDTA, 0.4M NaCl and 80% formamide and DEPC-treated ddH_2O). An initial denaturing step at 85°C of RNA for 10 minutes was followed by the annealing of the primer to RNA at 58°C over night. The RNA-primer complex was precipitated (as above) and air-dried. For cDNA synthesis, nucleic acid pellet was re-dissolved in 20µl reverse transcriptase buffer, which composed 5x M-MLV Reverse Transcriptase buffer (Promega), 10mM dNTPs (TaKaRa), 1mg/ml Acintomycin D (Sigma), 40U protector RNA inhibitor (Roche), 400U M-MLV RTase (Promega) and DEPC-treated ddH_2O. The reaction was performed at 42°C for 2 hours and stopped by adding 1µl EDTA (0.5M, pH 8). To remove remaining RNA, DNase free pancreatic RNase (Roche) was added (1µl of 0.5mg/ml) and incubated at 37°C for 30 minutes. Enzymatic impurities were removed by adding 150µl of TE (pH 7.6), 3µl 5M NaCl and 200µl phenol: chloroform (25:25). The organic phases and aqueous phase were mixed by brief vortexing and were separated by centrifugation at 12000xg for 5 minutes at RT. The cDNA transcript containing supernatant was transferred to fresh Eppendorf and precipitated with 100% ethanol at 0°C for 1 hour. The precipitate was collected by centrifugation, washed with 70% ethanol and reconstituted in 5µl DEPC-treated ddH_2O. The primer extension product was further analysed by Di James in the Department of Molecular and Cellular Biology, University of Cape Town, South Africa. ALFwin (Version 1.10, Pharmacia Biotech) was used to visualize the sequencing reaction and the primer extension product corresponding to the products of the appropriate sequencing reaction.

4.3. Results

4.3.1. Identification of transcription start site and promoter of *sul2* in strain RAM

No primer extension product was obtained with RNA from mid-exponential phase cultures of strain RAM. However, a product was obtained when RNA from mid-exponential phase cultures of strain RAM grown in the presence of sulfadimidine was used in the primer extension studies. The extension product mapped to an A located 48 nucleotides (start site A) upstream of the *sul2* start codon (Figure 4.1.B.). The hexamers designated $P_{70/S}$, TTAGAA (-35) and TTATTT (-10) separated by 16bp, are located upstream of the transcription start site A. These hexamers, showing similarity to the consensus sequences recognized by $E\sigma^{70}$ polymerase (Harley & Reynolds, 1987), were suggested to be the putative promoter for *ampC* in strain RAN (Segal *et al.*, 2004). The sequence immediately upstream of the -10 hexamer (TGACA) generates an extended -10 hexamer (TGACATTATTT) which shows similarity to sequences recognized by $E\sigma^{S}$ (Hengge-Aronis, 2002; Lacour & Landini, 2004). Using RNA from stationary phase cultures of strain RAM in the absence of sulfadimidine, the primer extension product mapped to a T, 50 nucleotides upstream of the *sul2* start codon (start site B; Figure 4.1.C.). Since the primer extension products mapped to nucleotides separated by 1 base, the promoter sequences ($P_{70/S}$) previously identified are also associated with this start site (Figure 4.1.D.).

4.3.2. Identification of transcription start site and promoter of *sul2* in *E. coli* (pSUL100)

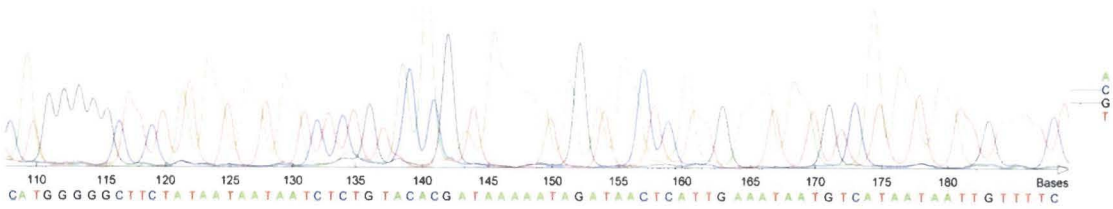
To test whether transcription signals recognized in *Acinetobacter* are different from their counterparts in *E. coli*, primer extension studies were carried using RNA isolated for *E. coli* (pSUL100). Using the RNA from mid exponential phase cultures of *E. coli* (pSUL100) in the absence of sulfadimidine, 8 transcriptional start sites were identified. They were designated 1 to 8 and mapped to T, G, A, A, T, T, T and A at 17bp, 22bp, 25bp, 40bp, 50bp, 54bp, 59bp, and 62bp, respectively, from the *sul2* start codon (Figure 4.2.B.). Based on the size of the peak, the transcript which accords to 5 (Figure 4.2.B.) was the major product and corresponds to the transcriptional start site (B) in strain RAM (Figure 4.1.C.).

Similarly, using RNA extracted from *E. coli* (pSUL100) mid-exponential phase cultures in the presence of sulfadimidine, a number of primer extension products (6) were obtained, which mapped to the previously described nucleotides (1, 2, 5, 6, 7 and 8). Primer extension products which mapped to nucleotides denoted 3 and 4 were absent (Figure 4.2.C.). In this experiment transcripts corresponding to 2 and 5 were the major products based on the size of the peaks.

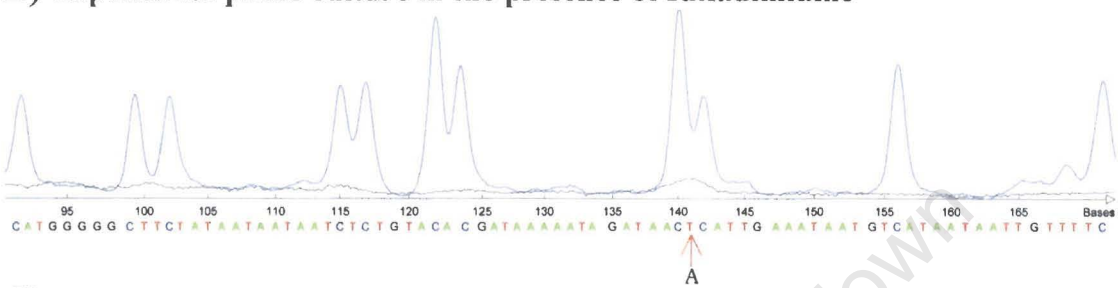
When RNA from stationary phase cultures of *E. coli* (pSUL100) grown in the absence of sulfadimidine was used in the experiment only one primer extension product, which mapped to a previously identified T (B in Figure 4.1.D. and 5 in Figure 4.2.D.), was located 50bp upstream of the *sul2* ATG start codon.

Analysis of the DNA sequence upstream of the start sites identified three promoter sequence within IS_{Aba-1} with similarity to the *E. coli* σ^{70} consensus, TTGACA (-35) $N_{16/17}$ TATAAT (-10) (Harleys & Reynolds, 1987). First, the denoted P_1 promoter, ATGACA (-35) N_{18} TATTTT (-10) was identified upstream of start sites 1, 2, and 3 (Figure 4.2.E.). The putative promoter ($P_{70/S}$) identified in the transcription of *sul2* in strain RAM is located upstream of start sites 4 and 5. A putative -10 hexamer (TATTAT) precedes start sites 6, 7 and 8. In relation to this hexamer, no obvious -35 sequence is apparent.

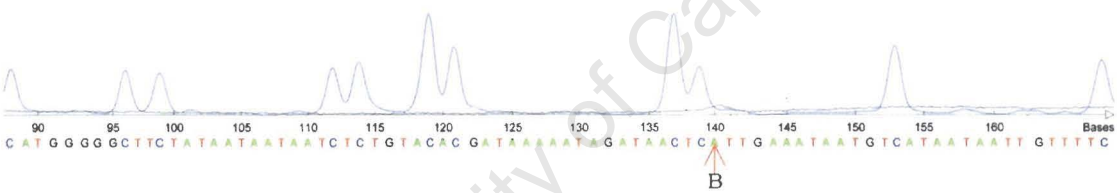
A) Sequence reaction



B) Exponential phase culture in the presence of sulfadimidine



C) Stationary phase culture in the absence of sulfadimidine



D)

TAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCAAAGC

CAP UP element

ACTTTAAATGTGACTTGTTCCATTTTAGATATTTGTTTAAGATAAGATATAACTCATTGAGAT

$P_{70/S}$ -35 $P_{70/S}$ -10

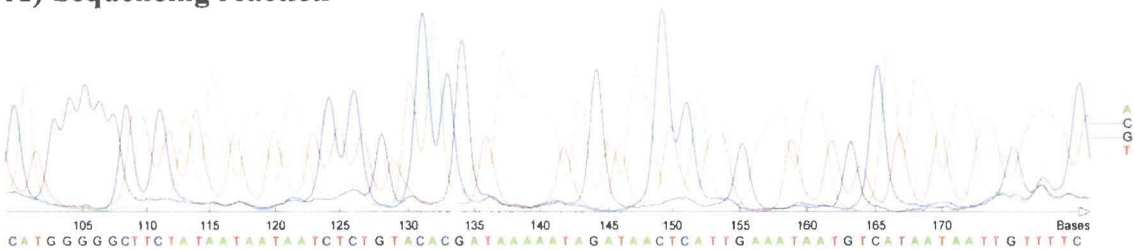
GTGT***CAT***AGTATTCGTCGTTAGAAAACAATTATTATGACATTATTTCAATGAGTTATCTATTT

B A

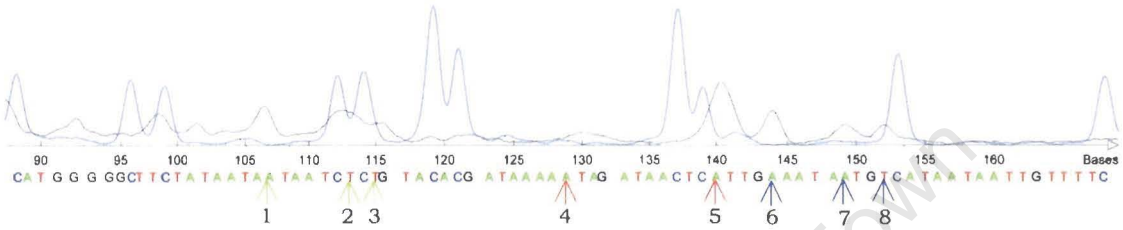
TTATCGTGTACAGAGATTATTATTAT**AGAAGCC**CCC***ATG***

Figure 4.1. Mapping of *sul2* transcription start site of strain RAM by primer extension analysis. A) DNA sequence of template strand 78bp upstream of *sul2* start codon, CAT. **B)** C sequencing reaction of strain RAM and the primer extension product (A) which was obtained mid exponential phase culture in the presence of sulfadimidine. **C)** C sequencing reaction of strain RAM and the primer extension product (B) which was obtained from stationary phase culture in the absence of sulfadimidine. **D)** Schematic representation of data shown in **B** and **C** on the nucleotide sequence (225bp) upstream *sul2* initiation codon. Putative ribosome binding site is bold and the right inverted repeat is underlined. Transcription start sites (A & B) mapped to nucleotides (red) and the promoter sequence similar to $E\sigma^{70}$ (red box). The $E\sigma^{S}$ recognized sequence is in the dotted red box. The start codon of open reading frames (*sul2* and transposase) are bold and italicised. Horizontal arrows indicate the direction of transcription. Yellow shaded boxes mark putative sequences of CAP (Segal *et al.*, 2003) and putative distal UP-element half-site (Germer *et al.*, 2001).

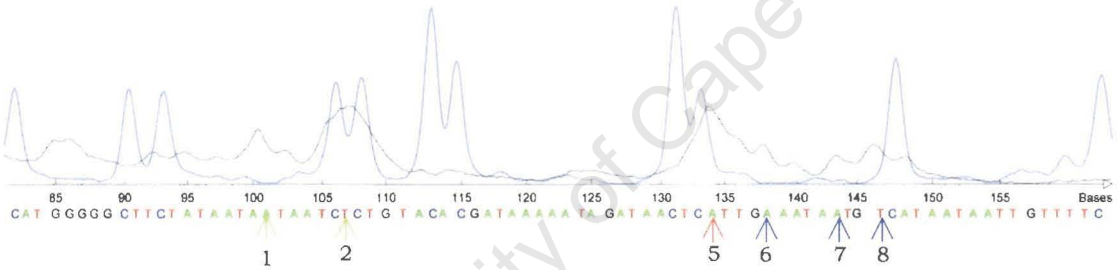
A) Sequencing reaction



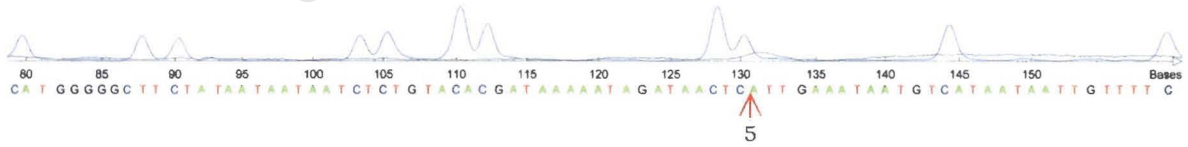
B) Exponential phase culture in the absence of sulfadimidine



C) Exponential phase culture in the presence of sulfadimidine



D) Stationary phase culture in the absence of sulfadimidine



E)

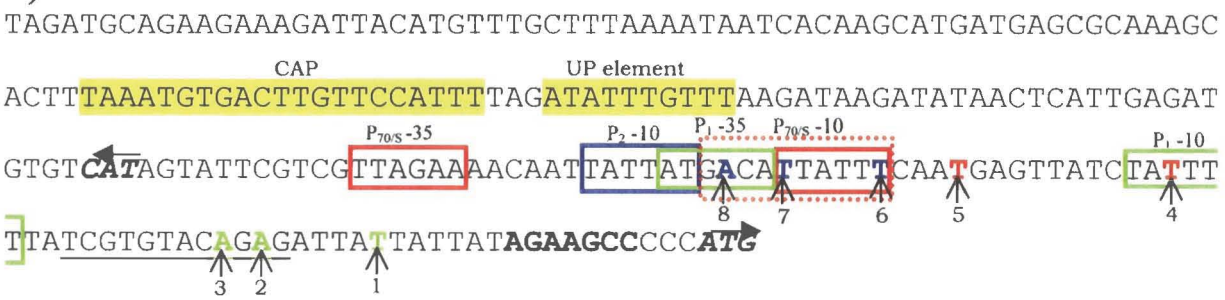


Figure 4.2. Mapping of *sul2* transcription start site in *E. coli* (pSUL100) by primer extension analysis. A) DNA sequence of template strand 78bp upstream of *sul2* start codon, CAT. **B)** C sequencing reaction of *E. coli* (pSUL100) and 8 primer extension products (indicated by vertical arrows) from mid exponential phase cultures of *E. coli* (pSUL100) in the absence of sulfadimidine. **C)** C sequencing reaction of *E. coli* (pSUL100) and 6 primer extension products obtained from mid exponential phase cultures of *E. coli* (pSUL100) in the presence of sulfadimidine. **D)** C sequencing reaction of *E. coli* (pSUL100) and the primer extension product obtained from stationary phase cultures of *E. coli* (pSUL100) in the absence of sulfadimidine. **E)** Schematic representation of data shown in **B** to **D** on the nucleotide sequence (225bp) upstream *sul2* start codon. Putative ribosome binding site is bold and the right inverted repeat is underlined. Transcription start sites mapped to nucleotides (1-8) and the corresponding promoter sequence are boxed with matching colour. The start codons of open reading frames (*sul2* and ORF1) are bold and italicised. Horizontal arrows indicate the direction of transcription. Yellow shaded boxes mark putative sequences of CAP (Segal *et al.*, 2003) and distal UP-element half-site (Germer *et al.*, 2001).

4.4. Discussion

Transcripts of *sul2* in exponential phase cultures of strain RAM were not obtained when the organism was grown in the absence of sulfadimidine. This accords with a previous study which showed that *ampC* transcripts were obtained only when *A. baumannii* strain RAN was cultured in presence of ampicillin (Segal *et al.*, 2004).

Single cDNA products were obtained from mid-exponential phase cultures and stationary phase cultures of strain RAM grown in the presence and absence of sulfadimidine, respectively. IS_{Aba-1} located promoter sequences, TTAGAA (-35) N_{16} TTATTT (-10), with characteristic features recognized by $E\sigma^{70}$ precede the start sites, which differed by one nucleotide (Figure 4.1.). This promoter was suggested to drive transcription of *ampC* in *A. baumannii* strain RAN (Segal *et al.*, 2004) and the constitutive expression of pho_{AC} in a unnamed *Acinetobacter* specie (Schembri *et al.*, 1995). A feature of the promoter is that it contains an extended -10 hexamer, showing similarity to sequences recognized by the stationary phase related sigma factor, σ^S (Hengge-Aronis, 2002; Lacour & Landini, 2004; Weber *et al.*, 2005). In this respect, the putative promoter of *sul2* is similar to that described in the transcription of *ampC* in *Vibrio fischeri* (Weng *et al.*, 2004). Several appropriately located sequences, such as the A/T rich sequence upstream of the extended -10 hexamer (Figure 4.1. and Figure 4.2.), a putative distal UP element half-site (TATTTGTTT) and a CRP box (TAAATGTGACTTGTTCCATTTTA), which could facilitate σ^S transcription (Germer *et al.*, 2001; Hengge-Aronis, 2002; Lacour & Landini, 2004; Weber *et al.*, 2005), were identified in IS_{Aba-1}. Considering, the similarity between $E\sigma^S$ and $E\sigma^{70}$ recognized promoters (Hengge-Aronis, 2002; Lacour & Landini, 2004) and that σ^{70} concentration decreases during transition to stationary phase (Nyström, 2004; Weber *et al.*, 2005), it is possible that transcription in the initial growth phase involved σ^{70} and that σ^S was responsible for the expression of *sul2* in stationary phase cultures of strain RAM.

Primer extension studies indicated that transcription of *sul2* in stationary phase cultures of *E. coli* (pSUL100) grown in the absence of sulfadimidine was similar to that observed in strain RAM. One cDNA product was obtained and the putative promoter is identical to the one identified in strain RAM (Figure 4.1. and Figure 4.2.). In the remaining cultures (mid exponential with and without selection) transcription of *sul2* in *E. coli* (pSUL100) was not the same as in strain RAM. In *E. coli* (pSUL100) multiple transcripts, proceeding from three putative promoters, were obtained. Similar findings were observed by Schembri *et al.* (1995); they identified primer extension products in *E. coli* that were not apparent in *Acinetobacter*. It has been suggested that the counterpart of σ^{70} is present in *Acinetobacter*, but that the association between this σ factor and the core enzyme is weak (Kleppe & Kleppe, 1976). This could be one explanation for the reduced promoter activity in strain RAM and the more frequent docking of σ^{70} at promoter sequences in *E. coli*.

Chapter 5

Summary and Conclusions

The focus of this study was IS_{Aba-1}, which was recently identified in *Acinetobacter baumannii* strain (Segal *et al.*, 2003; Corvec *et al.*, 2003). To investigate the distribution of this novel IS element several clinical isolates were screened for IS_{Aba-1}. Sequences hybridizing to the IS_{Aba-1} probe were identified in some, but not all of the *A. baumannii* strains and *A. lwoffii* strains investigated. IS_{Aba-1} was not identified in *A. calcoaceticus*, *E. coli*, *Klebsiella spp.*, *E. cloacae* and *P. aeruginosa.* Although this was a small study IS_{Aba-1} may be unique to some *Acinetobacter* species. As IS_{Aba-1} was identified in *A. baumannii* strains isolated in different hospitals indicates that this element is not confined to strains in a particular hospital.

Hybridization experiments were conducted to investigate the frequency of IS_{Aba-1} in the *A. baumannii* and *A. lwoffii* strains containing this element. The copy number of IS_{Aba-1} ranged from 6 to 19 copies, indicating the mobility of this IS element within the genome. Interestingly, the more recent isolates had more copies which might be a reflection of evolutionary bottlenecks (Nagy & Chandler, 2004).

IS_{Aba-1} was located upstream of several β-lactamase resistance genes, bla_{OXA23}, bla_{OXA27} and *ampC* (Donald *et al.*, 2000; Afzal-Shah *et al.*, 2001; Segal *et al.*, 2003; Corvec *et al.*, 2003), and to investigate its association with antibiotic resistance determinants of the other families, a partial genomic library was established from one *A. baumannii* strain (RAM) with multiple copies of IS_{Aba-1}. Several clones were identified which were positive for IS_{Aba-1}, but only one clone contained the IS element upstream of a resistance gene, which was identified as *sul2* and conferred resistance to sulphonamides. The plasmid was designated pSUL100. This is not a true reflection of the association of IS_{Aba-1} with antibiotic resistance genes in this strain as other genes have been identified downstream of this IS element since this study was completed.

Previous studies have identified several putative promoters located in IS_{Aba-1} (Segal *et al.*, 2004). Primer extension experiments were carried out to obtain the transcription start sites of *sul2* in strain RAM. Transcriptional products were obtained only when strain RAM was

cultured in the presence of sulfadimdine (mid-exponential) or from stationary phase cultures in the absence of selection. cDNA products were not obtained in exponential phase cultures of RAM in the absence of sulfadimidine. *sul2* was expressed from a promoter, TTAGAA (-35) N_{16} TTATTT (-10), which was suggested to drive transcription of *ampC* in *A. baumannii* (Segal *et al.*, 2004). The sequence upstream of the -10 hexamer shows features of an extended -10 hexamer which is selective for sigma S powered transcription in stationary phase cultures (Hengge-Aronis, 2002; Lacour & Landini, 2004; Weber *et al.*, 2005).

It has been suggested that transcription signals recognized in *Acinetobacter* are similar but may be different from their counterparts in *E. coli* (Segal & Elisha, 1999). Therefore primer extension studies were also carried out in *E. coli* (pSUL100). Expression of *sul2* in *E. coli* was independent of selection and growth phase. Expression of this gene was driven from three different promoters during exponential phase in both the presence and absence of sulfadimdine. However, the identification of one transcript from stationary phase cultures indicated that only one promoter drives expression during this phase. Interestingly, the promoter was the same as the one recognized in the transcription *sul2* in strain RAM. It is possible that in both organisms σ^S drives transcription in stationary phase cultures.

In conclusion, IS_{Aba-1} was in some *Acinetobacter* strains with most strains containing many copies of the element. This IS element was associated with a sulphonamide resistance gene (*sul2*) in one *A. baumannii* strain studied and transcription of this gene was dependent on a promoter within IS_{Aba-1}. Transcription of *sul2* cloned in *E. coli* was increased and different to that observed in *A. baumannii*. As most of the promoters in IS_{Aba-1} show similarity to $E\sigma^{70}$ sequences, the reduced promoter activity in *A. baumannii* may be due to the weak association of σ^{70} with the core enzyme in this organism (Kleppe & Kleppe, 1976).

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APPENDIX 1: MEDIA, BUFFERS AND SOLUTIONS

Media

Note: volumes were adjusted with ddH_2O, if not indicated otherwise

2x Yeast-Tryptone broth (2YT) (1l)
16g tryptone
10g yeast extract
5g NaCl
15g agar (for agar)
autoclaved

Solutions and Buffers

Note volumes are adjusted with ddH_2O, if not indicated otherwise. Final concentration is indicated in parenthesis

CTAB/NaCl (100ml)
4.1g NaCl (0.7M)
10g CTAB (10%)

Denaturing solution (1l)
87.66g NaCl (1.5M)
20.0g NaOH (0.5M)

Depurination solution (1l)
250mM HCl

Gel tracking dye (x6) (10ml)
25mg Bromophenol blue (0.25% w/v)
4.0g sucrose (40% w/v)
0.4ml 0.5M EDTA (20mM, pH 8)

Hybridization buffer (30µl) for Primary Extension
66µl 0.6M Pipes(40mM)
0.2µl 0.5M EDTA (1mM)
80µl 5M NaCl (0.4M)
800µl formamide (80%)
53.8µl DEPC-treated ddH_2O
made up fresh for each reaction

Hybridization buffer (60ml) for Southern Blotting
0.175g NaCl (0.5M)
3.0g ECL blocking agent (5% w/v)

Neutralization solution (1l)
87.66g NaCl (1.5M)
0.5M Tris-HCl

Phenol (500ml)
0.5g 8-hydroxyquinoline
500ml 50mM Tris-base
500ml 50mM Tris-Cl (pH 8)

Phenol/chloroform/isoamylalcohol
mixed the 3 compounds to in a ratio of 25:24:1

Primary washing buffer (1l)
4g SDS (0.4%)
25ml 20x SSC(0.5x SSC)

Reverse Transcriptase buffer (20µl) for Primer Extension
4µl RT buffer (Promega)
1µl 10mM dNTPs (TaKaRa)
2µl 1mg/ml Acintomycin D (Sigma)
1µl 40U protector RNA inhibitor (Roche)
400U M-MLV RTase (Promega)
12µl DEPC-treated ddH_2O

Secondary washing buffer (1l)
100ml 20x SSC (2x SSC)

Selection for transformed colonies
2YT agar containing:
100µg/ml ampicillin
100µg/ml IPTG
100µg/ml X-gal

20x SSC (1l)
88.23g Na_3Citrate (0.3M)
175.32g NaCl (3M)

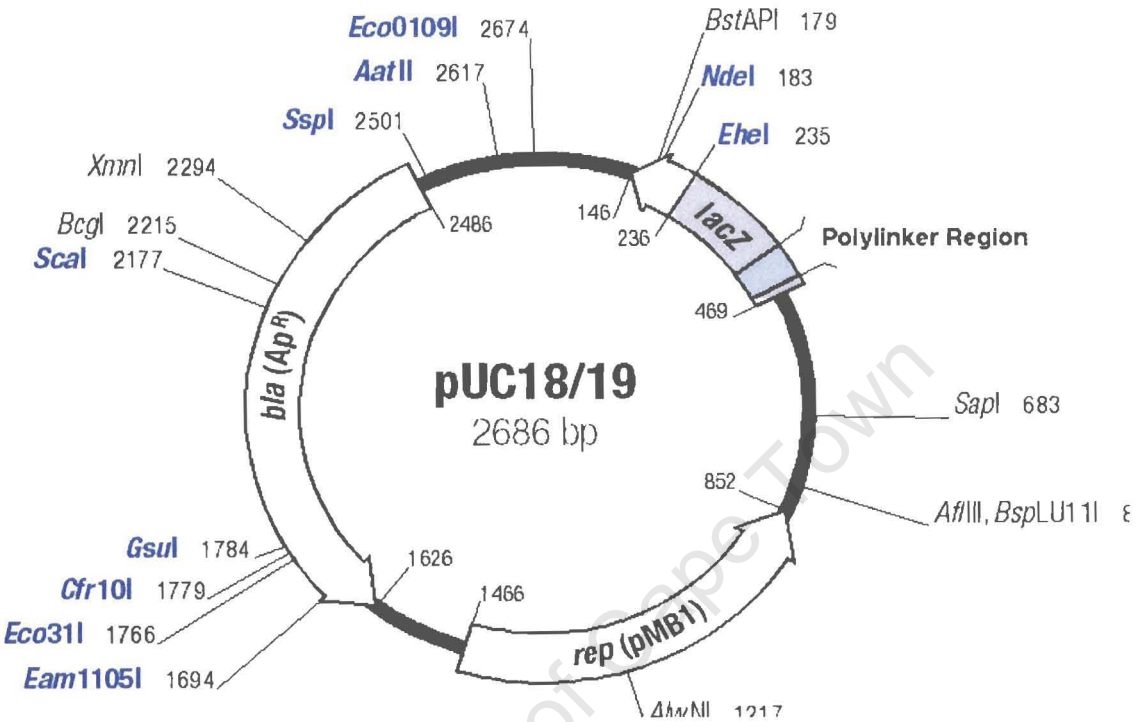
Tris-Cl (100ml)
12.1g Tris-Cl
pH 7.6

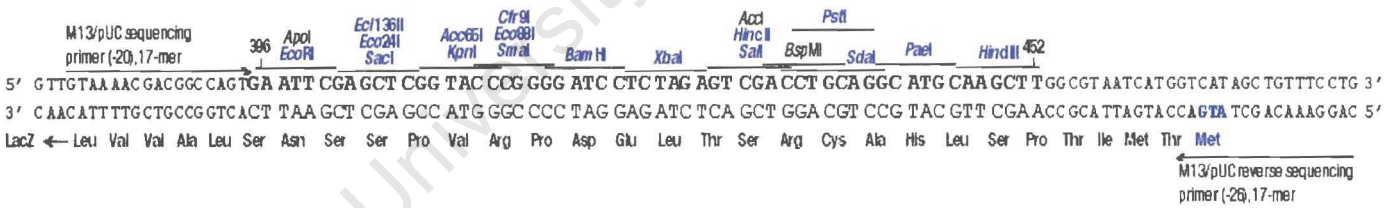
Tris-EDTA buffer (stock solution)
10mM Tris
1M M EDTA
pH 7.6

Tris-acetate-EDTA (TAE) buffer (50x) (1l)
242g Tris
18.6g EDTA
pH 8 – was adjusted with glacial acetic acid

X-gal (2ml)
40mg Xgal dissolved in 1ml DMSO
1ml ddH2O
Stored at -20°C

APPENDIX 2





Source of information:
http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm
GenBank/EMBL accession number L09137