INTEGRONS AND INTEGRON-RELATED ANTIBIOTIC RESISTANCE IN *ACINETOBACTER*

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Dedicated with love to my parents,
Charles and Sandra Thomas

DECLARATION

I, **ROBIN THOMAS**, hereby declare that the work on which this thesis is based is my original work (except where acknowlegdements 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

Acinetobacter baumannii is responsible for an increasing number of nosocomial infections in patients receiving intensive care and comprehensive antibiotic resistance of these organisms hampers treatment of infections due to *A.baumannii*. The molecular basis of antibiotic resistance in *A.baumannii* has not been extensively investigated. A few studies have demonstrated the role of plasmids and transposons in resistance in this organism, but there is little data on the role of integrons and integron-associated antibiotic resistance. This study was undertaken to determine the incidence of integrons in clinical isolates of *Acinetobacter* from Groote Schuur Hospital (GSH), Cape Town and Universitas Hospital (UH), Bloemfontein, and to characterise the resistance genes carried in the variable regions of these integrons.

Fifty-seven clinical isolates of *A.baumannii* were studied, the majority of which were multi-resistant. DNA-DNA hybridisation studies showed the presence of integrons in 63.2% (36/57) of the isolates (53.5% of the GSH isolates and 92.9% of the UH isolates). A polymerase chain reaction assay using primers directed at the 5`-conserved region and 3`-conserved region of class 1-type integrons was performed on genomic DNA from the 36 isolates. Twenty-four of these isolates yielded products. Products of 0.65kb, 0.7kb, 2.0kb, and 2.5kb were obtained. The 0.7kb and 2.0kb products were investigated further.

DNA sequence analysis of the 0.7kb product from one strain (*A.baumannii* strain CAR) showed that it carried an integron-related *dfrVII* gene, encoding resistance to trimethoprim. DNA-DNA hybridisation studies showed that all the 0.7kb products contained *dfrVII* sequences, suggesting that 22 of the isolates carried integron-associated trimethoprim resistance. Further DNA sequence analysis, of the regions flanking the *dfrVII* from strain CAR, suggested that it is carried in an integron similar to the one described for Tn*5086*, a Tn*21*-like integron containing a *dfrVII* cassette.

The two 2kb products were cloned into pUC19 and partially sequenced. *A.baumannii* strain G37 carried an *ant(2``)-Ia* (or *aadB*) gene cassette

(encoding AAD(2``)) and a putative cassette in the variable region of a class 1-type integron. The 5`-conserved region upstream of the *ant(2``)-Ia* and the 59 base element associated with the putative cassette were both atypical. *A.baumannii* strain G39 carried a putative gene cassette, *orfX*, and an *ant(3``)-Ia* (or *aadA* encoding AAD(3``)). The *ant(3``)-Ia* was also associated with an atypical 59 base element. The region upstream of *orfX* did not contain sequences related to the 5`-conserved region as expected. Instead, the 16bp of data contained a 14bp inverted repeat sequence.

Partial DNA sequence analysis of an integrase-related 6.5kb *Hin*dIII fragment from a multi-resistant *A.baumannii* isolate, strain PAU, revealed that it did not carry an integron as was expected. The fragment carried a truncated integrase gene which had been interrupted by an insertion sequence, IS*15-*⊿II. The insertion of this element also deleted the variable region and 3`-conserved region of the integron. Downstream of the truncated integrase gene was the *tnpM* gene (encoding the Tn*21* modulator protein) and the 5`-end of the *tnpR* gene (encoding the Tn*21* resolvase protein). Upstream of the insertion sequence and Tn*21*-related genes, was an *aac(3)-IIa* (or *aacC2*) encoding an AAC(3)-II aminoglycoside resistance gene. This gene was flanked by a portion of an insertion sequence, IS*1133* (on the 5`-end) and an ORF, the product of which has not been identified (on the 3`-end).

ABBREVIATIONS

59-be	59 base element
%	percent
AAC	aminoglycoside acetyltransferase
AAD	aminoglycoside adenyltransferase
ACE	*Acinetobacter* chromosomal enzyme
AGE	agarose gel electrophoresis
APH	aminoglycoside phosphotransferase
APS	ammonium persulphate
AME	aminoglycoside modifying enzyme
ANT	aminoglycoside nucleotidyltransferase
bp	base pair(s)
c	centi
^0C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CAP	catabolite activator protein
CTAB	Hexadecyltrimethylammonium bromide
ddATP/dATP/A	dideoxy/deoxyadenosine triphosphate
ddCTP/dCTP/C	dideoxy/deoxycytidine triphosphate
ddGTP/dGTP/G	dideoxy/deoxyguanosine triphosphate
ddNTP/dNTP/N	dideoxy/deoxynucleoside triphosphate
ddTTP/dTTP/T	dideoxy/deoxythymidine triphosphate
dH_2O	distilled water
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ESBL	extended spectrum β-lactamase
EtBr	ethidium bromide
GSH	Groote Schuur Hospital
ICU	intensive care unit

IPTG	isopropyl-β-D-thio-galactosidase
IS	insertion sequence
kb	kilobase
-l	litres
-m	metres
m	milli
M	molar
MCS	multiple cloning site
MD	megadaltons
MIC	minimum inhibitory concentration
mol%	mole percentage
n	nano
N	normal
NNB	Tris-Borate-EDTA
OD	optical density
ORF	open reading frame
p	pico
PBP	penicillin binding protein
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
spp	species
SSC	standard sodium citrate
TE	Tris-EDTA
TEMED	N,N,N`,N` tetramethyl ethylenediamine
Tn	transposon
μ	micro
U	units

UCT	University of Cape Town
UH	Universitas Hospital
UTI	urinary tract infection
UV	ultraviolet
V	volts
VCR	*Vibrio cholerae* repeated sequences
V.cholerae	*Vibrio cholerae*
v/v	volume per volume
W	watts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
YT	yeast tryptone

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CHAPTER 1: INTRODUCTION

1.A. General Description of the Genus

The genus *Acinetobacter* (Brisou and Prévôt, 1954) consists of non-motile, Gram-negative coccobacilli (Henriksen, 1973). These ubiquitous saprophytes are non-fastidious and are thus able to survive in relatively poor conditions, for example, in dry conditions (Bergogne-Bérézin, *et al.,* 1996). Acinetobacters are non-fermentative, strictly aerobic and grow at temperatures ranging from 20^0C to 37^0C (Bergogne-Bérézin and Towner, 1996). All members of the genus are oxidase-negative and catalase-positive (Bergogne-Bérézin and Towner, 1996).

In the last two decades, *Acinetobacter* spp., particularly *A.baumannii*, have emerged as significant nosocomial pathogens and have been implicated in the infection of patients, particularly those receiving intensive care (Bergogne-Bérézin and Towner, 1996). Recent investigations have shown that acinetobacters are responsible for an average of 5% of infections in certain hospitals (Bergogne-Bérézin, 1996). Nine to ten percent of nosocomial pneumonia can be attributed to acinetobacter (Struelens *et al.*, 1993). A problem with infection due to *Acinetobacter* is that treatment can be difficult due to the comprehensive resistance of these organisms and combination therapy is usually recommended (Bergogne-Bérézin and Towner, 1996).

1.B. Taxonomy

Acinetobacter has been through many taxonomic changes. Over the years they have been classified in various genera, including *Bacterium anitratum* (Schaub and Hauber, 1948), *Herella vaginicola, Mima polymorpha* (De Bord, 1939), *Achromobacter, Alcaligenes, Neisseria, Micrococcus calcoaceticus, Diplococcus,* 'B5W', and *Cytophaga* (Juni, 1972). The original concept (Brisou and Prévôt, 1954) of the genus included non-motile, Gram-negative, oxidase-negative and -positive saprophytes which lacked pigmentation. In 1971, the Subcommittee on the Taxonomy of Moraxella and Allied Bacteria proposed that the genus include only oxidase-negative strains (Lessel, 1971).

In Bergey's Manual of Systematic Bacteriology, the genus *Acinetobacter* was classified in the family *Neisseriaceae* (Juni, 1984) and consisted of one species, *Acinetobacter calcoacticus*, and two varieties, var *anitratus* (formerly *Herellea vaginicola*) and var *lwoffii* (formerly *Mima polymorpha*). In 1986, Bouvet and Grimont defined the *Acinetobacter* genomic species and divided them into their current classification. Rossau, *et. al.* (1991) proposed that members of the genus be classified in a new family, *Moraxellaceae*, consisting of *Moraxella*, *Acinetobacter*, *Psychrobacter*, and related organisms.

A critical feature which distinguishes the genus is that DNA extracted from members of the genus is able to transform the mutant strain BD413 *trpE27* to the wild-type phenotype (Juni, 1972). Classification using modern methods of taxonomy and identification such as genetic transformation, DNA hybridisation, and rRNA sequence comparisons, has *Acinetobacter* divided into 19 DNA-DNA homology groups or genomic species (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989, Tjernberg and Ursing, 1989, Nishimura, *et al.*, 1987; Nishimura, *et al.*, 1988). Of these, 7 have been named: *A. calcoaceticus, A. baumannii, A. haemolyticus, A. junnii, A. lwoffii,A. radioresistans,* and *A. johnsonii.* A definitive numbering scheme has not yet been decided on and Table 1.1. shows a comparison of the delineation of *Acinetobacter* genomic species by different laboratories (Towner, 1996).

Table 1.1.: Comparison of the delineation of *Acinetobacter* genomic species by different laboratories

(taken from Towner, 1996)

Species name	Genomic species number according to References 1 and 2	Reference 3	References 4 and 5	Type strain
A. calcoaceticus	1	1	1	ATCC 23055
A. baumannii	2	2	1	CIP 70.34
	3	3	nt	ATCC 19004
	ug	13	nt	ATCC 17903
A. haemolyticus	4	4	4	ATCC 17906
A. junii	5	5	nt	ATCC 17908
	6	6	4	ATCC 17979
A. johnsonii	7	7	3	ATCC 17909
A. lwoffii	8	8	2	ATCC 15309
	9	8	nt	ATCC 9957
	10	10	ug	ATCC 17924
	11	11	ug	ATCC 11171
A. radioresistens	(12)*	12	5	IAM 13186
	13	14	nt	ATCC 17905
	14	nt	nt	Bouvet 382
	15	nt	nt	Bouvet 240
	16	ug	nt	ATCC 17988
	17	nt	nt	Bouvet 942
	nt	15	nt	Tjernberg 151a

Note: ug, ungrouped; nt, not tested; *unpublished result.

References: (1) Bouvet and Grimont (1986); (2) Bouvet and Jeanjean (1989); (3) Tjernberg and Ursing (1989); (4) Nishimura et al. (1987); and (5) Nishimura et al. (1988).

The essential features of the genus are now clearly defined (Bergogne-Bérézin and Towner, 1996; Towner, 1996): Acinetobacters are Gram-negative coccobacilli with a DNA G+C content of 39-47 mol%. They are strictly aerobic, non-motile, catalase-positive, and oxidase-negative and grow at temperatures ranging from 20°C to 37°C. A simple minimal medium containing a single carbon source and ammonium and nitrate salts supports the growth of most *Acinetobacter* strains.

1.C. Habitats of *Acinetobacter* Species

Acinetobacter spp. can be isolated from virtually all soil and water samples, provided the correct enrichment cultures are used (Baumann, 1968; Joly-Guillou and Brun-Buisson, 1996). It was estimated that acinetobacters make up no less than 0.001% of the total heterotrophic aerobic population of the soil and water and tend to be the predominant species in some water samples (Baumann, 1968). The different *Acinetobacter* genomic species can be further divided on the basis of the environment they predominantly inhabit (Bouvet and Grimont, 1987; Tjernberg and Ursing, 1989). Clinically, *A. baumannii* is the most important species and accounts for 84 - 90% of acinetobacters isolated from hospitalised patients (Joly-Guillou *et al.*, 1991). Humans are the primary reservoir and no other natural habitat has been identified for this species (Joly-Guillou and Brun-Buisson, 1996). *A. calcoaceticus* inhabits the soil and is rarely involved in human infections (Baumann, 1968; Joly-Guillou and Brun-Buisson, 1996). *Acinetobacter* genomic species 3 has been recovered from both the soil and clinical samples (Joly-Guillou and Brun-Buisson, 1996). *A. haemolyticus* and genomic species 6 have been isolated from patients, the hospital environment, and activated sludge samples (Bouvet and Grimont, 1986), while *A. junii* has been isolated from both patients and the environment (Joly-Guillou and Brun-Buisson, 1996). *A. johnsonii* and *A. lwoffii* have been isolated from a variety of sources, namely, the skin of hospitalised patients and the hands of uninfected hospital staff, animals and animal products, soil, and activated sludge (Joly-Guillou and Brun-Buisson, 1996). They have also been isolated from clinical specimens, but only rarely. *A. radioresistans* has also been recovered from a number of different environments including irradiated poultry, cotton, soil, and, more recently, patients (Nishimura *et al.*, 1988; Tjernberg and Ursing, 1989; Christensen, *et al.*, 1991). The habitats of the remaining *Acinetobacter* species are poorly defined at present (Joly-Guillou and Brun-Buisson, 1996). What has become known as "the *A. baumannii- A. calcoaceticus* complex" consists of genomic species 1, 2, 3 and 13TU (Gerner-Smidt *et al.*, 1991; Kämpfer *et al.*, 1993). These species

are phenotypically similar and strains isolated from infected patients normally fall within this group.

1.D. Clinical Importance

1.D.1. Sources of Infection

Acinetobacters form a part of the normal bacterial flora of the skin, particularly the moist regions such as the axillae, groin, and toe webs (Taplin *et al.*, 1963; Joly-Guillou and Brun-Buisson, 1996). Studies conducted by Taplin *et al.* (1963) suggested that 25% of normal males carry *Herellea vaginicola* and 10% carry *Mima polymorpha*. The skin is often the source of contamination in outbreaks of infection and the organisms can be transmitted from patient to patient via the hands of healthy staff members (Taplin *et al.*, 1963; Al-Khoja and Darrell, 1979; French *et al.*, 1980; Holton, 1982). Acinetobacters have also been recovered from the oral cavities, respiratory tracts, and rectums of healthy adults (Rosenthal and Tager, 1975; Glew *et al.*, 1977, Allen and Green, 1987). The rectum does not appear to be an important reservoir, although Allen and Green (1987) reported a 33% incidence of rectal colonisation with *Acinetobacter,* which is higher than other reports. In addition to human reservoirs, the sources of various outbreaks have been traced to the inanimate environment. Strains causing outbreaks have been isolated from soaps, plastic urinals, blood-collection tubes, catheters, respirometers, ventillatory equipment, humidifiers, tap water, basins, and wet mattresses (Smith and Massanari, 1977; Abrutyn *et al.*, 1978; Ramphal and Kluge, 1979; Cunha *et al.*, 1980, McBride, 1984; Sheretz and Sullivan, 1985; Musa *et al.*, 1990; Pearse, 1995).

1.D.2. Virulence Factors

Although acinetobacters are considered to be low-grade pathogens they have certain inherent properties which enhance their virulence (Bergogne-Bérézin and Towner, 1996; Joly-Guillou and Brun-Buisson, 1996). A polysaccharide capsule increases the bacterium's invasiveness and helps protect it from phagocytosis (Rosenberg *et al.*, 1983; Kaplan *et al.*, 1985). They possess two types of fimbrae: the thin fimbrae are involved in adherence to epithelial cells

and the thick fimbrae mediate "twitching" motility (Henriksen and Blom, 1975; Rosenberg *et al.*, 1982). They have enzymes which cause damage to tissue lipids (Poh and Loh, 1985). Since iron is essential for the growth of bacteria, *Acinetobacter* strains producing siderophores, like aerobactin, which aid iron acquisition, are more virulent (Smith *et al.*, 1990). Acinetobacters also produce an endotoxin which is responsible for some of the symptoms of septicaemia experienced by the patient (Bergogne-Bérézin and Towner, 1996; Joly-Guillou and Brun-Buisson, 1996). Certain strains also produce slime, which has been reported to increase virulence (Obana *et al.*, 1985). The comprehensive antibiotic resistance of many clinical isolates of *Acinetobacter* can exacerbate virulence (Bergogne-Bérézin and Towner, 1996; Joly-Guillou and Brun-Buisson, 1996).

1.D.3. Role in Human Disease

1.D.3.a. Predisposing factors

Different predisposing factors for *Acinetobacter* infection are associated with specific cases but there are common factors. Patients with severe underlying disease, such as malignancy or burns, and patients who have undergone major surgery or are immunosuppressed are at particular risk (Rosenthal, 1974; Peacock *et al.*, 1988; Lortholary *et al.*, 1995). The setting plays an important role since most outbreaks occur in intensive care units, burn units, and renal wards (Bergogne-Bérézin *et al.*, 1987). Sites of entry include tubes, catheters, and artificial devices (Bergogne-Bérézin *et al.*, 1987).

Patients who have received previous antibiotic therapy are at increased risk since the antimicrobial selects for resistant strains (McGowan, 1983; Peacock *et al.*, 1988; Bergogne-Bérézin and Towner, 1996). Some studies have found that advanced age is also a predisposing factor, (Bergogne-Bérézin and Joly-Guillou, 1991) and that neonates are more at risk than older children (Ng *et al.*, 1989). Tobacco smoking and alcoholism have also been identified as risk factors in community-acquired *Acinetobacter* pneumonia (Rudin *et al.*, 1979; Levi and Rubinstein, 1996). It has also been noted by some authors (Retailliau, 1979; Smith, 1979) that there is a seasonal pattern to

Acinetobacter infections. The number of infections in late summer is greater than those in early winter. The exact reason for this observation has not been investigated but it has been suggested that the growth conditions for Acinetobacters are improved due to an increase in humidity, temperature, and perspiration (Retailliau, 1979; Smith, 1979).

1.D.3.b. Types of Infection

Since certain *Acinetobacter* spp. form a part of the normal skin flora, it is not always possible to differentiate between infection and colonisation (Streulens *et al.*, 1993). Acinetobacters are low-grade pathogens and they have been isolated from a variety of opportunistic infections, including septicaemia, pneumonia, endocarditis, meningitis, urinary tract infections, and skin and wound infections (Buxton *et al.*, 1978; French *et al.*, 1980; Bergogne-Bérézin *et al.*, 1987; Ng *et al.*, 1989;Levi and Rubinstein, 1996). Although infections occur predominantly in the intensive care setting, community-acquired infections have been reported and may be more significant than realised (Rudin *et al.*, 1979; Hoffman *et al.*, 1982). The existence of community-acquired infections does illustrate the potential primary pathogenicity of acinetobacters (Rudin *et al.*, 1979; Hoffman *et al.*, 1982; Bergogne-Bérézin *et al.*, 1996).

The main site of infection is the lower respiratory tract (Glew *et al.*, 1977; Retailliau *et al.*, 1979). *Acinetobacter* is responsible for about 9-10% of nosocomial pneumonias (Struelens *et al.*, 1993). The mortality rate associated with such infections ranges from 30-75% (average of 40%) which is higher than that for pulmonary infections caused by other Gram-negative (except *Pseudomonas*) and Gram-positive bacteria (Torres *et al.*, 1990; Bergogne-Bérézin and Joly-Guillou, 1991). Infection tends to be prevalent in patients with severe underlying disease requiring ventilation, intubation, or tracheostomies (Buxton *et al.*, 1978; Peacock *et al.*, 1988; Bergogne-Bérézin and Joly-Guillou, 1991; Struelens *et al.*, 1993; Lortholary *et al.*, 1995). Reports of community-acquired *Acinetobacter* pneumonia are infrequent. One

study, however, (Rudin *et al.*, 1979) did report a 10% frequency of community-acquired *Acinetobacter* pneumonia.

A. baumannii is the most common species involved in *Acinetobacter*-associated bacteraemia (Smego, 1985). The largest group of patients with *Acinetobacter* bacteraemia are immunocompromised patients (Bergogne-Bérézin and Towner, 1996; Levi and Rubinstein, 1996). In most cases, the source of the bacteraemia can be traced to a respiratory tract infection (Bergogne-Bérézin and Towner, 1996; Levi and Rubinstein, 1996). However, further risk factors have been identified: malignant disease, surgical wounds, burns, and vascular catheterisation (Bergogne-Bérézin and Towner, 1996; Levi and Rubinstein, 1996). *Acinetobacter* can present as either a single pathogen or as part of a polymicrobial bacteraemia (Bergogne-Bérézin and Towner, 1996; Levi and Rubinstein, 1996).

Acinetobacter is an infrequent cause of meningitis and it is normally as a result of secondary infection following neurosurgical procedures or head trauma (Berk and McCabe, 1981). Most cases of *Acinetobacter* meningitis are nosocomial, but a few community acquired cases have been reported (Levi and Rubinstein, 1996). As with bacteraemia, the predominant species in *Acinetobacter* meningitis is *A. baumannii* (Levi and Rubinstein, 1996). The mortality rate ranges from 20-27% (Levi and Rubinstein, 1996).

UTIs caused by *Acinetobacter* tend to be rare (Levi and Rubinstein, 1996). The portion of the population particularly at risk are elderly, debilitated males, patients confined to the ICU and patients with permanent indwelling catheters (Hoffman *et al.*, 1982). Again, community-acquired *Acinetobacter* UTIs are infrequent but have been reported (Hoffman *et al.*, 1982).

Other infections caused by *Acinetobacter* include peritonitis (Levi and Rubinstein, 1996), infective endocarditis (Gradon *et al.*, 1992), neonatal septacaemia (Ng *et al.*, 1989), and skin and wound infections (Glew *et al.*, 1977; Levi and Rubinstein, 1996).

1.D.4. Therapy for *Acinetobacter* Infections

Acinetobacters often exhibit multiple resistance to antibiotics and combination therapy is thus recommended (Bergogne-Bérézin and Towner, 1996). Normally a combination an aminoglycoside with imipenem or a broad spectrum cephalosporin is used (Levi and Rubinstein, 1996). In conjunction with combination therapy, strict measures need to be implemented which prevent the further dissemination of outbreak strains within hospitals (Pearse, 1995; Levi and Rubinstein, 1996).

1.E. Antibiotic Resistance in *Acinetobacter*

1.E.1. Introduction

A feature of *Acinetobacter*, particularly *A. baumannii*, is their ability to acquire and develop antibiotic resistance extremely rapidly (Amyes and Young, 1996; Bergogne-Bérézin and Towner, 1996). At least in part, the "success" of *A. baumannii* as a nosocomial pathogen can be attributed to its ability to respond rapidly to antibiotic challenge and the widespread use of antibiotics in hospitals, which selects for resistant strains (Amyes and Young, 1996; Bergogne-Bérézin and Towner, 1996).

In earlier times (up until the early 1970s), *Acinetobacter* infections were successfully treated with gentamicin, minocycline, nalidixic acid, ampicillin, or carbenicillin, either singly or as part of a combination of agents (Bergogne-Bérézin and Towner, 1996). Resistance to these and other drugs was first detected between 1971 and 1974 (Bergogne-Bérézin and Towner, 1996) and, since then, resistance to virtually all commercially available antibiotics has been documented in *A. baumannii*; including resistance to aminopenicillins, ureidopenicillins, cephalosporins, cephamycins, most aminoglycoside-aminocyclitols, chloramphenicol, trimethoprim, and tetracycline (Morohoshi and Saito, 1977; Dowding, 1979; Devaud *et al.*, 1982; Garcia *et al.*, 1983; Goldstein *et al.*, 1983). Although MICs of most drugs are increasing, partial susceptibility remains for some antibiotics such as broad-spectrum cephalosporins (like cefotaxime and ceftazidime), tobramycin, amikacin,

fluoroquinolones, and imipenem (Bergogne-Bérézin and Towner, 1996). Imipenem is the most active drug and 100% susceptibility was the norm until recently. Go *et al.* (1994) reported an *Acinetobacter* infection which was resistant to nearly all antibiotics, including imipenem, and could only be treated with polymixin B and sulbactams. Another cause for concern was the report by Schaife *et al.* (1995) describing a clinical isolate of *Acinetobacter*, which carries a transferable imipenem resistance. The nosocomial spread of imipenem resistance amongst strains of *Acinetobacter* poses a serious potential threat for the treatment of *Acinetobacter* infections (Bergogne-Bérézin and Towner, 1996).

Other strains, like *A. junii* and *A. lwoffii*, are isolated from the hospital environment but less frequently and they tend to be more susceptible to antibiotic treatment (Traub and Spohr, 1989). Strains of *A.lwoffii* are more susceptible to β-lactams than is *A.baumannii*, while *A.haemolyticus* isolates are normally resistant to aminoglycosides and rifampin (Bergogne-Bérézin and Joly-Guillou; 1985).

1.E.2. β-Lactam Resistance

The first β-lactamase identified in *Acinetobacter* spp. was the plasmid-encoded class A TEM-1 β-lactamase (Philippom *et al.*, 1980; Goldstein *et al.*, 1983). TEM-2 and CARB-5 β-lactamases were identified later and found to be plasmid-encoded (Devaud *et al.*, 1982). However, it has been reported that, despite the presence of these enzymes which have activity against penicillins, cephalosporinases are the predominant β-lactamases in *Acinetobacter* (Vila *et al.*, 1993).

Four chromosomal β-lactamases have been identified and designated ACE (*Acinetobacter* chromosomal enzymes) 1-4 (Hood and Amyes, 1991). All 4 enzymes are cephalosporinases, although some activity against penicillins was detected (Hood and Amyes, 1991). None of them had any detectable hydrolysing activity against aztreonam, caftazidime, or cefotaxime (Hood and Amyes, 1995). Their maximum activity was against cephaloridine and, except

for ACE-4, they had good activity against cephradine (Hood and Amyes, 1991). ACE-1 also showed some hydrolysis of cefuroxime (Hood and Amyes, 1991). It is a moot point as to whether these organisms are inducible (Joly-Guillou *et al.*, 1988; Hood and Amyes, 1991). It is thought that, although the contribution of these enzymes to the expression of β-lactam resistance is important, they work in concert with the inherent resistance conferred by permeability reduction and altered penicillin binding proteins (Obara and Nakae, 1991; Sato and Nakae, 1991).

Surprisingly, despite the presence of extended spectrum β-lactamase (ESBL) progenitors, TEM-1 and TEM-2 β-lactamases, the acquisition of plasmid-encoded β-lactamases does not appear to be of importance in acinetobacters (Amyes and Young, 1996; Bergogne-Bérézin and Towner, 1996). Until recently there had been only one suggestion of the presence of ESBLs in *Acinetobacter* (Amyes and Young, 1996). PER-1 is a non-TEM, non-SHV derived class A ESBL which was first described in *Pseudomonas*. The presence of this cephalosporinase in nosocomial *A. baumannii* was discovered by Vahaboglu *et al.* (1997). In the study, 46% of *Acinetobacter* strains were found to harbour PER-1-type β-lactamases. These strains were highly resistant to ceftazidime and gentamicin, intermediately resistant to amikacin, and susceptible or moderately susceptible to imipenem and meropenem. A plasmid location for the gene encoding the PER-1 enzyme could not be shown but was not ruled out. The authors suggest that, although the PER-1-type β-lactamases appear to be restricted to Turkey, their clonal diversity and high prevalence indicate a high spreading potential.

ARI-1, another novel β-lactamase, which mediates resistance to imipenem, has also been described in *Acinetobacter* spp (Paton *et al.*, 1993). Unlike other class C enzymes, ARI-1 is not a metallo-β-lactamase and is thought to be a serine-based enzyme, like class A enzymes. The gene encoding ARI-1 is carried on a 45kb plasmid, pUK1356, which is self-transferable within *Acinetobacter* and to other bacterial species (Schaife *et al.*, 1995). This was the first report of plasmid-encoded imipenem resistance in any bacterial

species isolated in Europe. In 1998, Brown *et al.* described another new β-lactamase, ARI-2, which was isolated from a multiresistant clinical isolate of *A.baumannii* from Argentina. ARI-2 is also a class A β-lactamase which hydrolyses imipenem. Evidence suggests that it is plasmid-encoded and preliminary studies have demonstrated its presence in a number of clinical isolates of *Acinetobacter* from Europe and south-east Asia. These studies augur badly for the future treatment of *Acinetobacter* infections. Previously, up to 100% of *Acinetobacter* isolates were sensitive to imipenem (Amyes and Young, 1996).

Vila *et al.* (1998) reported the presence of a gene encoding an oxacillinase in a clinical strain of *A.baumannii* (strain Ab41). The strain was resistant to all β-lactam antibiotics tested except ceftazidime, ceftriaxone, ceftizoxime, and imipenem. It produced 3 β-lactamases: a chromosomal cephalosporinase, a TEM-1-like β-lactamase, and a novel OXA-derived β-lactamase, designated OXA-21.

A summary of all the β-lactamases described in *Acinetobacter* spp. is listed in Table 1.2.

Table 1.2: Summary of the β-lactamases described in *Acinetobacter* spp.

(adapted from Amyes and Young, 1996)

Enzyme or strain	Location of gene	Predominant substrate	Molecular size (kDa)	Ref
TEM-1	Plasmid	Penicillin	29	1
TEM-2	Plasmid	Penicillin	29	2
CARB-5	Plasmid	Penicillin	29	3
ARI-1	Plasmid	Carbapenem	23	4
ARI-2	Plasmid	Carbapenem	Unknown	5
OXA-21	Integron	Oxacillin	Unknown	6
NCTC7844	Chromosome	Cephalosporin	30	7
ML4961	Chromosome	Cephalosporin	38	8
ACE-1	Chromosome	Cephalosporin	*ca.*500	9
ACE-2	Chromosome	Cephalosporin	60.5	9
ACE-3	Chromosome	Cephalosporin	32.5	9
ACE-4	Chromosome	Cephalosporin	>1000	9
PER-1	Chromosome	Cephalosporin	Unknown	10
SHV-like	Unknown	Penicillin	Unknown	11
SHV-like	Unknown	Penicillin	Unknown	12

References: 1. Philippon *er al.* (1980); 2. Devaud *et al.* (1982), 3. Bauernfeind (1985); 4 Paton *et al.* (1993); 5. Brown *et al.* (1998); 6. Vila *et al.* (1997); 7. Morohoshi and Saito (1977); 8. Hikida *et al.* (1989); 9. Hood and Amyes (1991); 10. Vahaboglu *et al.* (1997); 11. Joly-Guillou and Bergogne-Bérézin (1990); 12. Vila *et al.* (1993)

As briefly mentioned above, *Acinetobacter* also display an inherent high-level resistance to β-lactam antibiotics. Impermeability or reduced permeability of the outer membrane may play a role in this respect (Sato and Nakae, 1991). These authors reported that the permeability of *A. calcoaceticus* to cephalosporins was 2-7 fold lower than that of *Pseudomonas aeruginosa.* They show that the reason for this observation is due to *A. calcoaceticus* having a small number of small-sized porins. The penicillin binding proteins (PBPs) of *Acinetobacter* are unlike those in *E.coli* and *Pseudomonas* and are most similar to those in *Bacteroides* (Piddock and Wise, 1986). Six PBPs have been found in *Acinetobacter* (Obara and Nakae, 1991) and were designated PBP 1a, 1b, 1c, 2, 3, and 4. Resistance to β-lactams is enhanced by the presence of altered PBPs (Obara and Nakae, 1991). The PBPs have

an enhanced expression so that more antibiotic is required to bind before inhibition can occur and/or a reduced affinity for β-lactam which reduces uptake and effectiveness of the antibiotic (Obara and Nakae, 1991).

1.E.3. Aminoglycoside Resistance

The most common mechanism of aminoglycoside resistance is enzymatic inactivation (Shaw *et al.*, 1993). Three types of aminoglycoside modifying enzymes (AMEs) exist: acetyltransferases (AAC),adenylyl-or nucleotidyl-transferases (AAD or ANT, respectively), and phospho-transferases (APH). The AMEs are further classified according to site of modification on the aminoglycoside molecule. Several of the enzymes can be subdivided further according to their aminoglycoside resistance profile or their unique protein nature (Shaw *et al.,* 1993). All 3 types of AMEs have been identified in clinical strains of *Acinetobacter.* These are summarised in Table 1.3. Geographic variation has been observed in the incidence of particular genes and it was found that certain strains have as many as 6 different aminoglycoside resistance genes (Shaw *et al.*, 1993). Lambert *et al.* (1993) identified an *aac(6`)-Ig* responsible for amikacin resistance in an isolate of *A. haemolyticus*. Evidence from hybridisation studies led the authors to suggest that this gene was species specific and could be used for the identification of *A. haemolyticus*. Rudant *et. al.* (1997), showed that AAC(6')-Ig is not essential for the viability of *A.haemolyticus*, although the aac(6')-Ig was detected in all members of this species. Similarly, the *aac(6`)-Ij* was found in the chromosome of *Acinetobacter* genospecies 13 strains, but not in other genospecies (Lambert *et al.*, 1994) and Rudant *et al.* (1994) showed the presence of *aac(6`)-Ik* in the 3 strains of *Acinetobacter* genospecies 6. These results indicate that the genes may also be species specific.

Table 1.3.: Aminoglycoside-Modifying Enzymes in *Acinetobacter* spp.

(taken from Amyes and Young, 1996)

Enzyme	Resistance profile (AGRP)	Ref.
Acetylating:		
AAC(6')	Tob Dbk Ntl 6'Ntl 2'Ntl Siso Amk	Shannon et al. (1978) Murray and Moellering (1979) Lambert et al. (1990; 1993)
AAC(2')I	Tob Dbk Ntl 6'Ntl Gm	Dowding (1979)
AAC(3)I	Apr Lvdm Prm Rsm (But) (Neo)	Bergogne-Bérézin et al. (1980) Vila et al. (1993)
AAC(3)II	Tob Dbk Ntl 6'Ntl 2'Ntl Siso Gm	Murray and Moellering (1980)
[AAC(3)V]		Elisha and Steyn (1991a) Shaw et al. (1993)
AAC(3)IV	Tob Dbk Ntl 6'Ntl 2'Ntl Siso Gm Apr	Shaw et al. (1993)
Adenylating:		
ANT(3")I [AAD(3")(9)]	Sm Spcm	Shannon et al. (1978) Murray and Moellering (1980) Devaud et al. (1982) Goldstein et al. (1993) Vila et al. (1993)
ANT(2")I	Tob Dbk Siso Gm Km	Murray and Moellering (1980)
[AAD(2")]		Elisha and Steyn (1991a) Shaw et al. (1993)
Phosphorylating:		
APH(3')I	Km Neo Prm Rsm Lvdm GmB	Bergogne-Bérézin et al. (1980) Devaud et al. (1982) Goldstein et al. (1983) Shaw et al. (1993)
APH(3')II	Km Neo Prm Rsm But GmB (Amk)	Murray and Moellering (1979)
APH(3')III	Km Neo Prm Rsm Lvdm But GmB Amk Isp	Murray and Moellering (1979) Joly-Guillou et al. (1987)
APH(3')VI	Km Neo Prm Rsm But GmB Amk Isp	Martin et al. (1978) Lambert et al. (1988; 1990) Vila et al. (1993) Shaw et al. (1993)
APH(3")I	Sm	Elisha and Steyn (1989)

Note: Amk, amikacin; Apr, apramycin; But, butirosin; Dbk, dibekacin; Gm, gentamicin; GmB, gentamicin B; Isp, isepamicin; Km, kanamycin; Lvdm, lividomycin; Neo, neomycin; Ntl, netilmicin; 2'Ntl, 2'-*N*-ethylnetilmicin; 6'Ntl, 6'-*N*-ethylnetilmicin; Sm, streptomycin; Prm, paromycin; Rsm, ribostamycin; Siso, sisomicin; Spcm, spectinomycin; Tob, tobramycin. Parentheses indicate enzymic activity detectable only *in vitro*.

1.E.4. 4-Quinolone Resistance

DNA gyrase (topoisomerase II) is the target of the fluoronated 4-quinolone action and resistance to these antibiotics in other bacteria is attributed to changes in the structure of DNA gyrase subunits (mainly by mutations in the

gyrA) (Amyes and Young, 1996). After the introduction of the fluoroquinolones, *Acinetobacter* readily developed resistance to these antibiotics (Amyes and Young, 1996). Vila *et al.* (1995) studied mutations in *gyrA* of quinolone resistant *A. baumannii* isolates. In *E. coli*, substitutions of the residues Gly-81, Ser-83, Ala-84, and Gln-106 lead to resistance. They found these residues to be conserved in the sensitive isolates but found substitutions or Ser-83 to leucine and Ala-84 to proline in resistant isolates. The Ser-83 substitution was correlated to the observed resistance. The observed 4-quinolone resistance levels in a set of *A.baumannii* isolates studied by Vila *et al.* (1997) could not be solely attributed to the Ser-83 substitution in *gyrA* and, since ParC (from topoisomerase IV) is a secondary target of 4-quinolones in *E.coli,* it was thought that the reason for the high-level resistance in the *A.baumannii* isolates could be found there. The authors discovered Ser-80 to leucine and Glu-84 to lysine substitutions in the ParC of resistant isolates and it was concluded that the high-level resistance to ciprofloxacin displayed by these isolates was due to mutations in *gyrA* and *parC*.

Also, potentially involved in 4-quinolone resistance is the reduced permeability of the outer membrane described previously for β-lactam resistance (Amyes and Young, 1996). Since selection for resistance to 4-quinolones in *E. coli* and *P. aeruginosa* has been shown to result in cross-resistance to β-lactams (Neu, 1988). As is the case with *Staphylococcus aureus*, active efflux of quinolones has also been suggested as a mechanism of resistance but at this point it is no more than conjecture (Ng *et al.*, 1994; Amyes and Young, 1996). Amyes and Young (1996) also propose that, due to the selection pressure resulting from wide use of 4-quinolones, a plasmid-encoded resistance mechanism is likely to emerge.

1.E.5. Trimethoprim Resistance

Trimethoprim competitively inhibits the bacterial dihydrofolate reductase (DHFR) enzyme which is required for the reduction of dihydrofolate to the essential cofactor tetrahydrofolate (Bushby and Hitchings, 1968). Resistance

to trimethoprim in Gram-negative bacteria is mediated by an addtional DHFR which is resistant to the antibiotic (Amyes and Towner, 1990). A range of DHFRs have been identified - DHFR type Ia is the predominant one in Gram-negative pathogens – however, little is known about the types in *Acinetobacter* (Amyes *et al.*, 1992; Amyes and Young, 1996).

Acinetobacter spp. are inherently resistant to low levels of trimethoprim (MICs of 16-32mg/l). The first report on high-level (MIC of >1000mg/l) trimethoprim resistance in a clinical isolate of *Acinetobacter* was by Goldstein *et al.* (1983). The mechanism of resistance was not investigated but hybridization studies showed that resistance was not mediated by a type Ia DHFR nor was it carried on a Tn7-related transposon. A plasmid carrying resistance to trimethoprim and sulphamethoxazole was isolated from an isolate of *Acinetobacter* by Chirnside *et al.* (1985). However, the gene responsible for the resistance was not characterized. More recently, Amyes and Young (1996) describe a study of the genetic basis of high-level trimethoprim resistance in isolates of *A. baumannii* from Chile. In this case, the resistance gene was characterised and was found to be encoded by a *dfrIa* gene.

1.E.6. Chloramphenicol Resistance

Elisha and Steyn (1991b) cloned and sequenced a CAT1 structural gene from a clinical isolate of *A. baumannii* (strain SAK). CAT1 encodes a chloramphenicol acetyltransferase and mediates chloramphenicol resistance by the transfer of an acetyl group to the chloramphenicol molecule, thereby rendering it inactive. The CAT1 structural gene was shown to be on both the chromosome and a 120kb plasmid.

1.E.7. Location of Resistance Genes

In several studies, more than 80% of *Acinetobacter* isolates have been found to contain multiple indigenous plasmids (Gerner-Smidt, 1989; Seifert *et al.*, 1994). The plasmids are generally small in size (<23kb) and are often cryptic (Towner, 1996). Those whose functions have been elucidated usually encode unusual metabolic activities or antibiotic resistance genes (Towner, 1996).

However, the absence of a suitable detection system may hamper studies of plasmid-mediated resistance (Towner, 1991). Transposons and integrons are thought to play an important role in resistance by ensuring the establishment of novel genes from plasmids, which are unstable in *Acinetobacter,* in a new gene pool (Devaud *et al.*, 1982; Towner, 1991, Towner, 1996).

Both plasmid and transposon locations have been identified for aminoglycoside resistance genes in *Acinetobacter* (Murray and Moellering, 1980). Few studies have effectively investigated the genetic nature of aminoglycoside resistance mainly because of the lack of an efficient plasmid transfer model (Towner, 1991). It has been noted that *Acinetobacter* spp. readily acquire plasmids from Enterobacteriaceae but the reverse is not true (Towner, 1991).

Devaud *et al.* (1982) were the first to demonstrate transposon-encoded aminoglycoside resistance in *A. calcoaceticus*. Transfer of this resistance to *E. coli*, *P. aeruginosa*, or *A. calcoaceticus* could not be shown by conjugation but the plasmid RP4 effected the mobilization of resistance determinants to a wild-type strain. After mobilization it was shown that RP4 had increased in size by 16MD. This suggested the acquisition of a multi-resistance transposon which the authors proposed was chromosomally located in the host *Acinetobacter* strain.

Goldstein *et al.* (1983) described a multi-resistance plasmid, pIP1031 from a clinical isolate of *A. calcoaceticus*, which has 2 deletion derivatives of the insertion sequence, IS15. One of these deletion derivatives was shown to be on the same *Eco*RI restriction fragment as the gene encoding the APH(3`)-I enzyme. This gene has been found to reside on well-characterised compound transposons such as Tn*6*. It was therefore suggested that the APH(3`)-I gene of pIP1031 may also be within a compound transposon.

The *aac(6`)-Ih* of *A.baumannii* was found to be located on a 13.7kb non-conjugative plasmid (Lambert *et al.*, 1994). The most recent report of

plasmid-encoded aminoglycoside resistance in *Acinetobacter*, however, is by Segal and Elisha (1997). They report the identification and characterisation of an *ant(2``)-Ia* (or *aadB*) gene cassette on a plasmid, pRAY, from *Acinetobacter.*

High-level trimethoprim resistance in *Acinetobacter* has also been shown to be plasmid-mediated in certain instances [section 1.E.5.]. In the two cases described (Goldstein *et al.*, 1983; Chirnside *et al.*, 1985), the resistance gene was carried on a self-transferable plasmid but the gene responsible for the resistance was not characterised.

The CAT1 structural gene (Elisha and Steyn, 1991b) was shown to be on both the chromosome and a 120kb plasmid. These results suggested that the gene could be transposon-encoded. The transposon was thought to be Tn*21*-related and linked to IS*1* in a manner similar to the organisation of Tn*2670.* The significance of this is in the fact that Tn*21* contains recombinational hot-spots which may be involved in the acquisition of additional resistance genes.

Plasmid-mediated carbapenem resistance in *Acinetobacter* has also been described. A novel carbapenemase (ARI-2) isolated from a multiresistant clinical isolate of *A.baumannii* was plasmid-mediated (Brown *et al.*, 1998).

While there remains a paucity of data regarding the exact nature and extent of the role plasmids, transposons, and integrons play in antibiotic resistance in *Acinetobacter*, it is nonetheless clear that they are potentially very important factors in both the acquisition and dissemination of resistance genes (Towner, 1991, Bergogne-Bérézin and Towner, 1996).

1.F. Conclusions

The increasing threat posed by *Acinetobacter* spp. in the intensive care setting is not due to the inherent virulence of the organisms. The ability of *Acinetobacter* spp. to acquire antibiotic resistance genes, thereby posing a therapeutic problem, is where the real danger lies. The increased reliance on

antibiotics in hospitals due to advances in invasive surgical procedures has allowed *Acinetobacter* to fill an ecological niche left vacant by the elimination of its competitors (Bergogne-Bérézin *et al.*, 1996).

1.G. Aim of this work

Increased reliance on antibiotic therapy due to advances in invasive surgery has paved the way for the emergence of opportunistic pathogens. Numbered among the more important opportunistic pathogens is *Acinetobacter baumannii* (Joly-Guillou and Bergogne-Bérézin, 1996). This organism is responsible for an increasing number of nosocomial infections in patients receiving intensive care. Treatment of these infections is hampered by the comprehensive antibiotic resistance of *A. baumannii* (Amyes and Young, 1996). The molecular basis of antibiotic resistance has not been extensively investigated in *Acinetobacter* (Amyes and Young, 1996; Bergogne-Bérézin and Towner, 1996). A few studies have demonstrated the role of plasmids and transposons in resistance in this organism. In *Enterobacteriaceae*, gene cassettes and integrons have played an important role in the progressive acquisition and spread of resistance, however, little is known about their importance in *Acinetobacter*. A feature of integrons is their ability to effect the "capture" of resistance genes by the integron-encoded enzyme, integrase (Stokes and Hall, 1989). Resistance genes are inserted as cassettes consisting of the structural gene and sequences involved in recombination, at specific sites of integrons (Collis and Hall, 1992; Collis *et al.*, 1993). The role of integrons and integron-associated resistance genes in *Acinetobacter* has not been studied extensively.

The aim of this project was two-fold:

a) to determine the incidence of integrons in clinical isolates of *Acinetobacter* from two South African hospitals, Groote Schuur Hospital (GSH), Cape Town and Universitas Hospital (UH), Bloemfontein.

b) to characterise the antibiotic resistance genes carried in the variable regions of these integrons.

CHAPTER 2: SCREENING OF CLINICAL ISOLATES OF *ACINETOBACTER* FOR THE PRESENCE OF THE INTEGRASE GENE

2.A. INTRODUCTION

The term integron was originally coined to describe a family of novel mobile DNA elements (Stokes and Hall, 1989). Integrons have been defined as natural cloning and expression vectors containing the determinants for a site-specific recombination and expression system (Hall and Collis, 1995). Based on the DNA sequences of their associated integrases, 4 classes of integrons have been described (fig.2.1.). Fig. 2.1. shows an alignment of the 4 integrases.

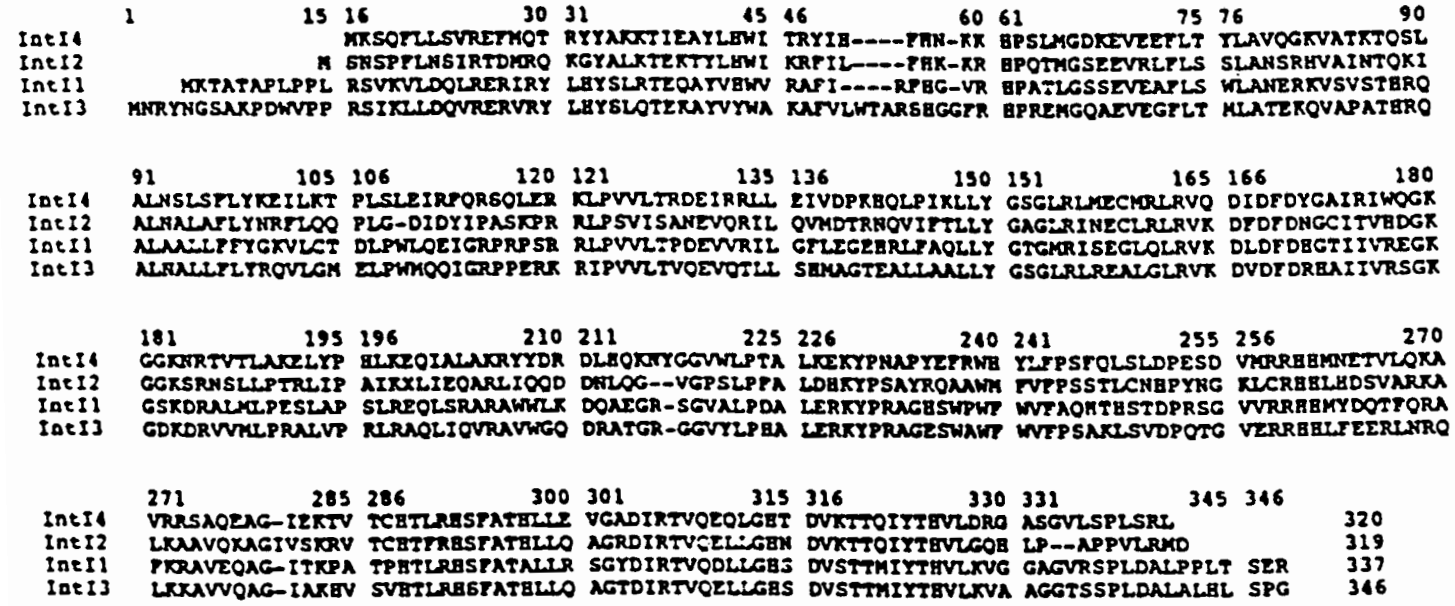


Fig. 2.1.: Alignment of the 4 integrases (taken from Mazel *et al.*, 1998)

Single letter abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; F, Phe, G, Gly; I, Ile, K, Lys; M, Met; P, Pro, R, Arg, S, Ser; T, Thr; and V, Val

Class 1-type integrons are made up of three regions: the 5 - onser ed segment (5 -C), the ariable region, and the 3 - onser ed segment (3 -C) (fig. 2.2.; tokes and Hall, 1989). The 5 -C ontains the gene for the Int1 integrase whi h is responsible for the re ombination of gene assettes into the ariable region of integrons (tokes and Hall, 1989; Hall and Collis, 1995). The ariable region onsists of the gene assettes whi h are made up of a stru tural gene, usually an antibioti resistan e gene (Re hia and Hall, 1995), and a re ombination site –the 59 base element or *attC* site (Hall and Collis, 1995; Collis and Hall, 1995; Hansson *et al.*, 1997). The gene assettes are mobile and their number and order in an integron does not appear to be restri ted. It an be altered by the insertion of new assettes and the ex ision or rearrangement of existing ones, through the a tion of integrase. (Re hia and Hall, 1995; Collis and Hall, 1992a). Most lass 1-type integrons ontain a *qacEΔ1* gene (en odes a quaternary ammonium ompound exporter protein whi h mediates resistan e to antisepti s and disinfe tants) and a *sull* gene (en odes a sulfonamide resistant dihydropteroate synthase) in the 3 -C (tokes and Hall, 1989; Re hia and Hall, 1995). The outer boundaries of the 5 -C and 3 -C of lass 1-type integrons ontain 25 base in erted repeats, alled Brown's repeats (Brown *et al.*, 1986).

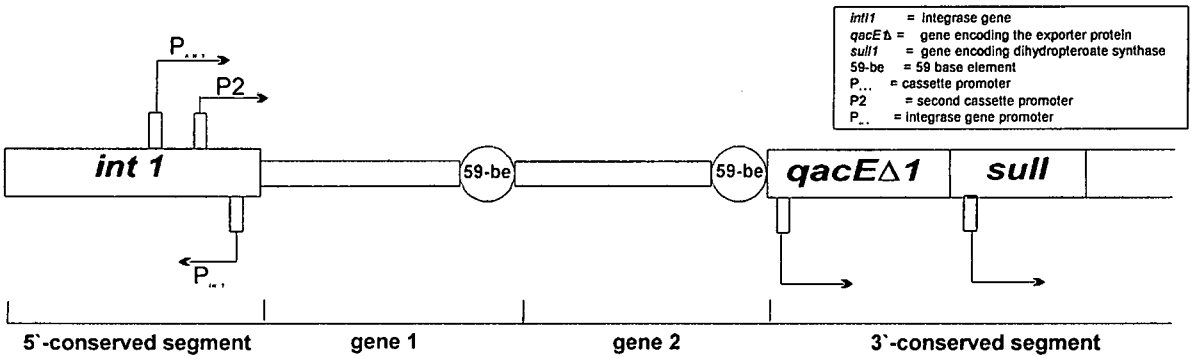


Fig. 2.2: A schematic diagram of a class 1-type integron showing 2 gene cassettes in the variable region The arrows indicate the direction in which the genes are transcribed (adapted from Bissonnette and Roy, 1992)

P_{ANT} is the common promoter from which all the inserted gene cassettes are transcribed (Collis and Hall, 1995). The gene transcripts all originate at this promoter which is located 214 bases from the inner boundary of the 5`-conserved segment (Collis and Hall, 1995). P_{ANT} has a strong similarity to the *E.coli* promoter consensus and sequence variations at the P_{ANT} location affects the relative strength of the promoter and thus the level of resistance (Hall and Collis, 1995; Collis and Hall, 1995). P2 is located 119 bases downstream of P_{ANT} in some integrons and, when present, transcription of the gene cassettes occurs from both promoters (Collis and Hall, 1995). P2, a weaker promoter, arises from the insertion of 3 G residues which increase the spacing between the -10 and -35 regions to the optimal 17 bases (Hall and Collis, 1995; Collis and Hall, 1995). The level of expression from a weak P_{ANT} is compensated for by combined expression from P2 (Collis and Hall, 1995). The *int1* is transcribed from P_{INT} in the opposite direction to P_{ANT} and P2 (Collis and Hall, 1995). The *qacEΔ1* and *sulI* genes are transcribed from their own promoters (Hall and Collis, 1995).

Class 1-type integrons are sometimes referred to as Tn*21*-like integrons. This is because the initial descriptions of integrons were of elements associated with Tn*21*. They were similar in structure to Tn*21* and included the *sulI* and the *ant(3``)-Ia* (or *aadA*; Hall and Stokes, 1993). If further resistance genes

were present they were located on either side of the *ant(3``)-Ia*, and in one case replaced it with an oxacillin resistance gene, *oxa2* (Hall and Stokes, 1993). Class 1-type integrons were further classified as In1, In2, In3, In4, In5, In6, In7, In8, In10, In13, In18, In30, and In0 for the elements from R46, Tn*21*, R388, Tn*1696*, pSCH884, pSa, pDGO100, Tn*2603*, pBP201, pLMO20, pLMO229, pBWH301, and pVS1, respectively (Stokes and Hall, 1989; Hall and Stokes, 1993; Recchia and Hall, 1995).

Tn7 and its close relatives make up the class 2 integrons. The general organisation of the left end of Tn7 is similar to that of class 1 integrons. There is a putative, but defective, *int1* gene (Hall and Vockler, 1987). This *int2* gene is interrupted by a termination codon (Hall and Vockler, 1987) and its product, Int2, is 40% identical to Int1 (Sundstrom *et al.*, 1991; Young *et al.*, 1994). Gene cassettes have been found downstream of the *int2* gene but the 3`-sequences flanking these cassettes are not related to those flanking the cassettes associated with class 1 integrons.

The putative integrase, Int3, of class 3 integrons is 61% identical to the Int1 integrase (Arakawa *et al.*, 1995). This is the only class 3 integron described thus far. The integrase gene is located 5` to the bla_{IMP} cassette (Arakawa *et al.*, 1995).

Class 4-type integrons have only recently been described. The integrase gene, *int4*, was identified in *Vibrio cholerae* (Mazel *et al.*, 1998) and the gene product was found to have 45 to 50% identity with the 3 previously described integrases. Interestingly, the gene cassettes associated with this class of integron do not encode antibiotic resistance genes. Rather, they are "gene-VCR" cassettes (VCRs are *V.cholerae* repeated sequences) similar to the well-characterised antibiotic resistance gene cassettes. A 90% sequence identity was found between the VCR sequences and the 59-base element associated with *blaP3*, which is an integron-associated antibiotic resistance gene encoding the carbenicillinase, CARB-4, isolated from *Pseudomonas* (GenBank accession number U14749). Their results have also shown that VCR cassettes have been in the *Vibrio* lineage since before the emergence of antibiotic resistance integrons.

Class 1 integrons have played an important role in the spread of antibiotic resistance genes and are the most prevalent integrons in clinical isolates of *Enterobacteriaceae* and pseudomonads (Recchia and Hall, 1995). To determine whether *Acinetobacter* isolates from Groote Schuur Hospital (GSH) and Universitas Hospital (UH) contain these elements, a portion of the *int1* integrase gene was used to screen a total of 57 clinical isolates of multi-resistant *A. baumannii* - 43 from GSH and 14 from UH.

2.B. MATERIALS AND METHODS

2.B.1. Bacterial isolates and plasmids

Acinetobacter spp. were isolated in the Clinical Microbiology Laboratory, Groote Schuur Hospital and the Department of Medical Microbiology, Universitas Hospital, Bloemfontein. The isolates were all identified as *Acinetobacter baumannii* by standard biochemical tests. The year of isolation of the acinetobacters is shown in Table 2.1.

Table 2.1.: Year of isolation of the acinetobacters

Isolate number	Year isolated	Isolate number	Year isolated
G1	1993	G31	1997
G2	1993	G32	1997
G3	1993	G33	1997
G4	1993	G34	1997
G5	1993	G35	1997
G6	1993	G36	1997
G7	1996	G37	1997
G8	1996	G38	1997
G9	1994	G39	1997
G10	1996	G58	1996
G11	1996	G60	1996
G12	1996	G61	1996
G13	1996	G95	1996
G14	1996	G98	1996
G15	1996	B1	1994
G16	1996	B2	1994
G17	1996	B3	1994
G18	1996	B4	1994
G19	1997	B5	1994
G20	1997	B6	1994
G21	1997	B7	1994
G23	1997	B8	1994
G24	1997	B9	1994
G25	1997	B10	1994
G26	1997	B11	1994
G27	1997	B12	1994
G28	1997	B13	1994
G29	1997	B14	1994
G30	1997		

Acinetobacter calcoaceticus BD413 C91 strain, which does not carry an integron or integron-related sequences, was used as a negative control in hybridisation and PCR experiments and was kindly supplied by A.Vivian (University of Western England, Bristol, U.K.).

Acinetobacter baumannii strain CW20 carries an integron with a gene cassette of 0.7kb in the variable region. This strain was used as a positive control in the PCR experiments and was kindly supplied by R. Seward and K.J. Towner (Department of Microbiology and PHLS Laboratory, University Hospital, Queen's Medical Centre, Nottingham).

E. coli DH5α (ϕ80d*lacZ*ΔM15) (Hanahan, 1983), which does not carry an integron or integron-related sequences, was used as a negative control in hybridisation experiments.

Plasmid pGSH108, which contains a 1.7kb *Bam*HI/*Hin*dIII insert encoding integrase and ANT(2``) activity, was used as the source of the *int1* probe and as a positive control in hybridisation experiments (Elisha, 1991).

2.B.2. Antibiotic sensitivity testing

Antibiotic susceptibility of the acinetobacters was determined by the Kirby Bauer disc diffusion test on Mueller Hinton agar at 37^0C for 18 hours. Resistance profiles were determined based on NCCLS criteria. Oxoid discs containing the following antibiotics (amount of antibiotic in μg) were used: amikacin (30), amoxicillin (10), cefapime (30), cefazolin (30), cefotaxime (30), cefoxatin (30), cefuroxine (30), chloramphenicol (30), coamoxiclav (amoxicillin (20), clavulanic acid (10)), cotrimoxazole (30), gentamicin (10), imipenem (10), meropenem (10), ofloxacin (5), oxacillin (1), penicillin (10 units), and piperacillin/tazobactam (piperacillin (100), tazobactam (10)).

2.B.3. Agarose gel electrophoresis (AGE)

AGE resolves DNA fragments on the basis of their differing rates of migration in an agarose gel (Ausubel *et al.*, 1987). Smaller fragments migrate faster than larger ones when an electrical field is applied across the gel and, since DNA is negatively charged, it migrates from the anode to the cathode. DNA was separated in horizontal gels of 1% (w/v) agarose dissolved in 0.4M Tris-acetate, 0.01M EDTA (1x TAE, Appendix A) using the standard submerged gel system described by Sambrook *et al.* (1989). Ethidium bromide (EtBr), a fluorescent dye containing a planar group that intercalates between the stacked bases of DNA, was included in the gels. The fluorescent yield of EtBr-DNA complexes is much greater than that of unbound dye, and DNA can thus be visualised by UV transillumination at 302nm (the wavelength at which the bound dye absorbs the ultraviolet radiation) (Sambrook *et al.*, 1989). The agarose was dissolved by heating the solution to boiling in a microwave oven.

The gel was poured into a mould and a comb was inserted. Once the gel had polymerised, the comb was removed and the gel was submerged in 1x TAE in the electrophoresis tank.

Before DNA samples were loaded into the wells formed by the comb, they were mixed with a gel tracking dye (Appendix A) which served 3 purposes: the sucrose increased the density of the sample ensuring that the DNA remained in the well, the bromophenol blue in the dye allowed one to monitor the rate of migration of the DNA, and the EDTA chelated Mg^{2+} ions thereby inhibiting enzymatic activity (ie. it stops the reaction). Molecular weight markers were included so that the DNA fragment sizes could be determined. Molecular weight marker VI (Boehringer Mannheim; Mannheim, Germany) and/or the 1kb ladder (Promega; Madison, USA) were used in this study (Appendix B).Electrophoresis was performed at 2-10V/cm for 1-2 hours.

2.B.4. Genomic DNA extraction

Genomic DNA was extracted using the method described by Ausubel *et al.* (1987). A single colony of each *Acinetobacter* isolate was inoculated into 10ml of 2X Yeast-Tryptone (YT) broth (Appendix A) and incubated aerobically at 37^0C for ±18 hours. The bacterial cells were harvested in a Beckman Model J2-21 Centrifuge at 6000 rpm for 10 minutes. The bacterial cell pellets were resuspended in 567µl of Tris-EDTA buffer (Appendix A) by repeated pipetting and the cell walls lysed following the addition of 30µl of the detergent SDS (Appendix A). Three microlitres of proteinase K (20mg/ml) was added to digest the cellular proteins released as a result of cell wall lysis. This suspension was mixed thoroughly and lysis and protein digestion were allowed to continue at 37^0C for 1 hour. After lysis, the solution became viscous and 100µl of 5M NaCl (Appendix A) and 80µl of CTAB/NaCl (Appendix A) were added and the solution was mixed thoroughly and incubated at 65^0C for 10 minutes. This removed the cell wall debris, denatured/digested protein, and polysaccharides, which became complexed to CTAB, by selective precipitation. The presence of the 5M NaCl prevents the formation of a CTAB-nucleic acid precipitate and the nucleic acids thus remain in solution. The CTAB-protein/polysaccharide complexes were then

extracted with an equal volume of chloroform-isoamylalcohol (Appendix A). After centrifuging the mixture for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C) at 14000 rpm a white interface was visible. The aqueous phase was removed to a clean microfuge tube and the DNA was further purified by extraction with an equal volume of phenol-chloroform-isoamylalcohol (Appendix A) and centrifugation for 10 minutes at 14000 rpm in a microfuge (Eppendorf Centrifuge 5415C). This step was repeated until the aqueous phase was completely clear of the CTAB-protein/polysaccharide complexes. Once the aqueous phase was clear, the DNA was precipitated with 0.6 volumes of isopropanol. Shaking the tube back and forth allowed visualisation of stringy white DNA precipitate. This was collected by centrifugation for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C) at 14000rpm. With certain preparations, no stringy precipitate was visible since the DNA had been sheared during the extraction process. However, DNA could still be pelleted by centrifugation. DNA pellets were washed with 70% ethanol to remove residual CTAB and recentrifuged. The supernatant was carefully removed and the DNA was allowed to dry for 10-15 minutes at 37^0C. The DNA was then resuspended in 70-100µl TE buffer at 4^0C overnight.
The concentration of DNA in each genomic DNA preparation was determined using fluorescent quantitation. Ethidium bromide intercalates between DNA bases [section 2.B.3] and there is a relationship between the amount of fluorescence and the quantity of DNA in the sample –the more DNA in a given sample, the more it will fluoresce. To estimate the concentration of DNA in a sample it was subjected to AGE [section 2.B.3] alongside a sample of known DNA concentration. The fluorescence emitted by the sample of unknown DNA concentration was compared with that of the DNA of known concentration.

2.B.5 DNA-DNA hybridisation

2.B.5.a. DNA transfer

Ten micrograms of genomic DNA from each of the *Acinetobacter* isolates was transferred to a Hybond^{TM}-N^+ membrane (Amersham International; Buckinghamshire, UK) using the Minifold II Slot-Blot Apparatus (Schleicher

and Schuell; Germany). Each sample was made up to a 50µl volume with distilled water (dH_2O). The DNA was then denatured at 95^0C for 10 minutes and placed on ice to prevent renaturation. Fifty microlitres of transfer buffer, 20X SSC (Appendix A), which provides the ionic strength necessary for the binding of DNA to the nylon membrane (Sambrook *et al.*, 1989), was added to the denatured DNA. The slot-blot apparatus was then assembled according to the manufacturer's instructions with the Hybond^{TM}-N^+ membrane (prewet with 10x SSC) resting on filter paper (also prewet with 10X SSC). The samples were then carefully loaded into separate slots and a vacuum was applied until transfer to the membrane was complete. The apparatus was dismantled and the Hybond^{TM}-N^+ membrane with the DNA was removed. To denature the DNA, the membrane was placed on filter paper soaked in a solution of 1.5M NaCl and 0.5M NaOH for 5 minutes. Neutralisation was achieved by soaking it in a solution of 0.5M Tris pH 7.2, 1.5M NaCl, and 1mM EDTA for 5 minutes. After allowing the membrane to air-dry, the DNA was cross-linked to the membrane using UV light for 30 seconds at a wavelength of 254nm in a Hoefer Scientific Instruments (California, USA) UV Cross-linker. The membrane was stored in a sealed plastic sleeve at 4^0C until required.

2.B.5.b. Preparation of the *int1* probe

pGSH108 contains a 1.7kb insert which carries genes encoding ANT(2``) and integrase. Immediately downstream of the *int1* stop codon is a *Bam*HI site (G↓GATCC) and inside the structural gene are 2 *Sph*I sites (GCATG↓C). These two enzymes were used to cleave a 589bp *Bam*HI/*Sph*I fragment containing a portion of the *int1* structural gene (Elisha, 1991) from pGSH108 for use as a probe (Fig. 2.3.). This portion of the *int1* shares a 52% homology with the same portion of the *int2*. Four micrograms of pGSH108 were digested at 37^0C with 30 units (U) of *Bam*HI (Boehringer Mannheim; Mannheim, Germany) and 30U of *Sph*I (Boehringer Mannheim; Mannheim, Germany) in 1x buffer B (Boehringer Mannheim; Mannheim, Germany). This was allowed to continue for ±18 hours to ensure complete digestion of the plasmid.

*Bam*HI STOP

G↓GATCCCTAC CTCTCACTAG TGAGGGGCGG CAGCGCATCA AGCGGTGAGC GCACTCCGGC 60

ACCGCCAACT TTCAGCACAT GCGTGTAAAT CATCGTCGTA GAGACGTCGG AATGGCCGAG 120

CAGATCCTGC ACGGTTCGAA TGTCGTAACC GCTGCGGAGC AAGGCCGTCG CGAACGAGTG 180

GCGGAGGGTG TGCGGTGTGG CGGGCTTCGT GATGCCTGCT TGTTCTACGG CACGTTTGAA 240

GGCGCGCTGA AAGGTCTGGT CATACATGTG ATGGCGACGC ACGACACCGC TCCGTGGATC 300

GGTCGAATGC GTGTGCTGCG CAAAAACCCA GAACCACGGC CAGGAATGCC CGGCGCGCGG 360

ATACTTCCGC TCAAGGGCGT CGGGAAGCGC AACGCCGCTG CGGCCCTCGG CCTGGTCCTT 420

CAGCCACCAT GCCCGTGCAC GCGACAGCTG CTCGCGCAGG CTGGGTGCCA AGCTCTCGGG 480

TAACATCAAG GCCCGATCCT TGGAGCCCTT GCCCTCCCGC ACGATGATCG TGCCGTGATC 540

*Sph*I

GAAATCCAGA TCCTTGACCC GCAGTTGCAA ACCCTCACTG ATCCGCATG↓C CCGTTCCATA 600

CAGAAGCTGG GCGAACAAAC GATGCTCGCC TTCCAGAAAA CCGAGGATGC GAACCACTTC 660

ATCCGGGGTC AGCACCACCG GCAAGCGCCG CGACGGCCGA GGTCTTCCGA TCTCCTGAAG 720

CCAGGGCAGA TCCGTGCACA GCACCTTGCC GTAGAAGAAC AGCAAGGCCG CCAATGCCTG 780

ACGATGCGTG GAGACCGAAA CCTTGCGCTC GTTCGCCAGC CAGGACAGAA ATGCCTCGAC 840

TTCGCTGCTG CCCAAGGTTG CCGGGTGACG CACACCGTGG AAACGGATGA AGGCACGAAC 900

CCAGTGGACA TAAGCCTGTT CGGTTCGTAA GCTGTAATGC AAGTAGCGTA TGCGCTCACG 960

START

CAACTGGTCC AGAACCTTGA CCGAACGCAG CGGTGGTAAC GGCGCAGTGG CGGTTTT**CAT** 1020

GGCTTGTTAT GACTGTTTTT TTGGGGTACA GTCTATGCCT CGGGCATCCA AGCAGCAAGC 1080

GCGTTACGCC GTGGGTCGAT GTTTGATGTT ATGGAGCAGC AACGATGTTA CGCAGCAGGG 1140

CAGTCGCCCT AAAACAAAGT TAGGCCGCAT GGACACAACG CAGGTCACAT TGATACACAA 1200

AATTCTAGCT GCGGCAGATG AGCGAAATCT GCCGCTCTGG ATCGGTGGGG GCTGGGCGAT 1260

CGATGCACGG CTAGGGCGTG TAACACGCAA GCACGATGAT ATTGATCTGA CGTTTCCCGG 1320

CGAGAGGCGC GGCGAGCTCG AGGCAATAGT TGAAATGCTC GGCGGGCGCG TCATGGAGGA 1380

GTTGGACTAT GGATTCTTAG CGGAGATCGG GGATGAGTTA CTTGACTGCG AACCTGCTTG 1440

GTGGGCAGAC GAAGCGTATG AAATCGCGGA GGCTCCGCAG GGCTCGTGCC CAGAGGCGGC 1500

TGAGGGCGTC ATCGCCGGGC GGCCAGTCCG TTGTAACAGC TGGGAGGCGA TCATCTGGGA 1560

TTACTTTTAC TATGCCGATG AAGTACCACC AGTGGACTGG CCTACAAAGC ACATAGAGTC 1620

*Sph*I

CTACAGGCTC GCATG↓CACCT CACTCGGGGC GGAAAAGGTT GAGGTCTTGC GTGCCGCTTT 1680

CAGGTCGCGA TATGCGGCCT AACAATTCGT CCAAGCCGAC GGCTTCGCGG CGCGGCTTAA 1740

CTCAGGTGTT AGGCCGCATG GACACAGGCA TGCAAG 1776

Fig. 2.3.: Sequence of the pGSH108 insert showing the 589bp *int1* probe. The *Bam*HI and *Sph*I sites and the start and stop codons of the integrase gene are indicated. The blocked off area corresponds to the 589bp *int1* probe sequence (Elisha, 1991; Elisha and Steyn, 1991a).

Ten microlitres of gel tracking dye was added and the digest was subjected to AGE [section 2.B.3] for 75 minutes at 80V. The band corresponding to 589bp was excised and the DNA was eluted from the agarose: the agarose was cut into very small pieces and placed in a microfuge tube. An equal volume of phenol was added to solubilize the agarose (Sambrook *et al.*, 1989) as described by Seth (1984) and mixed by vortexing the tube. The tube was placed at -70^0C for 15 minutes and then centrifuged at 14000rpm for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C). After centrifugation, the upper aqueous phase containing the DNA was removed and the DNA was further purified by extraction with phenol-chloroform-isoamylalcohol as described by Sambrook *et al.* (1989). An equal volume of phenol-chloroform-isoamylalcohol was added and, after mixing, the solution was centrifuged as before to remove any residual agarose and phenol. The DNA in the resulting supernatant was concentrated by precipitation with 1/10 volume of 4M LiCl (Appendix A) and 3 volumes of 100% ethanol. After placing the tube at -70^0C for ~10 minutes to allow for precipitation, the DNA was collected by centrifugation as before. The resulting DNA pellet was washed in 70% ethanol, dried at 37^0C for 10-15 minutes, and resuspended in 20µl of dH_2O. The DNA was allowed to resuspend for ≥30 minutes and, following quantitation [section 2.B.4], 500ng of the 589bp *Bam*HI/*Sph*I *int1* fragment was labelled using the ECL™ direct nucleic acid labelling and detection system (Amersham International; Buckinghamshire, UK), according to the manufacturer's instructions (Fig. 2.4.): the DNA fragment was denatured by boiling for 5 minutes, snap cooled on ice for a further 5 minutes and labelled with horseradish peroxidase (which is responsible for the loose attachment formed between the label and the DNA), complexed with a positively charged polymer. Addition of glutaraldehyde resulted in the formation of chemical cross-links between the DNA and the peroxidase-polymer complex so that the enzyme was covalently attached to the fragment. The labelled fragment was used in hybridisation experiments.

2.B.5.c DNA-DNA hybridisation

The 589bp *int1* probe was hybridised to the genomic DNA immobilized on the Hybond^{TM}-N^{+} membrane [section 2.B.5.a] using a modification of the protocol for the ECL direct nucleic acid labelling and detection system: a volume of hybridisation buffer(Appendix A) equivalent to 0.125ml/cm^2 of membrane was used. A blocking agent which is designed to pre-coat all the sites on the membrane to which the probe would bind non-specifically, was added to the buffer to a final concentration of 5% (w/v). NaCl was also added to a final concentration of 0.5M. The hybridisation was performed in a Hybridiser HB-1D oven (Techne; Cambridge, UK) at 42^{0}C.

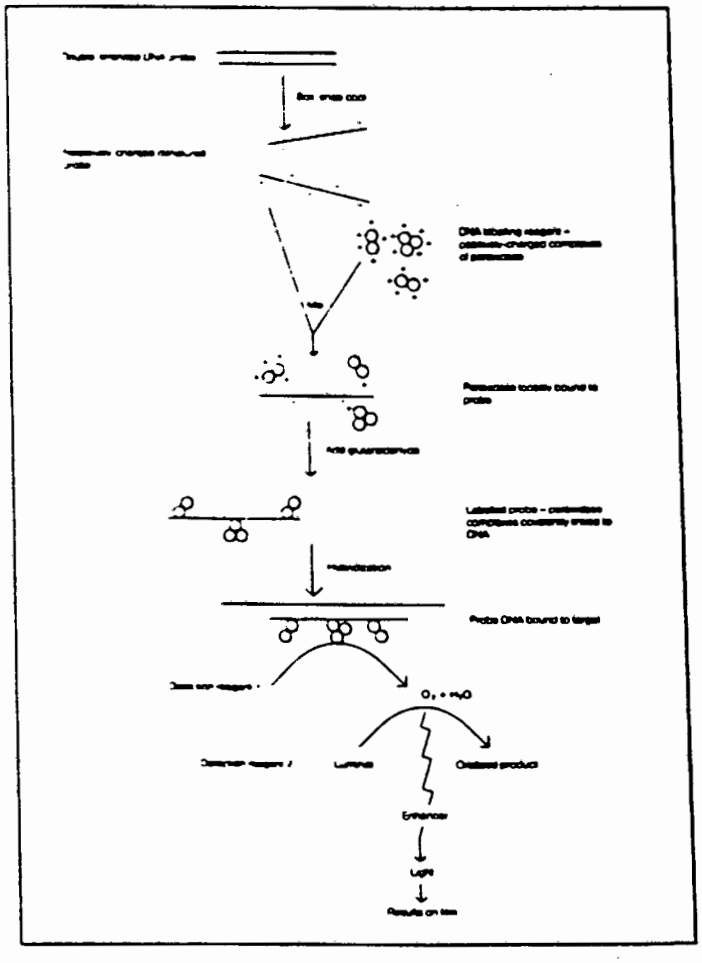


Fig. 2.4.: Principles of the ECL direct nucleic acid labelling and detection system (taken from the kit manual)

Prehybridisation was allowed to continue for ~1 hour, during which time the non-specific binding sites on the membrane were blocked. Thereafter, the probe was added to the buffer and hybridisation was allowed to continue overnight at 42^0C.

After hybridisation, the membrane was washed to remove unbound, excess probe. Firstly, the membrane was rinsed in 50-100ml of 5X SSC at 42^0C. Thereafter it was washed twice in 100ml of wash buffer (Appendix A) containing 6M urea, 0.4% SDS, and 0.1x SSC, at 55^0C for 10 minutes. Using a buffer containing urea and a low concentration of SSC and washing at 55^0C increased the stringency of the wash, thereby creating conditions which allow for the formation of only well-matched hybrids of probe and target DNA. The third wash was performed twice at room temperature (RT) on a Stovall Low Profile Roller (Stovall Life Science, Inc; North Carolina, USA) for 5 minutes in ±100ml 2X SSC.

Signal generation and detection were performed following the protocol supplied by the manufacturers of the ECL kit. Detection reagents 1 and 2 were mixed in equal volumes to give enough solution to cover the membrane. On completion of the post-hybridisation washes, excess buffer was drained from the membrane, which was then flooded with the detection reagent mix and allowed to incubate for 1 minute at room temperature. Excess detection reagent was drained from the membrane, which was sealed between two pieces of plastic. Detection reagent 1 decays to hydrogen peroxide, the substrate for peroxidase - with which the probe was labelled [section 2.B.5.c]. Reduction of the hydrogen peroxide by peroxidase is coupled to a light-producing reaction catalysed by detection reagent 2, which contains luminol and produces blue light on oxidation. An enhancer prolongs and increases the light output which can be detected on a blue-light sensitive film. The membrane was exposed to X-ray film (AGFA CP-BU; Mortsel, Belgium) for 2 hours and the autoradiograph was developed.

2.C. RESULTS

2.C.1. Antibiotic sensitivity testing

The antibiotic resistance profile of the 57 *Acinetobacter* isolates are shown in Table 2.2.

Table 2.2.:Antibiotic resistance profile of the *Acinetobacter* isolates

Isolate number	Antibiotic resistance profile	Blot[a]	PCR[b]
G1	Gent, Amox, Coam, Chlo, Cfox, Crox	+	+
G2	Amik, Gent, Amox, Coam, Ctri, Chlo, Cfox, Crox	+	+
G3	Amik, Gent, Amox, Coam, Ctri, Chlo, Cfox, Crox	+	+
G4	Amik, Gent, Amox, Ctri, Chlo, Cfox, Crox	+	+
G5	Amik, Gent, Amox, Coam, Pip, Ctri, Chlo, Cfox, Crox	+	+
G6	Amik(I), Gent, Amox, Ctri, Chlo, Cfox, Crox	+	+
G7	Amik	-	-
G8	Gent, Pip, Ctri, Chlo, Oflo, Cpim(I), Ctaz(I)	-	-
G9	Amik, Gent, Amox, Coam, Ctri, Chlo, Cfox, Crox	-	-
G10	Amik, Gent, Chlo, Cpim	-	-
G11	Amik, Gent, Chlo, Cpim	-	-
G12	Gent, Chlo	+	+
G13	Amik, Oflo	-	-
G14	Amik, Gent, Chlo	-	-
G15	Gent, Pip, Ctri, Chlo, Cpim(I), Ctaz	+	+
G16	Amik	-	-
G17	Amik, Gent, Chlo, Cpim, Ctaz(I)	-	-
G18	Oflo	-	-
G19	Amik, Gent, Chlo, Cpim	-	-
G20	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo	+	+
G21	Gent(I), Pip, Oflo, Cipr, Ctaz	-	-
G23	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo, Cpim, Ctaz(I)	+	+
G24	Amik, Gent, Ctri, Chlo, Cipr, Oflo	+	-
G25	Gent, Chlo	+	-
G26	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo	+	-
G27	Amik, Gent, Chlo, Cpim	-	-
G28	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo	+	-
G29	Gent, Pip, Chlo	+	-
G30	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo	+	-
G31	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo	-	-
G32	Amik, Gent, Pip, Ctri, Chlo, Oflo, Cpim, Ctaz, Mero	+	-
G33	Amik, Gent, Cpim, Ctaz	-	-
G34	Gent, Pip, Ctri, Chlo, Cipr, Oflo, Cpim, Ctaz	+	-
G35	Amik, Gent, Pip, Ctri, Chlo, Oflo, Cpim	+	-
G36	Amik, Gent, Pip, Ctri, Chlo, Oflo, Cpim	+	-
G37	Amik, Gent, Pip, Ctri, Chlo, Oflo, Cpim	+	+
G38	Amik(I), Gent, Pip, Ctri, Chlo, Cipr(I), Oflo(I), Ctaz	+	-
G39	Amik, Gent, Pip, Ctri, Chlo, Cipr, Oflo, Cpim, Ctaz(I)	+	+
G58	Amik, Gent	-	-
G60	Amik	-	-
G61	Amik	-	-
G95	Amik	-	-
G98	Amik, Gent	-	-
B1	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax, Ctaz	+	+
B2	Gent, Amp, Pip, Chlo, Cfox, Ctaz	+	+
B3	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax, Ctaz	+	+
B4	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax, Ctaz	+	+
B5	Gent, Amp, Pip, Ctri, Chlo, Ctax	+	-
B6	Amp, Ctri, Chlo, Cfox, Ctax, Ctaz	+	+
B7	Gent(I), Amp, Chlo, Cfox Ctax	-	-
B8	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax	+	+
B9	Gent, Amp, Pip, Ctri, Chlo, Ctax	+	+
B10	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax, Ctaz	+	+
B11	Gent, Amp, Pip, Ctri, Chlo, Ctax	+	+
B12	Gent, Amik, Amp, Pip, Chlo, Cfox, Ctax, Ctaz	+	+
B13	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax, Ctaz(I)	+	+
B14	Gent, Amp, Pip, Ctri, Chlo, Cfox, Ctax(I), Ctaz	+	+

Amik, amikacin; Amox, amoxicillin; Amp, ampicillin; Cpim, cefapime; Cpri, ciprofloxacin; Czol, cefazolin; Ctax, cefotaxime; Ctaz, ceftazidime, Cfox, cefoxatin; Crox, cefuroxine; Chlo, chloramphenicol; Coam, coamoxiclav; Ctri, cotrimoxazole; Gent, gentamicin; Impn, imipenem; Mero, meropenem; Oflo, ofloxacin; Oxa, oxacillin; Pen, penicillin; Pip, piperacillin/tazobactam; I, Intermediate resistance; [a] Results of the hybridisation (p36); + = signal, - = no signal; [b] Results of the PCR assay (p49); + = product, - = no product

2.C.2. DNA-DNA hybrisation with the *int1* probe

The *int1* probe gave no signal with the DNA from the integron-free strains, *E.coli* DH5α and *A.calcoaceticus* BD413 C91. A signal was obtained with pGSH108, the source of the probe. The probe hybridised to genomic DNA from 23/43 acinetobacters from GSH and 13/14 isolates from USH, suggesting the presence of integrons in these strains (Fig. 2.5.)

	A	B	C
1	NT	G19	G58
2	pGSH108	G20	G60
3	NT	G21	G61
4	NT	G23	G95
5	G1	G24	G98
6	G2	G25	NT
7	G3	G26	B1
8	G4	G27	B2
9	G5	G28	B3
10	G6	G29	B4
11	G7	G30	B5
12	G8	G31	B6
13	G9	G32	B7
14	G10	G33	B8
15	G11	G34	B9
16	G12	G35	B10
17	G13	G36	B11
18	G14	G37	B12
19	G15	G38	B13
20	G16	G39	B14
21	G17	NT	NT
22	G18	NT	NT
23	NT	NT	NT
24	NT	DH5α	C91

pGSH108 = positive control

DH5α and C91 = negative controls

NT = lane left blank

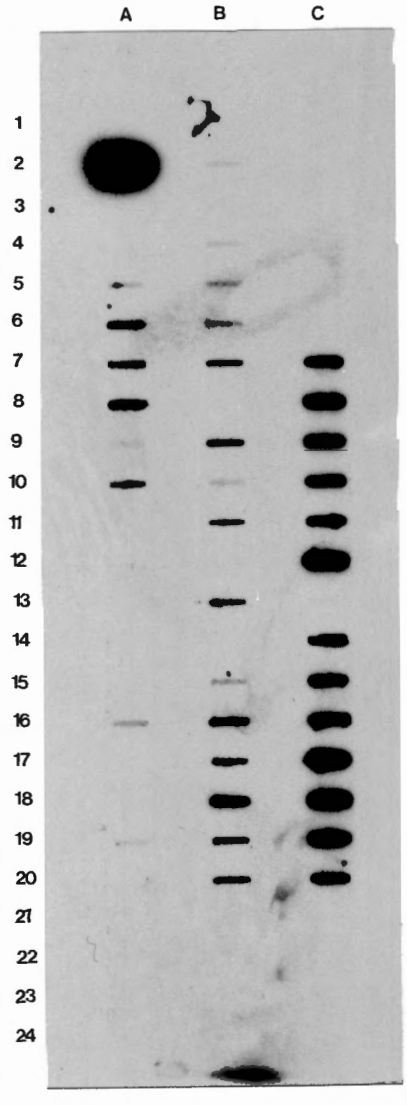


Fig. 2.5.: Autoradiograph of the slot blot of the genomic DNA from the clinical *A.baumannii* isolates hybridised to the *int1* probe.
pGSH108 plasmid DNA from which the probe was derived was used as a positive control. Genomic DNA from *E.coli* DH5α and *A.calcoaceticus* BD413 C91 were used as negative controls. Autoradiography was for 2 hours.

2.D. DISCUSSION

The hybridisation studies indicate the presence of integrons in 53.5% of the GSH isolates and 92.9% of the UH isolates (63.2% overall). As a number of antibiotic resistance patterns were observed in the strains it was not possible to associate a particular phenotype with the presence of an integron. Nevertheless, 35 of the isolates that were resistant to gentamicin gave a signal with the *int1* probe (see Table 2.2.). The incidence of *int1* observed in this study is higher than that found in acinetobacter isolates investigated by Tait and Amyes (1994). They investigated the presence of Tn*21*-like transposons and the integron elements (class 1-type integrons) associated with this family of transposons in various isolates of *Acinetobacter* spp. collected in Britain and the Netherlands. They performed colony- and dot-blot hybridisations on these isolates using a probe for the integrase gene. Of the 108 isolates (67 blood culture isolates and 41 isolates collected from various sites) from Edinburgh Royal Infirmary, none of the blood culture isolates hybridised to the integrase probe, however, a positive signal was obtained with the DNA from 2 of the other isolates. This set of isolates thus has a 1.8% incidence of *int1*. Further hybridisation studies on the integrase positive isolates, using probes directed against Tn*21*-associated genes (*tnpA*, *tnpR*, and *tnpM*) were carried out. These studies suggested that the integron in one of the *Acinetobacter* isolates is associated with Tn*21*, since a signal was obtained with each of the probes. The other isolate had only *tnpM* in addition to *int1*.

Acinetobacter strains from outbreaks in Aberdeen, Glasgow, Basildon and Utrecht were also examined. A total of 11 strains gave a positive signal with the *int1* probe: 1 from Glasgow, 3 from Aberdeen, 2 from Basildon, and 5 from Utrecht. Hybridisation studies indicated that the integrons from only 2 of the acinetobacters (1 from Glasgow and 1 from Basildon) are associated with Tn21-like transposons. In addition, 2 isolates, provided by W. Noble (the origins of which were unknown), were also studied. Both were found to be positive for integrase and were Tn*21*-associated.

In a recent study, Gonzalez *et al.* (1998) determined the presence of class 1- and class 2-type integrons in 100 isolates of multi-resistant isolates of *A.baumannii,* by hybridisation with probes specific for the *int1* and *int2* genes. They found *int1* in only 5.1% of the isolates (biotype 9) -much lower than the incidence observed for this study (63.2%) - and *int2* in 52.6% of biotype 9 and 16.7% of biotype 8 isolates. Positive hybridisation for both *int1* and *int2* was obtained for 23.7% of biotype 9 isolates. *A.baumannii* biotype 9 was the most prevalent biotype in Chilean hospitals. The biotype 9 isolates were multi-resistant and had the highest frequency of integron carriage. The authors suggest that the biotype 9 isolates may be favouring the spread of and act as a reservoir for resistance gene cassettes in the hospital environment.

CHAPTER 3: POLYMERASE CHAIN REACTION ASSAY TO DETERMINE THE PRESENCE OF INTEGRON-ASSOCIATED ANTIBIOTIC RESISTANCE GENES

3.A. INTRODUCTION

To study the contents of the variable regions of integrons, Levesque *et al.* (1995) designed primers directed at the 5`- and 3`-conserved regions of class 1-type integrons for use in polymerase chain reaction (PCR) assays. Using these primers (5`-CS and 3`-CS, respectively), they identified integron-related antibiotic resistance genes in 75% of aminoglycoside resistant strains of *Enterobacteriacea* (including *Klebsiella pneumoniae, Enterobacter cloacae, Serratia marcescens, Salmonella typhimurium, Escherichia coli, and Proteus mirabilis*) and *Pseudomonas aeruginosa.* They obtained amplification products of ~1kb to ~3.5kb. The larger products encoded as many as 4 antibiotic resistance genes (Table 3.1.).

Table 3.1.: PCR amplification products obtained by Levesque *et al.* (1995) using the 5`-CS and 3`-CS primers
(adapted from Levesque *et al.*, 1995)

Bacterial strain	Length of product (bp)	Gene order in variable region
K.pneumoniae	1000	*ant(3``)-Ia*
E.cloacae	1600	*ant(2``)-Ia – ant(3``)-Ia*
S.marcescens I	1600	*ant(2``)-Ia – oxa2*
S.marcescens II	1600	*dfrI – ant(3``)-Ia*
S.typhimurium	2000	*aac(6`)-Ib – ant(3``)-Ia*
E.coli	3000	*aac(3)-Ia – orfE – ORF – ant(3``)-Ia*
P.mirabilis	3500	*aac(3)-Ia – orfE – ant(3``)-Ib - cmlA*

In another study, Vila *et al.* (1997) used the same primers to amplify a PCR product of ~1.5kb from genomic DNA isolated from *Acinetobacter* (strain Ab41). DNA sequencing of the product showed that the integron contains an *ant(2``)-Ia* (aminoglycoside adenylyltransferase) gene followed by a novel OXA-derived β-lactamase gene.

The 36 *Acinetobacter* isolates (23 from GSH and 13 from UH) which were positive for the presence of the integrase gene in hybridisation studies [Chapter 2] were subjected to a polymerase chain reaction (PCR) assay using the primers described by Levesque *et al.* (1995).

3.B. MATERIALS AND METHODS

3.B.1. Isolation of DNA

Genomic DNA was isolated as described [section 2.B.4]. Alternatively, DNA was prepared as follows: a bacterial colony was suspended in 100µl distilled water. To release the genomic DNA, the supension was boiled vigorously for 10-15 minutes. Cellular debris was removed by centrifugation in a microfuge (Eppendorf Centrifuge 5415C) for 2 minutes at 14000rpm. Five microlitres of the lysate was used in the PCR (Grimberg *et al.*, 1989).

3.B.2. PCR primers

The sequences of the primers directed against the 5`- and 3`-conserved sequences of type 1 integrons were obtained from R.Seward and K.J. Towner (Department of Microbiology and PHLS Laboratory, University Hospital, Queen's Medical Centre, Nottingham) and have been described by Levesque *et al.* (1995). The 5`-CS primer, 5`-GGCATCCAAGCAGCAAG-3` (corresponding to nucleotides 1190 to 1206 in the sequence published by Bissonnette and Roy, 1992), and the 3`-CS primer, 5`-AAGCAGACTTGACCTGA-3` (nucleotides 1342 to 1326 in the same sequence), flank the variable region which contains the gene cassettes (fig.3.1.).

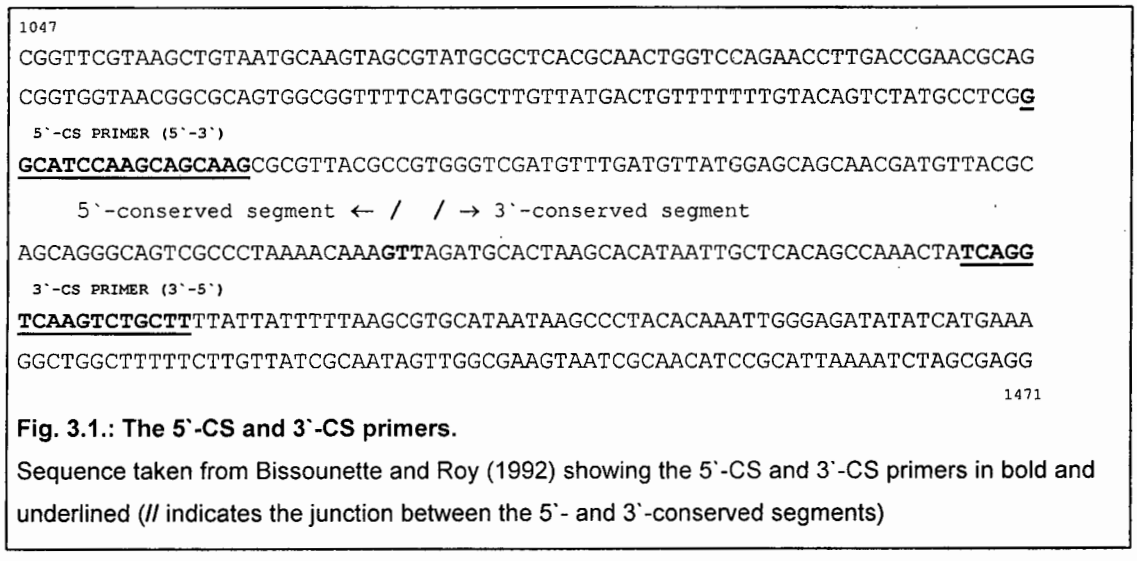


Fig. 3.1.: The 5`-CS and 3`-CS primers.

Sequence taken from Bissounette and Roy (1992) showing the 5`-CS and 3`-CS primers in bold and underlined (*//* indicates the junction between the 5`- and 3`-conserved segments)

3.B.3. PCR Assay

The polymerase chain reaction (PCR) is the primer-directed enzymatic amplification of DNA using a thermostable DNA polymerase isolated from *Thermus aquaticus*, called *Taq* DNA polymerase (Saiki *et al.*, 1988). The reaction involves cycles of template denaturation, primer annealing, and DNA strand extension by the polymerase (Saiki *et al.*, 1988). Thermostable DNA polymerase is used since it is not denatured or inactivated at the high temperatures required to denature the double-stranded template (Saiki *et al.*, 1988). Fig. 3.2. illustrates the principles of PCR

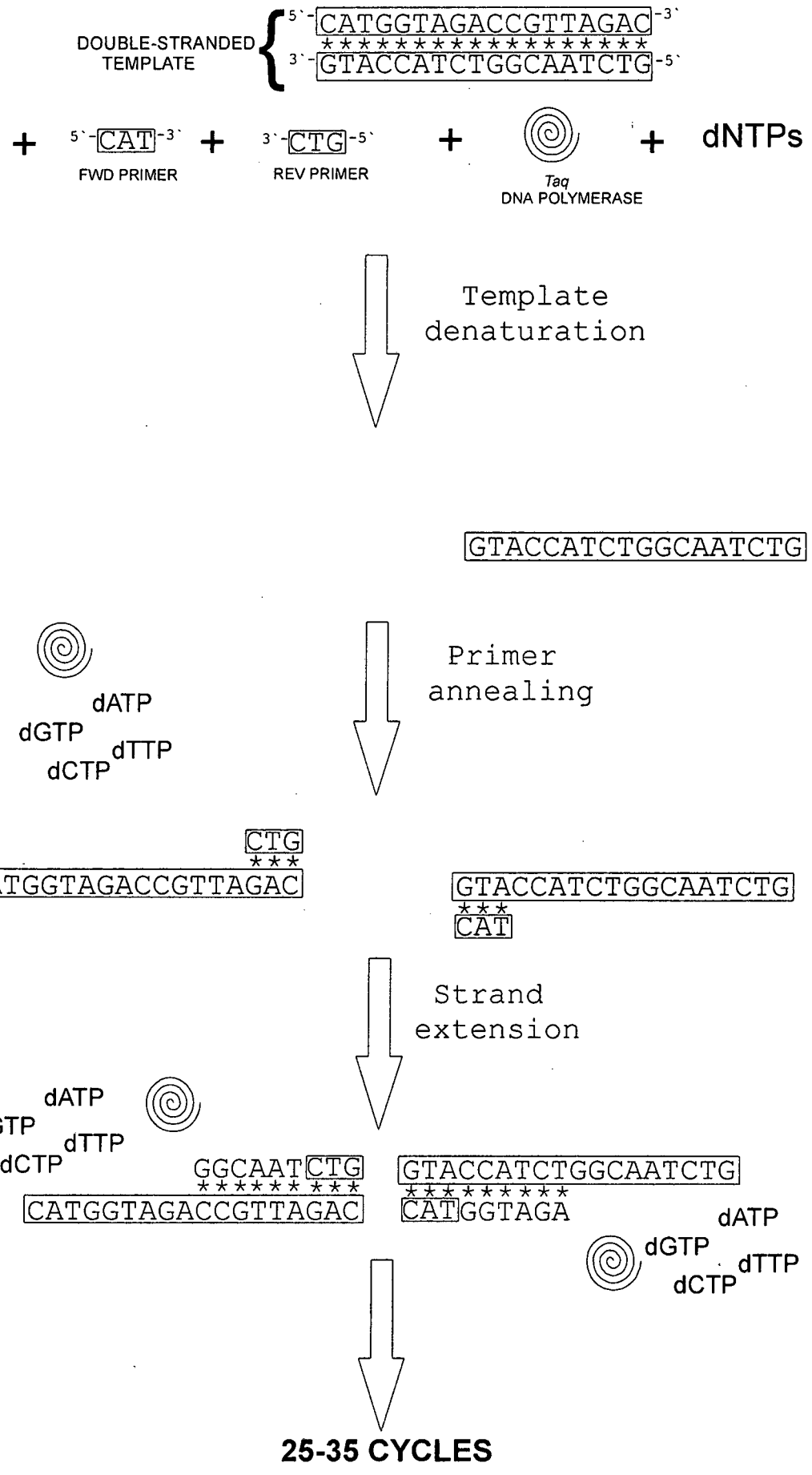


Fig. 3.2: Schematic diagram of the polymerase chain reaction (PCR) (adapted from Boehringer Mannheim PCR Applications Manual)

The initial reaction components and thermocycle profile were those recommended by Seward and Towner and consisted of a 25μl amplification mix with 1x *Taq* DNA polymerase buffer (Boehringer Mannheim; Mannheim, Germany) containing 1.5mM $MgCl_2$, 0.2mM of each dNTP (Boehringer Mannheim; Mannheim, Germany), 1U *Taq* DNA polymerase (Boehringer Mannheim; Mannheim, Germany) and 2.5pmol of each primer. DNA obtained using the crude boiling method of preparation was utilised as template (Grimberg *et. al.*, 1989). The thermocycle profile consisted of three steps: 1 minute of denaturation at 94^0C, 1 minute of annealing at 55^0C, and 5 minutes of extension at 72^0C; for 35 cycles. The reactions were performed in a Perkin Elmer (California, USA) Model 24600 PCR machine. Inconsistent results were obtained using these parameters making it necessary to optimise both the reaction components and the thermocycle profile.

Amplification products were analysed by electrophoresis [section 2.B.3]. Five to ten microlitres of gel tracking dye were added to the PCR products and 20μl of this mix was loaded onto the gels.

3.C. RESULTS

3.C.1. Optimisation of the PCR assay

Using the conditions recommended by Seward and Towner, the assay was not reproducible and often yielded multiple products. The reaction components and thermal cycle were optimised. The optimisation strategy is summarised in Table 3.2.

Table 3.2.: PCR assay optimisation strategy

	Total volume (µl)	Primer conc (pmol)	$MgCl_2$ conc (mM)	DNTP conc (mM)	Taq pol. (U)	Pol buffer	Template	Thermocycle Denat	Anneal (T^0C/min)	Extension	Cycles
A	25	2.5	1.5	0.2	1	1X	Crude	94/1	55/1	72/5	35
B	50	5	1.5	0.2,0.3,0.4	1	1X	Crude	94/1	55/1	72/1	35
	50	5	1.75	0.2,0.3,0.4	1	1X	Crude	94/1	55/1	72/1	35
	50	5	2.0	0.2,0.3,0.4	1	1X	Crude	94/1	55/1	72/1	35
	50	5	2.25	0.2,0.3,0.4	1	1X	Crude	94/1	55/1	72/1	35
	50	5	2.5	0.2,0.3,0.4	1	1X	Crude	94/1	55/1	72/1	35
C	50	5	2.25	0.2	1	1X	Crude	94/1	57.5/1	72/1	35
	50	5	2.25	0.2	1	1X	Crude	94/1	60/1	72/1	35
	50	5	2.25	0.2	1	1X	Crude	94/1	62/1	72/1	35
D	50	5	2.25	0.2	1	1X	Genomic	94/1	60/1	72/1	35

A = Recommended conditions
B = $MgCl_2$ and dNTP titrations
C = Annealing temperature changes
D = Conditions used in the PCR assays
Genomic template = 500ng

Template obtained from *A.baumannii* strain CW20, using the "boiling method," was used for optimisation reactions. Empirical changes were to double the reaction volume and increase the primer concentration to 5pmol for each primer. These changes did not have an effect on the results. $MgCl_2$ and dNTP titrations were performed to determine the optimum concentration of these components. The extension time was reduced to 1 minute in order to speed up the amplification and to test whether a 5 minute extension period was necessary – reducing the time did not affect the results. The $MgCl_2$ was titrated across the following concentrations: 1.5mM, 1.75mM, 2mM, 2.25mM, and 2.5mM. For each of these $MgCl_2$ concentrations, dNTP concentrations of 0.1mM, 0.2mM, and 0.3mM were used. As can be seen in Fig. 3.3., although amplicons were obtained for all $MgCl_2$ and dNTP concentrations, the highest yield was at concentrations of 2.25mM $MgCl_2$ and 0.2mM dNTPs (lane 10). The annealing temperature was raised to increase the specificity of the primers. Temperatures of 57.5^{0}C, 60^{0}C and 62^{0}C were tested. The highest yield was obtained at 60^{0}C (Fig. 3.4., lane 2). Although the optimisation was performed using DNA prepared by the "boiling method", when the PCR was performed on clinical isolates using a crude template, amplicons were not obtained. Genomic DNA was therefore used as a template in test assays.

Lane	[$MgCl_2$] mM	[dNTP] mM
1	1.5	0.1
2	1.75	0.1
3	2.0	0.1
4	2.25	0.1
5	2.5	0.1
6	1.5	0.2
7	1.75	0.2
8	2.0	0.2
9	2.25	0.2
10	2.5	0.2
11	1.5	0.3
12	1.75	0.3
13	2.0	0.3
14	2.25	0.3
15	2.5	0.3

Lane 16 = molecular weight marker VI
Lane 17 = dH_2O negative control
[$MgCl_2$]=1.5mM; [dNTP]=0.2mM

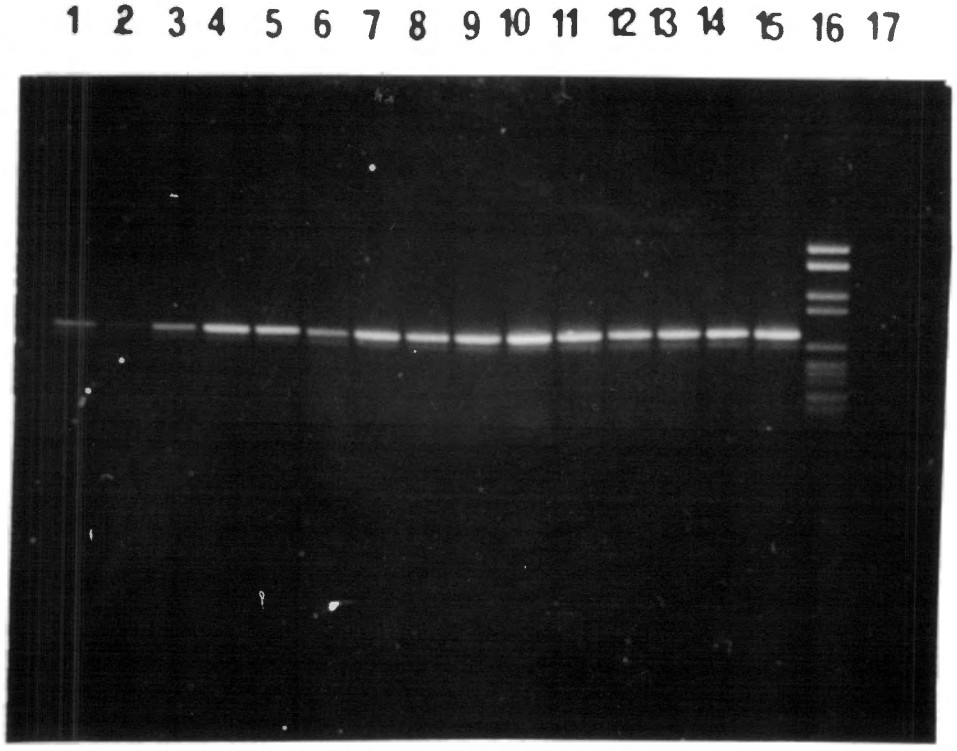


Fig. 3.3.: Optimisation of the PCR assay: $MgCl_2$ and dNTP titrations.

PCR was performed in 50µl volumes containing 5pmol of each primer, 1U of *Taq* DNA polymerase, 1X polymerase buffer, and 5µl of crude template (*A.baumannii* CW20 genomic DNA). The $MgCl_2$ and dNTP concentrations were varied as indicated. The thermocycle involved 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, and extension at 72°C for 1min. Products were electrophoresed on a 1% agarose gel at 80V for 1 hour. See Appendix B for sizes of the bands from molecular weight marker VI.

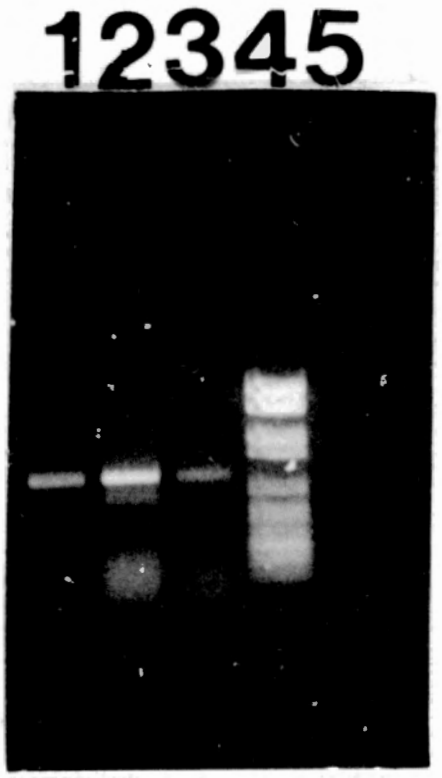


Fig. 3.4.: Optimisation of the PCR assay: annealing temperature

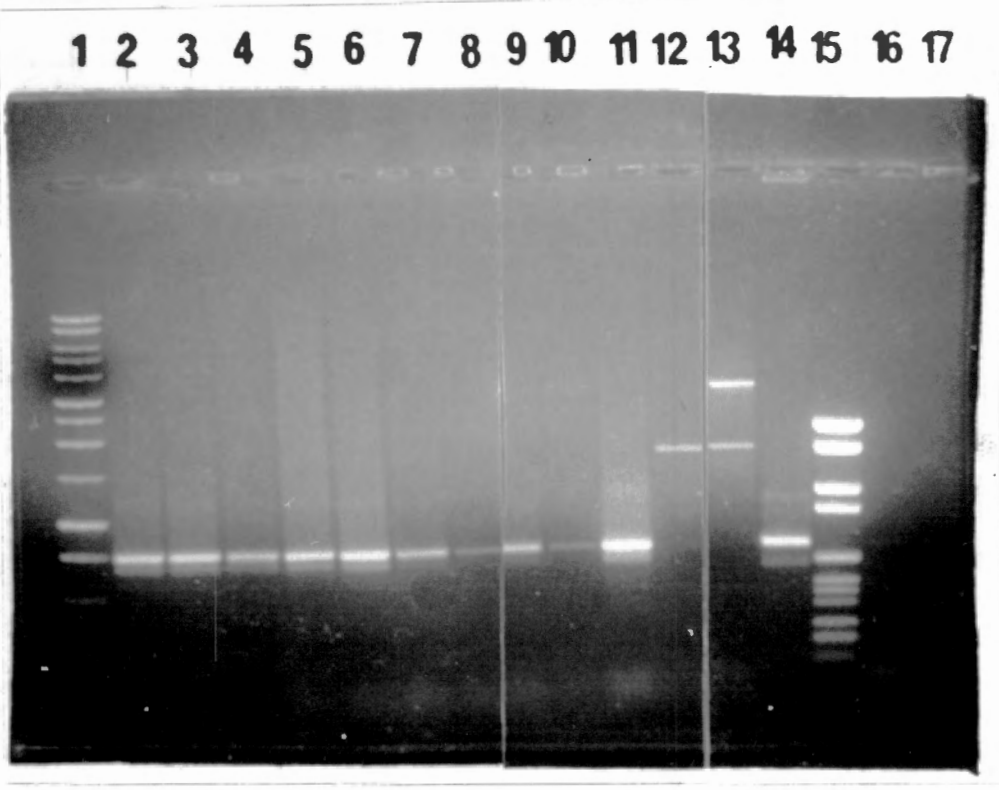
Lane 1, 57.5^{0}C; lane 2, 60^{0}C; lane 3, 62^{0}C; lane 4, molecular weight marker VI; and lane 5, dH_2O at 60^{0}C. PCR was performed in 50µl volumes containing 5pmol of each primer, 2.25mM $MgCl_2$, 0.2mM dNTPs, 1U *Taq* DNA polymerase, 1X polymerase buffer, 5µl crude template (*A.baumannii* CW20 genomic DNA). The thermocycle consisted of 35 cyces of denaturation at 94^{0}C for 1min, annealing at 57.5^{0}C, 60^{0}C,or 62^{0}C for 1min, and extension at 72^{0}C for 1min. Products were electrophoresed on a 1% agarose gel at 80V for 1 hour. See Appendix B for sizes of the bands of molecular weight marker VI.

On completion of optimisation, assay conditions and the thermocycler profile were as follows: 50µl volumes containing 1x BioTaq^{TM} Buffer (Bioline; London, UK) without $MgCl_2$, 1U BioTaq ^{TM} DNA polymerase (Bioline, London, UK), 5pmol of each primer, 0.2mM deoxynucleoside triphosphate mix (Boehringer Mannheim; Mannheim, Germany), 2.25mM $MgCl_2$ (Bioline; London, UK) and 500ng of genomic DNA. The reaction profile consisted of 5 minutes of initial denaturation at 95^{0}C followed by 1 minute of denaturation at 94^{0}C, 1 minute of annealing at 60^{0}C, and 1 minute of extension at 72^{0}C for 35 cycles.

3.C.2. PCR assay

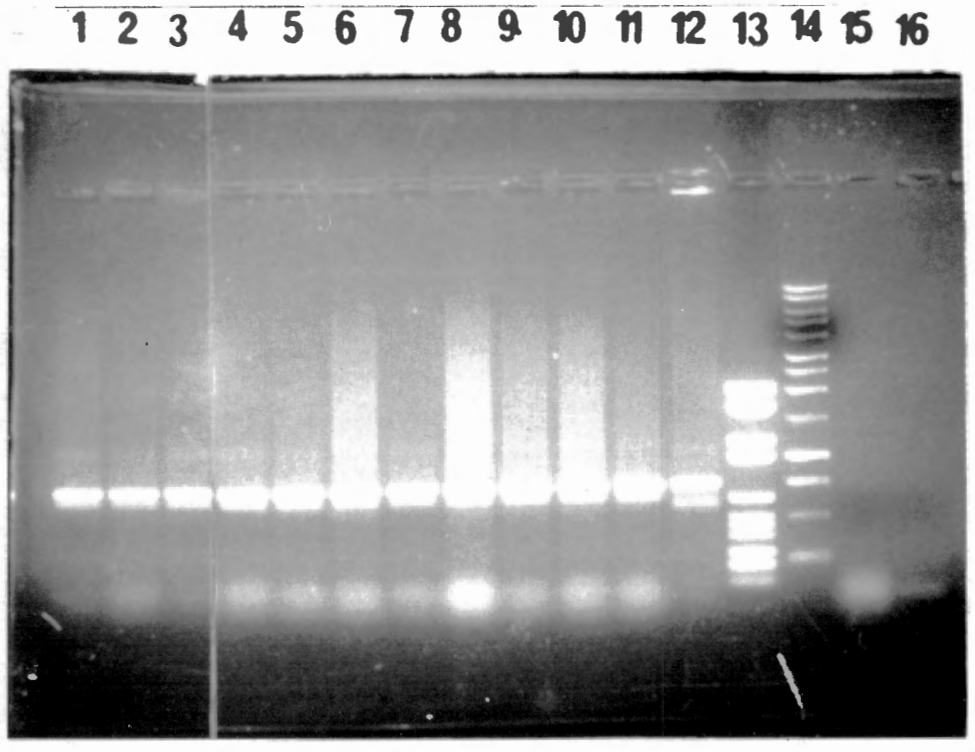
Assays were carried out on the strains which gave a signal with the *int1* probe. Using primers directed at the 5`- and 3`-conserved sequences of integrons, a product of the correct size (0.7kb) was obtained from the control strain *A.baumannii* CW20 (Fig 3.5.A; Table 2.2.). Of the 36 isolates positive for the presence of the integrase gene (Table 2.2), 24 (67%) yielded PCR products.

A product of 0.7kb was obtained from 10/23 and 12/13 of the acinetobacters from GSH and UH, respectively, indicating the presence of a single cassette in the variable region. A product of 2kb was obtained from 1 GSH strain (fig.3.5.A., lane 12); suggesting that this acinetobacter has an integron carrying at least 2 gene cassettes. Two isolates, one from GSH and one from UH, yielded 2 amplicons. The isolate from GSH (fig.3.5.A., lane 13), yielded a 2kb and a 3.5kb product, suggesting that this strain contains 2 integrons; one with 2 gene cassettes and one with 3. The isolate from UH (fig.3.5.B., lane 12) yielded a 0.65kb product in addition to its 0.7kb product, suggesting that it contains 2 integrons with one gene cassette in each.



A.

Lane	Sample
1	1kb ladder
2	G1
3	G2
4	G3
5	G4
6	G5
7	G6
8	G12
9	G15
10	G20
11	G21
12	G37
13	G39
14	CW20
15	MW VI
16	DH5α
17	dH_2O



B.

Lane	Sample
1	B1
2	B2
3	B3
4	B4
5	B6
6	B8
7	B9
8	B10
9	B11
10	B12
11	B13
12	B14
13	MW VI
14	1kb ladder
15	DH5α
16	dH_2O

Fig.3.5.: PCR products obtained from the genomic DNA of the clinical *A.baumannii* isolates. (A) Isolates from GSH; (B) Isolates from UH

The products were electrophoresed on 1% agarose gels at 80V for 1.5 hours. PCR was performed in 50µl volumes containing 5pmol of each primer, 2.25mM $MgCl_2$, 0.2mM dNTPs, 1U *Taq* DNA polymerase, 1X polymerase buffer, and 500ng genomic DNA. The thermocycle profile consisted of 35 cycles denaturation at 94°C for 1min, annealing at 60°C for 1min, and extension at 72°C for 1min. See Appendix B for the sizes of the bands of molecular weight marker VI and 1kb ladder.

3.D. DISCUSSION

Twenty-four of the 36 (67%) of the *Acinetobacter* isolates shown to contain *int1* by hybridisation studies, yielded amplification products using primers directed at the 5`- and 3`-conserved regions of class 1-type integrons (see also Table 2.2.). A single product of 0.7kb was obtained from 22/36 strains, suggesting that these acinetobacters contain integrons with one cassette in the variable region. One of the strains which yielded a 0.7kb product also yielded a 0.65kb product, suggesting that this strain contains 2 integrons. A product of 2.0kb was obtained from 1 strain, suggesting the contents of the variable region of this integron consist of at least 2 cassettes. Two products (2.0kb and 3.5kb) were obtained from 1 strain, suggesting that this acinetobacter contains at least 2 integrons.

No PCR products were obtained with the DNA from 12 acinetobacters which had hybridised to the *int1* probe. Interestingly, of these 12 isolates, G26, G28, and G30, isolated in 1997, have the same resistance profile as G20 (also isolated in 1997). However, G20 did yield a PCR product. A number of reasons could account for this result. Firstly, the isolates may contain an In0-like integron. In0 lacks a variable region, and the 5`-conserved region is contiguous with the 3`-conserved region in this integron (Bissonnette and Roy, 1992). A second and more likely possibility is that the variable regions of these integrons contain a number of cassettes, resulting in variable regions too large to be amplified using the PCR assay described in this study. A final reason could be that the isolates contain only a portion of the integrase gene - hence the positive hybridisation signal with the *int1* probe - but lack other conserved integron-related sequences (see Chapter 6).

In a recent study of the molecular epidemiology of aminoglycoside resistance in *Acinetobacter,* Seward *et al.* (1998) used the 5`-CS primer in combination with various other primers specific for aminoglycoside resistance genes (including *aac(6`)-Ia, aac(6`)-Ib, aac(3)-Ia, aac(3)-IIa, ant(3``)-Ia, ant(3``)-Ib,* and *ant(2``)-Ia*), in a PCR analysis to determine the association of these resistance genes with class 1-type integrons. They found that 6 of the 24

(25%) *Acinetobacter* isolates carried an integron-associated *aac(3)-Ia* and 3 of these isolates also carried an integron-associated *ant(3``)-Ia*. No integron-associated products were detected using the 5`-CS primer with any of the other primers (specific for *aac(6`)-Ia, aac(6`)-Ib, aac(3)-IIa, ant(3``)-Ib,* and *ant(2``)-Ia*).

Class 2-or Tn*7*-type integrons have been described in *Acinetobacter* strains from Chile (Amyes and Young, 1996). The investigators studied the genetic basis of high level trimethoprim resistance of 30 isolates. Initial hybridisation studies on total cellular DNA from 13 isolates, using a probe for *dfrIa* (encoding a dihydrofolate reductase which mediates resistance to trimethoprim), indicated the presence of this gene in each isolate. Subsequent hybridisation experiments using probes for *int2* and the transposition gene (*tnsE*) from Tn*7* suggested that *dfr* was part of a Tn*7*-like transposon in these isolates. To confirm the genetic surroundings of the *dfrIa* and to extend the analysis to the remaining strains, all 30 isolates were subjected to PCR using primers directed at the N-terminal sequences of *dfrIa* and the Tn7 *int*. Twenty-seven (90%) of the isolates yielded the expected 700bp product indicative of a *dfrIa* in a classic Tn7 integron structure (Sundström *et al.*, 1991). A second PCR product of about 2.5kb was found in 11 of the isolates (usually in conjunction with the smaller 700bp product but on 4 occasions the 2.5kb amplicon was the sole product), but it was not consistently obtained. Subcloning and partial sequence analysis of the ~2.5kb product revealed the presence of 3 gene cassettes inserted between the integrase gene and the *dfrIa –sat, ant(2``)-Ia,* and *cat* gene cassettes (encoding streptothricin resistance, aminoglycoside resistance, and chloramphenicol resistance, respectively). Interestingly, although previous investigators have observed that the Tn7-related integrase gene (*int2*) is interrupted by a termination codon and is thus non-functional, the results described here suggest the presence of a Tn7-related integron which is active in the integration of resistance gene cassettes. Another feasible explanation for these observations is that the strains may contain a Tn*21* (Int1) integrase which was responsible for the recombination of cassettes into the Tn*7*-like

structure –Tn*21* integrase has been shown to function *in trans* to effect such insertions and deletions. However, no signal was obtained with the DNA from the isolates when they were hybridised with probes specific for Tn*21* integrase and the authors concluded that the integrase of the transposon described in their study may be active. This assumption may be incorrect. Segal and Elisha (1997) described an *ant(2``)-Ia* gene cassette recombined at a secondary site in a plasmid (pRAY) isolated from *Acinetobacter* strain SUN. They suggest that the gene cassette might have been inserted into pRAY by integrase encoded by an integron on a plasmid, resident long enough for such events to occur, before being lost from strain SUN.

CHAPTER 4: CHARACTERIZATION OF THE 0.7kb AMPLICONS

4.A. INTRODUCTION

Using primers for the amplfication of the variable region of type 1 integrons, a PCR product of 0.7kb was obtained from the DNA of 22 isolates. As a first step to the characterisation of these products, one of the 0.7kb amplicons obtained from an *A.baumannii* strain, designated CAR, was cloned and sequenced.

4.B. MATERIALS AND METHODS

4.B.1. Bacterial isolates and plasmids

E.coli LKIII (*lacI*$^-$, *lacZ*ΔM15, *lacY*$^+$) (Zabeau and Stanley, 1982) and *E.coli* DH5α were used as recipients in transformation experiments, while pUC19 (Norrander *et al.*, 1983) was used as a vector.

4.B.2. Isolation of plasmid DNA

4.B.2.a. Small-scale extraction

Plasmid DNA was extracted from putative *E.coli* DH5α transformants using a protocol based on those of Ish-Horwicz and Burke (1981) and Sambrook *et al.* (1989). Six to eighteen white colonies were inoculated, separately, into 5ml of 2X YT broth containing 50μg/ml of ampicillin (Ranbaxy; South Africa) and grown, with vigorous shaking, at 37^{0}C overnight. One and a half millilitres of each culture was decanted into microfuge tubes and the cells were harvested at 14000rpm for 5 minutes in a microfuge (Eppendorf Centrifuge 5415C). Cell pellets were resuspended in 0.2ml of Solution I (25mM Tris-HCl pH 8.0, 50mM EDTA, 1%w/v Glucose; Appendix A). The glucose in Solution I weakens the cell walls and the EDTA chelates Mg^{2+} ions making them unavailable to degradative enzymes (Ausubel *et al.,* 1987). After resuspension, 0.4ml of Solution II (0.2N NaOH, 1% w/v SDS; Appendix A) was added, the tubes shaken to ensure thorough mixing, and held on ice for 5 minutes. This is a denaturation step: the SDS denatures bacterial proteins causing the cells to lyse and release their contents, while the NaOH

denatures both chromosomal and plasmid DNA (Ausubel *et al.*, 1987). Pre-cooled Solution III (5M potassium acetate, 3M glacial acetic acid; Appendix A) –0.3ml- was added and the tubes were kept on ice for ≥10 minutes. This step neutralises the preparation and selectively renatures the plasmid DNA. The chromosomal DNA, bacterial proteins, and SDS are complexed with the potassium and precipitate (Ausubel *et al.*, 1987). The precipitates were pelleted by centrifugation in a microfuge (Eppendorf Centrifuge 5415C) at 14000rpm for 10 minutes and the plasmid DNA in the recovered supernatants was purified further by phenol-chloroform-isoamylalcohol precipitation [section 2.B.5.b] and then concentrated by precipitation with 100% ethanol, washed in 70% ethanol, dried at 37^0C for 10-15 minutes, and resuspended in 50µl TE buffer (Appendix A).

4.B.2.b. Large-scale extraction

A large-scale extraction of plasmid DNA was performed on certain of the *E.coli* transformants using a method based on those described by Ish-Horwicz and Burke (1981) and Sambrook *et al.* (1989). A single colony was inoculated into 200ml of 2X YT broth containing 20µg/ml of trimethoprim (Roche; Mannheim, Germany) and allowed to grow at 37^0C for ±18 hours. The cells were then harvested by centrifugation (5000rpm) at 4^0C in a Beckman Model J2-21 Centrifuge for 5 minutes. Plasmid DNA was extracted using the same procedure and reagents as the small-scale plasmid extraction in section 4.B.2.a., except that the volumes of the reagents were increased proportionally. After lysis, cell debris and chromosomal DNA was removed by centrifugation at 10000rpm in a Beckman Centrifuge for 10 minutes and the clear lysate was recovered to a fresh tube. Plasmid DNA was precipitated by adding 0.6 volumes of isopropanol, incubating at RT for 10 minutes, and centrifugation at 10000rpm as described. After discarding the supernatant, the plasmid DNA pellet was washed in 70% ethanol, drained thoroughly, allowed to air dry, and resuspended in 7.5ml of TE buffer.

Plasmid DNA was further purified by CsCl-EtBr gradient centrifugation (Sambrook et al., 1989). This method depends on differences between the amounts of EtBr that can be bound to linear and closed circular DNA. EtBr

unwinds DNA helices by intercalating between the bases (1 EtBr molecule for every 2 base pairs) (Cantor and Schimmel, 1980). Unwinding leads to an increase in the length of linear DNA molecules and to the introduction of compensatory superhelical turns in closed circular plasmid DNA. The density of the superhelical turns eventually becomes so great that further intercalation of EtBr molecules is not possible. Linear molecules, however, bind dye until saturation is reached. Due to this differential binding of dye, the buoyant densities of the linear and closed circular DNA molecules are different and they can be separated in CsCl gradients containing saturating amounts of EtBr.

In the protocol used (Sambrook et al., 1989), 8.3g of CsCl was dissolved in the 7.5ml DNA solution. A volume (0.8ml) of 10mg/ml EtBr was added, the solution was mixed and centrifuged at 10000rpm as before. The supernatant was recovered and the refractive index was adjusted to 1.390 using a refractometer (ABBE; London, UK). Two Vti65 tubes (13X51mm) were filled with the solution, the tubes were sealed and centrifuged at 60000rpm at 20^0C for 7hours in a Beckman L-70 Ultracentrifuge.

With visualisation under UV (310nm), 1 major plasmid band and a minor band (residual chromosomal DNA) was seen. The tops of the sealed tubes were cut off, the bottom of the tube was pierced with a needle, and the flow of the tube contents was controlled with a finger. The excess solution below the plasmid band was allowed to drip out and the plasmid was collected in a 2ml microfuge tube. The remaining solution was discarded. The EtBr was removed by extracting (3X) with salt-saturated isopropanol (Appendix A) – the isopropanol containing the EtBr was discarded after each extraction. Two volumes of dH_2O were added and the solution was mixed. A volume of isopropanol, equivalent to the new total volume, was added, mixed, the solution incubated at RT for 10 minutes, and centrifuged for 15 minutes in a microfuge (Eppendorf Centrifuge 5415C) at 14000rpm. The DNA pellet was washed in 70% ethanol, allowed to dry at 37^0C, and resuspended in a suitable volume of TE buffer. The concentration of the DNA was determined by fluorescent quantitation (section 2.B.4).

4.B.3. DNA-DNA hybridisation studies

4.B.3.a. Preparation of probe

An internal 300bp *Eco*RV fragment of the *dfrVII* gene (Fig. 4.1.) was used as a specific probe. After restriction enzyme digestion, the 300bp *Eco*RV fragment was purified [section 2.B.5.b.] and labelled [section 2.B.5.c.].

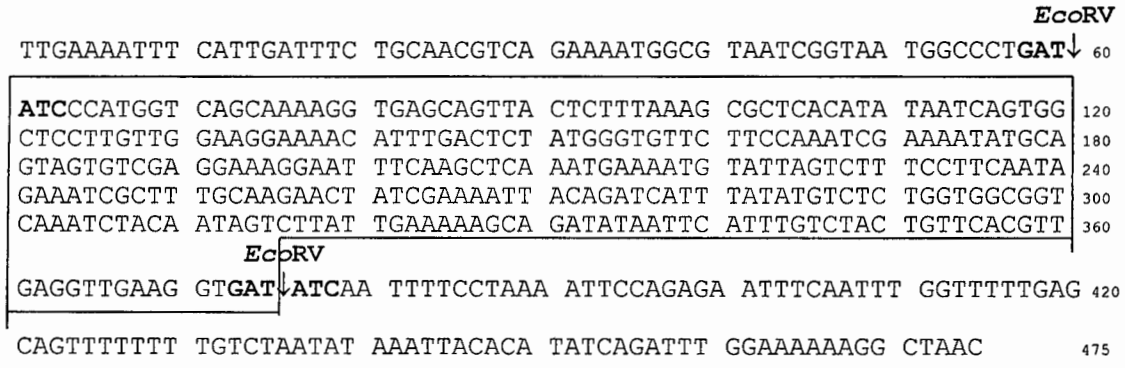


Fig.4.1.: DNA sequence of the *dfrVII* gene showing the internal 300bp *Eco*RV probe fragment (blocked off)
(Towner and Carter, 1990)

4.B.3.b. DNA transfer

DNA was transferred from agarose gels to Hybond^{TM}-N^{+} membranes, using a mono-directional transfer based on the method of Smith and Summers (1980). Before the transfer was performed, the gel was soaked twice in 2-3 gel volumes of 0.25M HCl (Appendix A) for 15 minutes at RT. This partially hydrolyses the DNA by acid depurination and causes double-stranded breaks, allowing larger fragments of DNA to transfer more efficiently. The gel was then soaked twice in 2-3 gel volumes of 1.5M NaCl, 0.5M NaOH (Appendix A) at RT for 15 minutes to denature the DNA. Finally, it was neutralised by soaking it once in 2-3 gel volumes of 0.5M Tris pH 7.2, 1.5M NaCl, 1mM EDTA (Appendix A) at RT for 30 minutes. Between each step and after neutralisation the gel was rinsed with water.

On completion of the pre-transfer treatment, DNA was transferred from the gel to a membrane. The gel was placed face down on a piece of glass on a flat surface. A piece of Hybond^{TM}-N^{+} membrane was cut to size and layered

carefully onto the gel, ensuring that no air bubbles were trapped beneath it. Three pieces of Whatmans No. 1 filter paper were soaked in the neutralisation solution and layered on the Hybond^{TM}-N^{+}. About 50mm of dry paper towel was placed on filter paper, followed by a piece of perspex and a brick to compress the sandwich. Transfer was allowed to continue for 4.5 hours. The different materials were removed and the Hybond^{TM}-N^{+} membrane was peeled off. After the DNA had been fixed to the membrane by UV cross-linking at 254nm in a UV Cross-linker (Hoefer Scientific Instruments; California, USA) for 30 seconds, it was hybridised to the *dfrVII* probe [section 4.B.3.a].

DNA-DNA hybridisations, post-hybridisation washes and signal detection were performed as described in section 2.B.5.c.

4.B.4. Cloning experiments

4.B.4.a. Amplification product

The amplification product was cloned using the DNA blunting kit from Amersham International (Buckinghamshire, UK) following the protocol supplied by the manufacturer.

Preparation of pUC19 vector

For cloning the amplication product, pUC19 vector DNA was digested with *Sma*I (CCC↓GGG), which cleaves the plasmid within the multiple cloning site (Appendix C) to generate a linear plasmid with blunt ends. Two micrograms of pUC19 DNA were digested with 20U of *Sma*I (Boehringer Mannheim; Mannheim, Germany) in 1X buffer A (Boehringer Mannheim; Mannheim, Germany) in a total volume of 20μl (adjusted with dH_2O) at 25^0C for 1 hour. The linearized plasmid DNA was purified and precipitated as in section 2.B.5.b. The DNA was resuspended in 20μl of dH_2O to give a final concentration of 100ng/ul.

Preparation of insert

The amplification product was purified by electrophoresis on a 1% agarose gel at 80V for ±1 hour, excised and extracted with phenol [section 2.B.5.b] and the DNA was quantitated [section 2.B.4.].

Taq DNA polymerase has template-independent terminal transferase activity which adds dATP residues to the 3`-ends of amplification products (Haqqi, 1992). For this reason, it was necessary to remove the dATP residues (blunting) from the amplicon before it could be cloned into pUC19 digested with *Sma*I. The blunting reaction was performed using a DNA blunting kit (Amersham International; Buckinghamshire, UK) following the protocol supplied by the manufacturer. Eight microlitres of the purified product (500-800ng) was mixed with 1μl of the 10X blunting buffer and incubated at 70^0C for 5 minutes. Thereafter 1μl of T4 DNA polymerase was added. T4 DNA polymerase has 5`-3` polymerase activity (blunts the DNA by adding complimentary bases to an overhang) and 3`-5` exonuclease activity (blunts the DNA by removing the overhang). For blunting the amplification product the exonuclease activity was utilised. The reaction was mixed by gentle pipetting, incubated at 37^0C for 5 minutes, vortexed to deactivate the polymerase, and placed on ice for immediate use in the ligation reaction.

4.B.4.b. Enriched cloning

Preparation of pUC19 vector

pUC19 vector DNA was prepared as in section 4.B.4.a., except that 20U of *Bam*HI and 20U of *Hin*dIII were used for the restriction endonuclease digestion. Both these endonucleases cleave within the multiple cloning site (Appendix C) and generate a linear plasmid with 5`-overhangs ("sticky" ends).

Identification and preparation of insert for enriched cloning

Approximately 10μg of genomic DNA [section 2.B.4] was digested with either *Bam*HI + *Eco*RI, *Bam*HI + *Hin*dIII, *Bam*HI + *Sph*I, *Eco*RI + *Hin*dIII, *Eco*RI + *Xba*I, or *Hin*dIII + *Xba*I (all Boehringer Mannheim; Mannheim, Germany) and electrophoresed on a 1% agarose gel at 60V for 4 hours [section 2.B.3.]. The DNA was then transferred to a Hybond-N^+ membrane as described [section 4.B.3.b.] and hybridised with the *dfrVII* probe [section 2.B.5.c.].

4.B.5. Ligations

Ligation involves the formation of new bonds between phosphate residues located at the 5`-termini of double-stranded DNA and adjacent 3`-hydroxyl moieties (Sambrook et al., 1989).

The blunted amplification product was ligated to *Sma*I-digested pUC19 using a DNA blunting kit (Amersham International; Buckinghamshire, UK). The protocol supplied with the kit was followed: 500-800ng of the blunted amplification product was mixed with 100ng of *Sma*I-digested pUC19 in a microfuge tube. Ligation solution A (4X volumes), which is the reaction buffer, and an equal volume of ligation solution B, containing T4 DNA ligase, which catalyses ligation (Sgaramella and Ehrlich, 1978) were added and mixed gently but thoroughly. The ligation reaction was allowed to continue at 16^0C overnight.

The restriction fragments identified for enriched cloning [section 4.B.4.b.] were ligated to appropriately digested pUC19 using a DNA ligation kit from Amersham International (Buckinghamshire, UK). The manufacturers's protocol was followed and was similar to the ligation reaction for the DNA blunting kit. The ligation was allowed to proceed at 16^0C for about 4 hours.

4.B.6. Transformation studies

4.B.6.a. Preparation of competent *E.coli* cells

In 1970, Mandel and Higa showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then heated briefly could be transfected with bacteriophage λ DNA. Most current methods of bacterial transformation are based on these observations. The treatment induces a transient state of "competence" in the recipient bacteria during which they can take up DNA from various sources (Mandel and Higa, 1970; Sambrook et al., 1989).

E.coli DH5α cells were made competent by a $CaCl_2$-shock procedure based on the method described by Dagert and Ehrlich (1979). A single colony of the bacterium was inoculated into 5ml of 2X YT broth (Appendix A) and grown at 37^0C overnight with shaking. This starter culture was diluted 1/100 in 100ml of 2X YT broth and cultured to early logarithmic phase, corresponding to an optical density (OD) of ±0.3. The cells were harvested by centrifugation in a Beckman Model J2-21 Centrifuge for 5 minutes at 4^0C and were kept at 4^0C during all subsequent procedures. Cells were resuspended in 50ml of ice-cold 0.1M $CaCl_2$ (Appendix A) and kept on ice for 1 hour. They were harvested as before and resuspended in 10ml of ice-cold 0.1M $CaCl_2$, thereby inducing the transient state of competence. Ice-cold, sterile glycerol was added to the cells to a final concentration of 10% (v/v), mixed and held on ice for 30 minutes. Aliquots of the competent cells were stored at -70^0C until required in transformation experiments.

4.B.6.b. Transformation studies and screening for putative recombinants

Transformation studies were carried out using a modification of the protocols of Cohen *et al.* (1972) and Dagert and Ehrlich (1979). Twenty microlitres of the ligation mix [section 4.B.5.] was added to 100μl of competent *E.coli* DH5α

cells and kept on ice for ≥30 minutes to enable the DNA to bind to the cell surface. To facilitate the uptake of DNA by the bacterial cells (ie. transformation) the transformation mix was heat-shocked at 42^0C for 90 seconds. To allow for the expression of the resistance markers carried by the vectors and/or the insert (ampicillin resistance in pUC19, Appendix C), 0.9ml 2X YT was added to the transformed cells and they were incubated at 37^0C for 1 hour. The transformations were plated onto appropriate selective media and incubated at 37^0C overnight.

When selecting for recombinants containing the amplification product, 200μl of the transformation mix was plated on 2X YT agar (Appendix A) containing 50μg/ml of ampicillin (Ranbaxy; South Africa), 100μg/ml of IPTG (Appendix A), and 200μg/ml of X-gal (Appendix A). Ampicillin was added to ensure the growth of only those bacteria that had been transformed with pUC19. X-gal was included as a colour indicator of β-galactosidase (β-gal) activity, which is induced by IPTG. pUC vectors carry the amino-terminal (α-peptide) of β-gal. When this plasmid is introduced into bacteria containing the carboxy-terminal (eg. DH5α), the two portions can associate (α-complementation) to form an active enzyme. X-gal is hydrolysed by β-gal and releases a blue, non-diffusable dye, giving rise to blue colonies on media containing X-gal. On the other hand, if the sequence encoding the α-peptide is interrupted by cloning into the multiple cloning site (MCS, Appendix C), α-complementation cannot occur and colonies grown on similar media will be white. Thus, putative recombinants will appear as white colonies on media containing X-gal, IPTG and ampicillin.

Positive selection was used to isolate transformants resulting from the enriched cloning experiment by plating the transformation mixes on media containing trimethoprim (30μg/ml).

4.B.6.c. Ligation and transformation controls

Controls were included in all ligation and transformation experiments. They are summarised in Table 4.1.

Table.4.1.: Ligation and transformation controls

CONTROL	RATIONALE	EXPECTED RESULT
Ligation	To determine whether ligase was functioning, pUC19 was digested with *Sma*I and religated	Numerous blue colonies are expected if ligase is functioning
pUC19 digestion	Linear DNA is not taken up by *E.coli*, so linearised vector was used to determine if pUC19 had been digested completely	Zero blue colonies indicate complete digestion; a few = near-complete digestion; numerous indicate very Little or no digestion
Transformation efficiency of competent cells	Determined by transforming the competent cells with circular, undigested pUC19	Numerous blue colonies indicate a high efficiency of transformation
Contamination Of competent Cells	To ensure that the competent cells are not contaminated with pUC19, heat-shocked cells were plated onto selective media without transforming them.	Zero colonies indicate that there is no contamination; any blue colonies indicate contamination with pUC19

4.B.7. DNA Sequencing

4.B.7.a. DNA sequencing protocol

DNA sequencing data were generated by the dideoxy chain termination method (Sanger *et al.*, 1977) using either a ^{T7}Sequencing Kit (Pharmacia Biochemicals; Uppsala, Sweden), the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies; Wisconsin, USA), or rapid automated flourescence in an automatic DNA sequencer (373A, Applied Biosystems; Perkin Elmer; California, USA). The latter was performed in the Department of Chemical Pathology, University of Cape Town. The dideoxy chain termination method of sequencing (Sanger, *et al.*, 1977) is illustrated in Fig. 4.2.

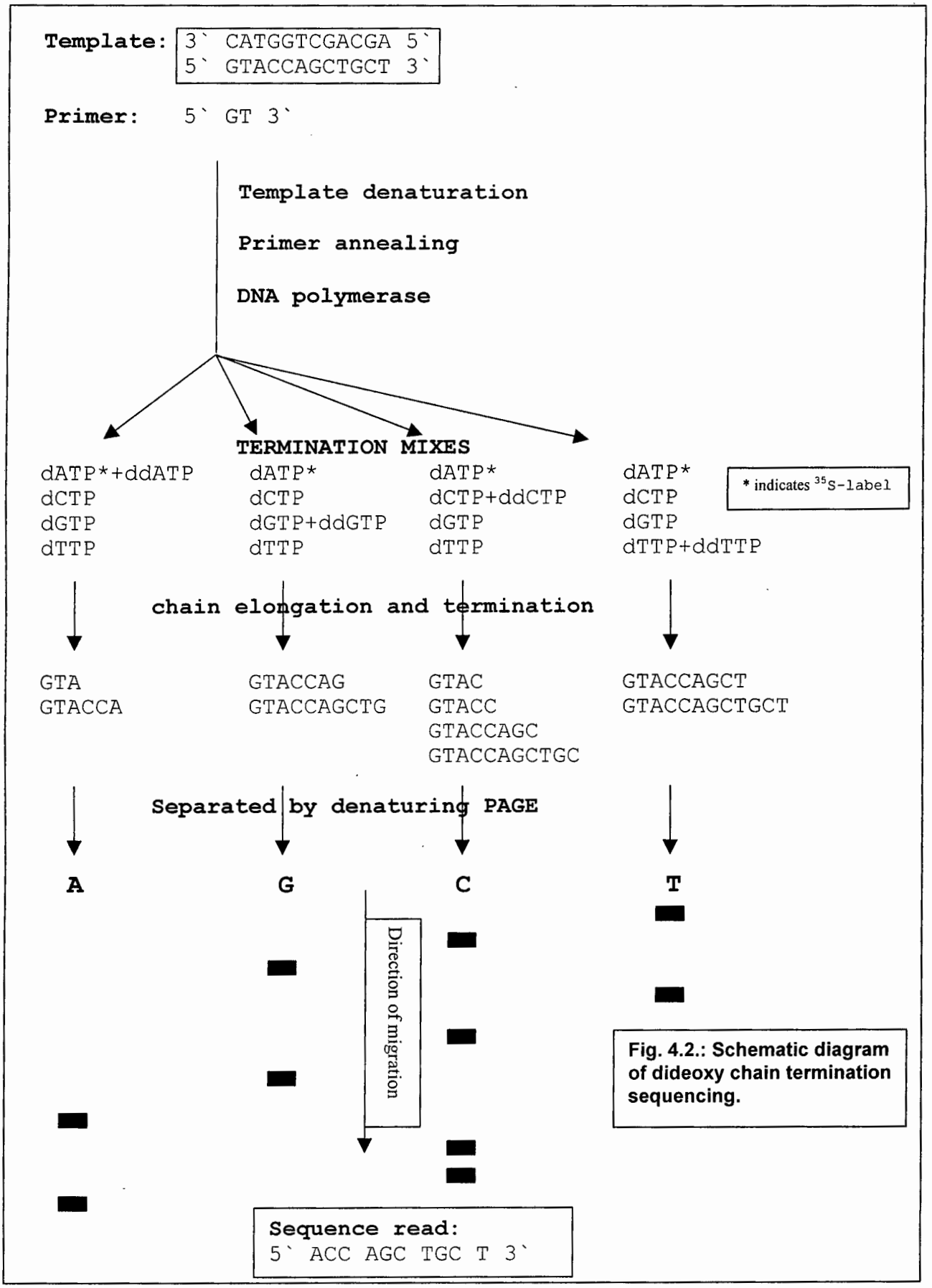


Fig. 4.2.: Schematic diagram of dideoxy chain termination sequencing.

The rationale behind the dideoxy chain termination method is as follows (Fig. 4.2.): DNA polymerase incorporates dNTPs into growing DNA chains through phosphodiester bonds at the 3`-end of the chain. One of the dNTPs is labelled to produce a signal which is captured on autoradiographic film. The termination mixes contain dideoxynucleotides (ddNTPs) which differ from dNTPs in that they lack a 3`-hydroxyl group. Once they have been incorporated into the growing DNA chain the absence of the 3`-hydroxyl group prevents the formation of a further phosphodiester bond with the succeeding dNTP and chain elongation terminates. There is thus a termination corresponding to each base in the sequence and when the reactions are electrophoresed on a denaturing polyacrylamide gel, there will be a band corresponding to each termination. The sequence of the new strand can be "read" directly from an autoradiograph of the gel – it will be complementary to that of the template DNA.

DNA sequencing using the ^{T7}Sequencing Kit (Pharmacia Biochemicals; Uppsala, Sweden) was performed according to the manufacturer's instructions: Approximately 2μg of double-stranded DNA template [prepared in section 4.B.2.b] (in a total volume of 16μl) was denatured in 4μl 2M NaOH (Appendix A) at RT for 10 minutes. Six microlitres 3M sodium acetate (Appendix A) was added to stop denaturation and prevent renaturation (Sambrook et al., 1989). The volume was made up to 40μl with dH_2O and the single-stranded DNA was precipitated on dry ice for 15 minutes by the addition of 3 volumes of 100% ethanol. The DNA was collected by centrifugation at 14000rpm for 10 minutes in a microfuge (Eppendorf Centrifuge 5415C). The DNA pellet was washed in 70% ethanol, dried at 37^0C for 10-15 minutes, and resuspended in 10μl of dH_2O.

Two to ten picomoles of primer (2μl) and 2μl of annealing buffer were added to the denatured template. The microfuge tube was vortexed, centrifuged briefly, and incubated at 60^0C for 10 minutes to allow the primer to anneal to the template. The tube was left at RT for ≥10 minutes and centrifuged briefly to collect the contents at the bottom of the tube.

Three microlitres of labelling mix (containing dCTP, dGTP, and dTTP but not dATP), 1μl of ^{35}S-labelled dATP, and 2μl of T7 DNA polymerase (diluted 1 in 5 with the enzyme dilution buffer) were added to the tube containing the annealed primer and template. The components were mixed by gentle pipetting and the contents were collected at the bottom of the tube by brief centrifugation. The labelling and chain elongation was allowed to proceed at RT for 5 minutes. Thereafter, 4.5μl of the labelling reaction was transferred to each of the four termination mixes (prewarmed to 37^{0}C) containing 2.5μl of the ddATP-mix, ddCTP-mix, ddGTP-mix, or ddTTP-mix. The components were mixed by gentle pipetting and the termination reactions were incubated at 37^{0}C for 5 minutes. The reactions were stopped by the addition of 5μl of stop solution and the contents were collected at the bottom of the tubes by brief centrifugation. The reactions were stored at –20^{0}C until required. Stop solution contains EDTA which chelates Mg^{2+} ions thereby inhibiting the polymerase enzyme. It also contains bromophenol blue which served as a tracking dye during electrophoresis.

Alternatively, DNA sequencing was performed using the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies; Wisconsin, USA), which is based on the same principal as the ^{T7}Sequencing Kit. Sequencing was carried out according to the manufacturer's protocol: the premix consisted of 5μl 5X sequencing buffer, 1μl prelabelling mix, 5-10pmoles primer, 5μCi of [α-^{35}S]-dATP, 500-1000ng DNA template, 1μl DNA polymerase and deionised water to a total volume of 17μl. The tube containing the premix was heated at 95^{0}C for 5-10 minutes to denature the template DNA. The tube was then placed at RT and primer annealing and incorporation of the labelled nucleotide was allowed to continue for 20-30 minutes. Two microlitres of each termination mix (C, A, T, and G) were added to 4 appropriately labelled tubes. After annealing and labelling/chain elongation, 4μl of the premix were added to each of the 4 tubes containing the termination mixes and termination reactions were carried out at 65^{0}C for 2-5 minutes. The reactions were stopped with 3μl stop/loading buffer.

4.B.7.b. Denaturing polyacrylamide gel electrophoresis

Sequencing reactions were electrophoresed on denaturing polyacrylamide gels based on a method described by Sambrook *et al.* (1989).

The apparatus consisted of two glass plates of differing lengths (cleaned with detergent and 70% ethanol and dried with acetone), two spacers made of thin, flexible plastic, a strip of filter paper, clamps, a shark's tooth comb, and electrophoresis equipment. The apparatus was assembled as illustrated in Fig. 4.3.

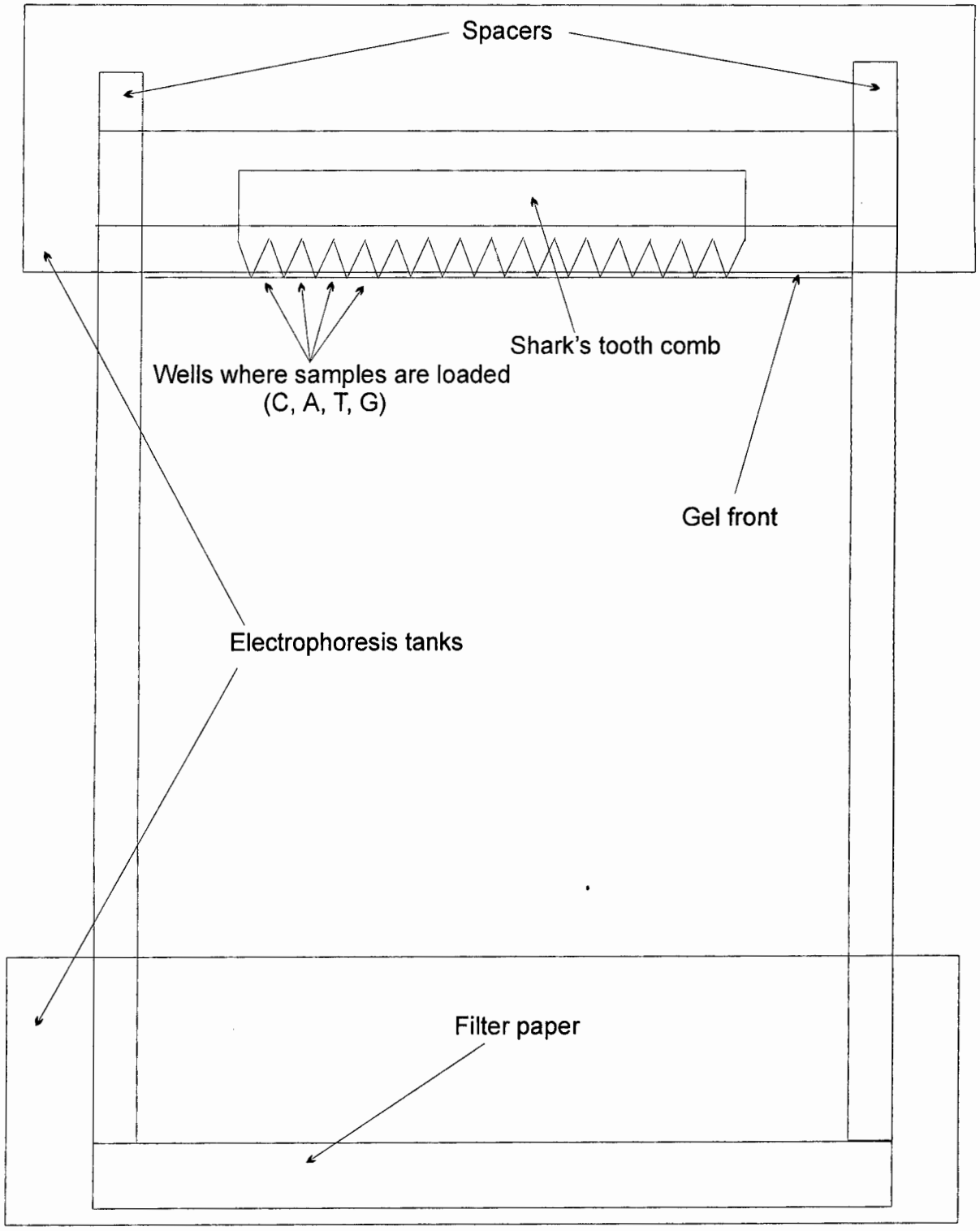


Fig. 4.3: Schematic diagram of sequencing apparatus (adapted from Sambrook, *et al.*, 1989)

Six percent polyacrylamide was prepared as follows (Appendix A): 50% acrylamide, 2.5% bis-acrylamide (6ml), 10X NNB (5ml), dH_2O (21ml), and urea (24g) were mixed and dissolved. The polymerisation process was initiated by the addition of 200µl 20% ammonium persulphate (APS, Appendix A) and 40µl of TEMED. The polyacrylamide was immediately poured between the two glass plates (see Fig.4.3.) and allowed to polymerise completely.

In preparation for loading and electrophoresis of samples, the gel was clamped to the electrophoresis apparatus (Fig.4.3.). 1x NNB buffer was added to the upper and lower tanks. The shark's tooth comb was removed and reinserted with the teeth just touching the gel surface to form the wells. In order to help maintain the denatured state of the DNA samples, the gel was prewarmed for approximately 30 minutes at 35-40W prior to electrophoresis of the samples. DNA in the sequencing reactions was denatured by heating at 80^0C for 2 minutes, during which time excess urea was washed out of the wells with 1XNNB using a syringe. The latter was carried out since excess urea masks the signal generated by ^{35}S, causing the bands to become diffuse. Three to four microlitres of each reaction was loaded, in separate wells, in the sequence CATG, and electrophoresed at a constant power of 40W. The length of time of electrophoresis was 2-4 hours and was monitored by tracking the rate of migration of the dye in the stop solution. When the gels were electrophoresed for 4hours, 100ml 3M sodium acetate was added to the bottom tank (to give a final concentration of 1M sodium acetate) after 2hours. This was to create a buffer gradient to improve resolution of the larger DNA bands.

After electrophoresis, the apparatus was dismantled and the plates were carefully separated. The gel was removed from the glass plate by placing a sheet of Whatman 3MM filter paper on top of it, applying gentle pressure over the surface to ensure the gel attached to the filter paper, and then carefully peeling the filter paper, holding the gel, from the plate. The gel was covered with cling film and dried for 1.5-2 hours under vacuum on a Slab Gel Drier (Model SE1160; Hoefer Scientific Instruments; California, USA). After drying, the cling film removed and the gel was exposed to X-ray film (Hyperfilm MP,

Amersham Life Science; Buckinghamshire, UK) for 16-24 hours at RT. The autoradiograph was then developed.

4.B.7.c. Computer analysis of sequencing data

The DNA sequences were analysed using the Basic Local Alignment Search Tool, BLAST, (Altschul *et al.*, 1997) and Genepro version 6.1 (Riverside Scientific).

4.C. RESULTS

4.C.1.Characterisation of the amplification product from strain CAR

The 0.7kb amplicon from strain CAR was sequenced directly using the 5`-CS primer. Of the 426bp of data generated, the first 68 nucleotides were identical to integron-related sequences (Fig. 4.4.A.). The remaining 358bp were identical to the *dfrVII* gene encoding dihydrofolate reductase type VII (Fig. 4.4.B.). The amplicon was cloned into pUC19 and introduced into *E.coli* DH5α; the recombinant plasmid was designated pG5DR.

```
1 = PCR product from strain CAR (bases 1-68)
2 = Integrase-related region (Bissonnette and Roy, 1992)

2  TGTTATGACT GTTTTTTTGG GGTACAGTCT ATGCCTCGGG CATCCAAGCA GCAAGCGCGT   1080
                                              5`-CS PRIMER
1                                  GG AGCAGCAACG ATGTTACGCA GCAGGGCAGT   32
                                   ** ********** ********** **********
2  TACGCCGTGG GTCGATGTTT GATGTTATGG AGCAGCAACG ATGTTACGCA GCAGGGCAGT   1140

1  CGCCCTAAAA CAAAGTTAGC CATTACGGGG GTTGAA                             68
   ********** ********** ********** ******
2  CGCCCTAAAA CAAAGTTAGC CATTACGGGG GTTGAA                             1158
```

Fig.4.4.A.: Comparison of the integrase-related region of the PCR product from strain CAR with the corresponding region described for *int1* (Bissonnette and Roy, 1992)

The first 68 bases of the PCR product from strain CAR generated by direct sequencing with the 5`-CS primer are compared to the corresponding region for *int1* (Bissonnette and Roy, 1992). Identical nucleotides are indicated with an asterisk (*). The 5`-CS primer annealing site is indicated in bold and underlined.

```
1 = PCR product from strain CAR (bases 69 - 426)
2 = dfrVII from E.coli (Sundstrom et al., 1993)

1  TTGAAAATTT CATTGATTTC TGCAACGTCA GAAAATGGCG TAATCGGTAA TGGCCCTGAT     60
   ********** ********** ********** ********** ********** **********
2  TTGAAAATTT CATTGATTTC TGCAACGTCA GAAAATGGCG TAATCGGTAA TGGCCCTGAT     60
              →dfr start
1  ATCCCATGGT CAGCAAAAGG TGAGCAGTTA CTCTTTAAAG CGCTCACATA TAATCAGTGG     120
   ********** ********** ********** ********** ********** **********
2  ATCCCATGGT CAGCAAAAGG TGAGCAGTTA CTCTTTAAAG CGCTCACATA TAATCAGTGG     120

1  CTCCTTGTTG GAAGGAAAAC ATTTGACTCT ATGGGTGTTC TTCCAAATCG AAAATATGCA     180
   ********** ********** ********** ********** ********** **********
2  CTCCTTGTTG GAAGGAAAAC ATTTGACTCT ATGGGTGTTC TTCCAAATCG AAAATATGCA     180

1  GTAGTGTCGA GGAAAGGAAT TTCAAGCTCA AATGAAAATG TATTAGTCTT TCCTTCAATA     240
   ********** ********** ********** ********** ********** **********
2  GTAGTGTCGA GGAAAGGAAT TTCAAGCTCA AATGAAAATG TATTAGTCTT TCCTTCAATA     240

1  GAAATCGCTT TGCAAGAACT ATCGAAAATT ACAGATCATT TATATGTCTC TGGTGGCGGT     300
   ********** ********** ********** ********** ********** **********
2  GAAATCGCTT TGCAAGAACT ATCGAAAATT ACAGATCATT TATATGTCTC TGGTGGCGGT     300

1  CAAATCTACA ATAGTCTTAT TGAAAAAGCA GATATAATTC ATTTGTCTAC TGTTCACG       358
   ********** ********** ********** ********** ********** ********
2  CAAATCTACA ATAGTCTTAT TGAAAAAGCA GATATAATTC ATTTGTCTAC TGTTCACGTT     360

2  GAGGTTGAAG GTGATATCAA TTTTCCTAAA ATTCCAGAGA ATTTCAATTTG GTTTTTGAG     420

2  CAGTTTTTTT TGTCTAATAT AAATTACACA TATCAGATTT GGAAAAAAGGCT AAC           475
```

Fig.4.4.B. Comparison of the *dfrVII* region of the PCR product from *A.baumannii* strain CAR with the corresponding region of the published *dfrVII* gene (Sundstrom *et al.*, 1993)

The remaining 358bp of the PCR product from strain CAR generated by direct sequencing with the 5`-CS primer are compared to the *dfrVII* gene described by Sundstrom *et al.* (1993). Identical nucleotides are indicated with an asterisk (*). The *dfrVII* start codon is shown in bold.

To further characterize the regions abutting *dfrVII,* the structural gene and its flanking sequences were cloned. To identify the DNA fragment carrying *dfrVII*, genomic DNA from strain CAR was digested with *Bam*HI + *Eco*RI, *Bam*HI + *Hin*dIII, *Bam*HI + *Sph*I, *Eco*RI + *Hin*dIII, *Eco*RI + *Xba*I, or *Hin*dIII + *Xba*I, electrophoresed, transferred to a Hybond^{TM}-N^{+} membrane, and hybridised with the *dfrVII* probe (Fig. 4.5.). A 2kb *Bam*HI/*Hin*dIII fragment, which hybridised to the probe, was cloned into pUC19 and expressed in *E.coli.* The recombinant plasmid was designated pDS100 and conferred resistance to trimethoprim.

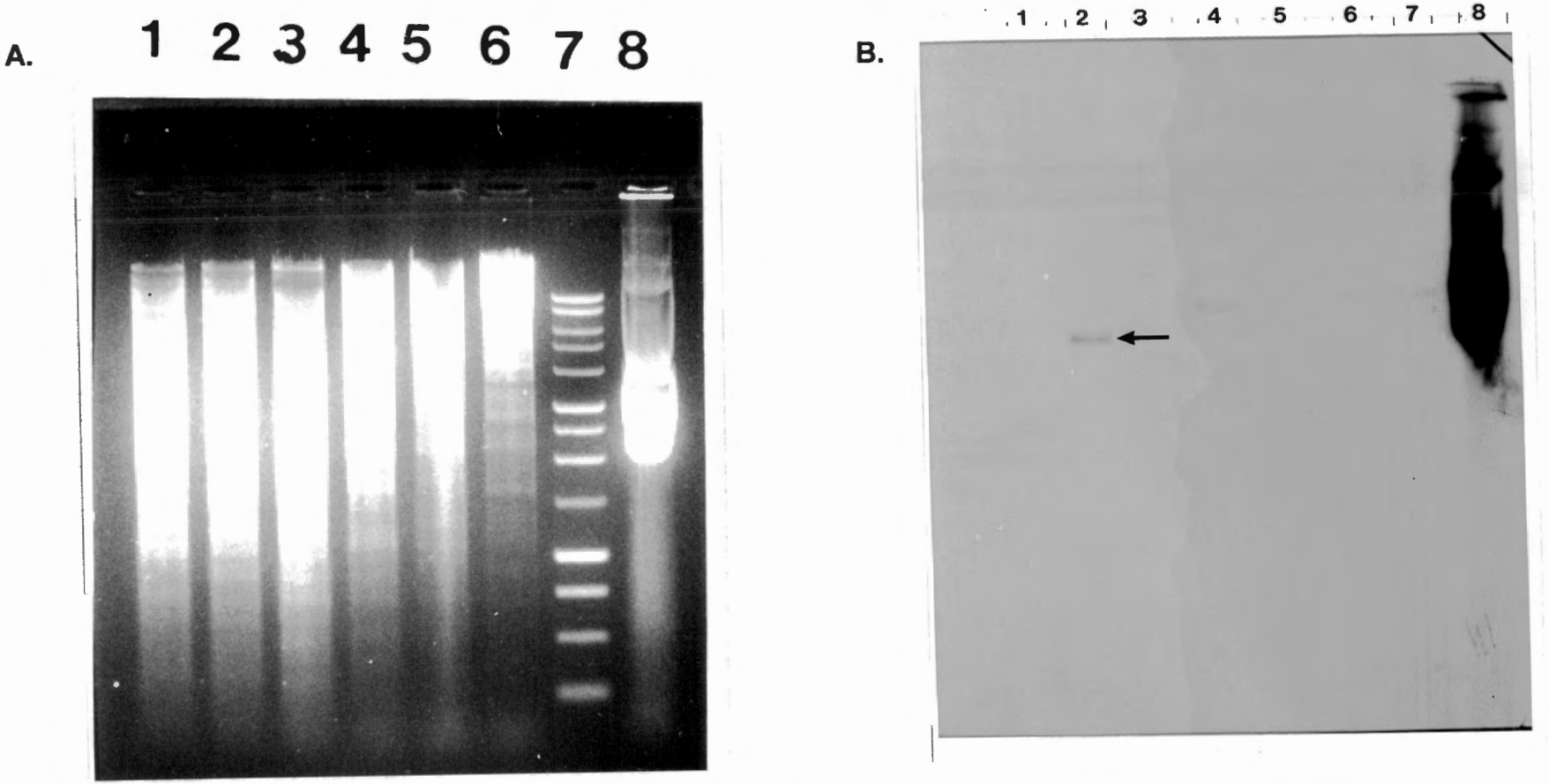


Fig. 4.5. Identification of the DNA fragment from *A.baumannii* strain CAR carrying the *dfrVII* gene

(A) Digests of genomic DNA from strain CAR. Lane 1, *Bam*HI/*Eco*RI digest; lane 2, *Bam*HI/*Hin*dIII digest; lane 3, *Bam*HI/*Sph*I digest; lane 4, *Eco*RI/*Hin*dIII digest; lane 5, *Eco*RI/*Xba*I digest, lane 6, *Hin*dIII/*Xba*I digest; lane 7, 1kb ladder (Appendix B), lane 8, undigested pG5DR plasmid DNA. Electrophoresis was on a 1% agarose gel at 60V for 4 hours. **(B)** Autoradiograph of the Southern blot of the DNA shown in (A) hybridised to the 300bp *dfrVII* probe. Autoradiography was overnight. The 2kb *Bam*HI/*Hin*dIII fragment is indicated with an arrow.

4.C.2. Nucleotide sequence analysis of *dfrVII* and its flanking sequence

The DNA sequencing strategy is shown in Fig.4.6. The DNA sequence of the 2kb insert is shown in fig.4.7. and was confirmed on both strands.

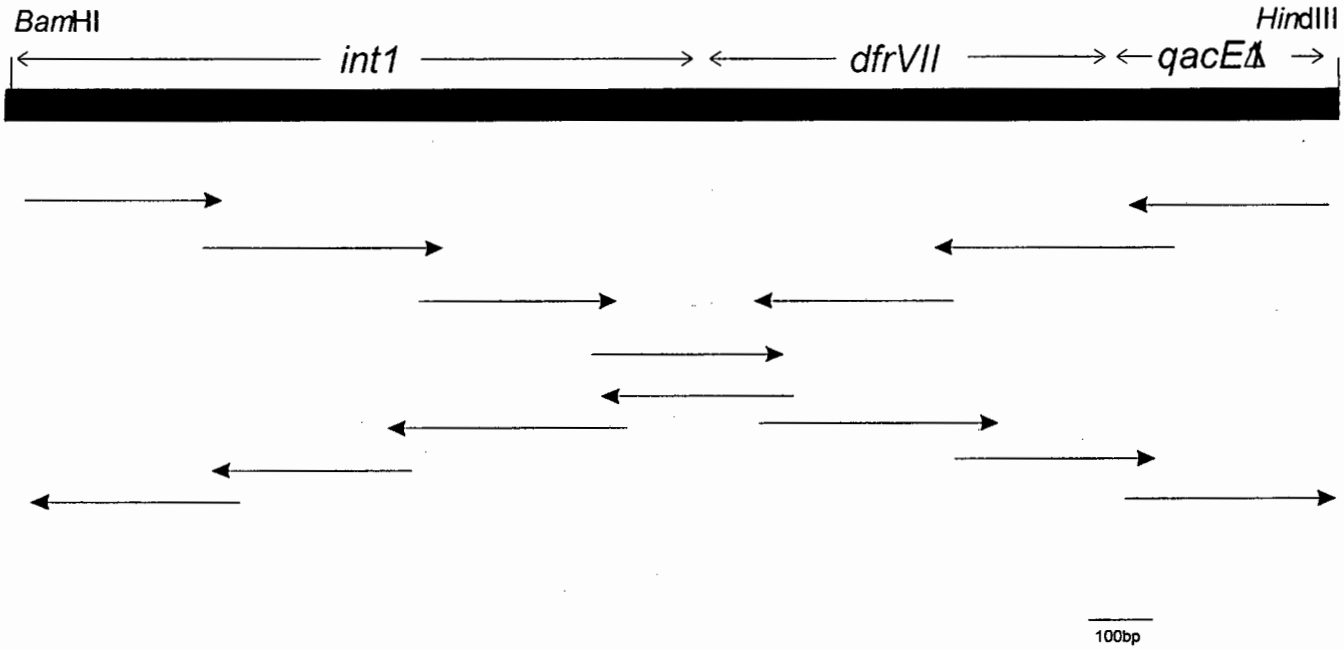


Fig. 4.6.: DNA sequencing strategy for the 2kb *Bam*HI/*Hin*dIII fragment carrying the *dfrVII* gene from *A.baumannii* strain CAR

The arrows below the bar indicate the direction and extent of the sequenced regions. The location of the *dfrVII*, *int1*, and *qacEΔ1* genes are indicated by arrows above the bar.

***Bam*HI**

GGATCCATCAGGCAACGACGGGCTGCTGCCGGCCATCAGCGGACGCAGGGAGGACTTTCC 60

GCAACCGGCCGTTCGATGCGGCACCGATGGCCTTCGCGCAGGGGTAGTGAATCCGCCAGG 120

ATTGACTTGCGCTGCC**CTA**CCTCTCACTAGTGAGGGGCGGCAGCGCATCAAGCGGTGAGC 180

***int1* stop**

GCACTCCGGCACCGCCAACTTTCAGCACATGCGTGTAAATCATCGTCGTAGAGACGTCGG 240

AATGGCCGAGCAGATCCTGCACGGTTCGAATGTCGTAACCGCTGCGGAGCAAGGCCGTCG 300

CGAACGAGTGGCGGAGGGTGTGCGGTGTGGCGGGCTTCGTGATGCCTGCTTGTTCTACGG 360

CACGTTTGAAGGCGCGCTGAAAGGTCTGGTCATACATGTGATGGCGACGCACGACACCGC 420

TCCGTGGATCGGTCGAATGCGTGTGCTGCGCAAAAACCCAGAACCACGGCCAGGAATGCC 480

CGGCGCGCGGATACTTCCGCTCAAGGGCGTCGGGAAGCGCAACGCCGCTGCGGCCCTCGG 540

CCTGGTCCTTCAGCCACCATGCCCGTGCACGCGACAGCTGCTCGCGCAGGCTGGGTGCCA 600

AGCTCTCGGGTAACATCAAGGCCCGATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCG 660

TGCCGTGATCGAAATCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGC 720

CCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGAGGATGC 780

GAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACGGCCGAGGTCTTCCGA 840

TCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCG 900

CCAATGCCTGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAA 960

ATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGA 1020

AGGCACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTA 1080

-35 -10

G

TGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAACGGTGGTAACGGCGCAGTGG 1140

CGGTTTT**CAT**GGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGC 1200

←*int1* start

AGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGC 1260

AGCAGGGCAGTCGCCCTAAAACAAA**GTTAGCC**ATTACGGGGGTTGAA**TTG**AAAATTTCAT 1320

att1 **--RBS-----** **→*K I S L***

***dfrVII* start**

TGATTTCTGCAACGTCAGAAAATGGCGTAATCGGTAATGGCCCTGATATCCCATGGTCAG 1380

I S A T S E N G V I G N G P D I P W S A

CAAAAGGTGAGCAGTTACTCTTTAAAGCGCTCACATATAATCAGTGGCTCCTTGTTGGAA 1440

K G E Q L L F K A L T Y N Q W L L V G R

GGAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGAAAATATGCAGTAGTGTCGAGGA 1500

K T F D S M G V L P N R K Y A V V S R K

Fig.4.7.: DNA sequence analysis of the 2kb *Bam*HI/*Hin*dIII fragment carrying the *dfrVII* gene from *A.baumannii* strain CAR

The *Bam*HI and *Hin*dIII sites at the ends of the insert are indicated in bold. Dots above the sequence correspond to every 10 nucleotides. The first 1307 bases of sequence are 99.9% homologous to the *int1* gene (and its flanking regions) as described for Tn*5086* (Sundström *et al.*, 1993). The G→A transition is indicated by a **G** above the sequence. The *int1* start and stop codons, the recombination site, *attI*, and the ribosomal binding site (RBS) are indicated in bold. The –35 and –10 sites of the weak P_{ANT} promoter are underlined. Bases 1308 to 1781 are identical to the *dfrVII* gene from Tn*5086* (Sunström *et al.*, 1993). The start and stop codons are indicated in bold and the derived amino acid sequence is indicated below the nucleotide sequence. The 59-bpe associated with the *dfrVII* is identical to the one from Tn*5086* except for the C→T transition indicated in bold (Sunström *et al.*, 1993) – the core site of the 59-bpe is indicated in bold. The sequence of the *qacEΔ1* gene (start codon in bold) downstream of the *dfrVII* gene is identical to the published sequence (Sundström *et al.*, 1993). The arrows indicate the direction in which the genes are transcribed. Single letter abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; F, Phe, G, Gly; I, Ile, K, Lys; M, Met; P, Pro, R, Arg, S, Ser; T, Thr; and V, Val.

The pDS100 insert was 2102 nucleotides in length (fig.4.7.). The first 1307 bases were 99.9% homologous to the *int1* gene and the regions flanking the gene as described for Tn*5086* (nucleotides 1 to 714; Sundström *et al.*, 1988; and nucleotides 1 to 593; Sundström *et al.*, 1993). A transition of G→A accounts for the difference in homology. This transition does not change the amino acid sequence but results in the preferred TTG for leucine in *Acinetobacter* (White *et al.*, 1991). The first 136 nucleotides are the region upstream of the *int1* stop codon and the nucleotides from position 1151 to 1307 (fig.4.7) are downstream of the *int1* start codon. The downstream region includes the integron-associated *attI* recombination site (position 1286 to 1292; fig.4.7.) and the ribosomal binding site (position 1295 to 1303; fig.4.7.). The weak P_{ANT} promoter, TGGACA(-35)N_{17}TAAGCT(-10) (Collis and Hall, 1995) was identified at positions 1035-1040 and 1058-1063 (fig.4.7.). The promoter (P2) TTGTTA(-35)N_{17}TACAGT(-10), usually associated with P_{ANT} was not present (Collis and Hall, 1995). The pDS100 sequence lacks the three G-residues between nucleotides 1173 and 1174 (fig.4.7.), which alter the space between the hexamers of P2 to 17, creating an alternative promoter. In this respect, pDS100 resembles the Tn*21*-like integron on Tn*5086* (Sundstrom *et al.*, 1993).

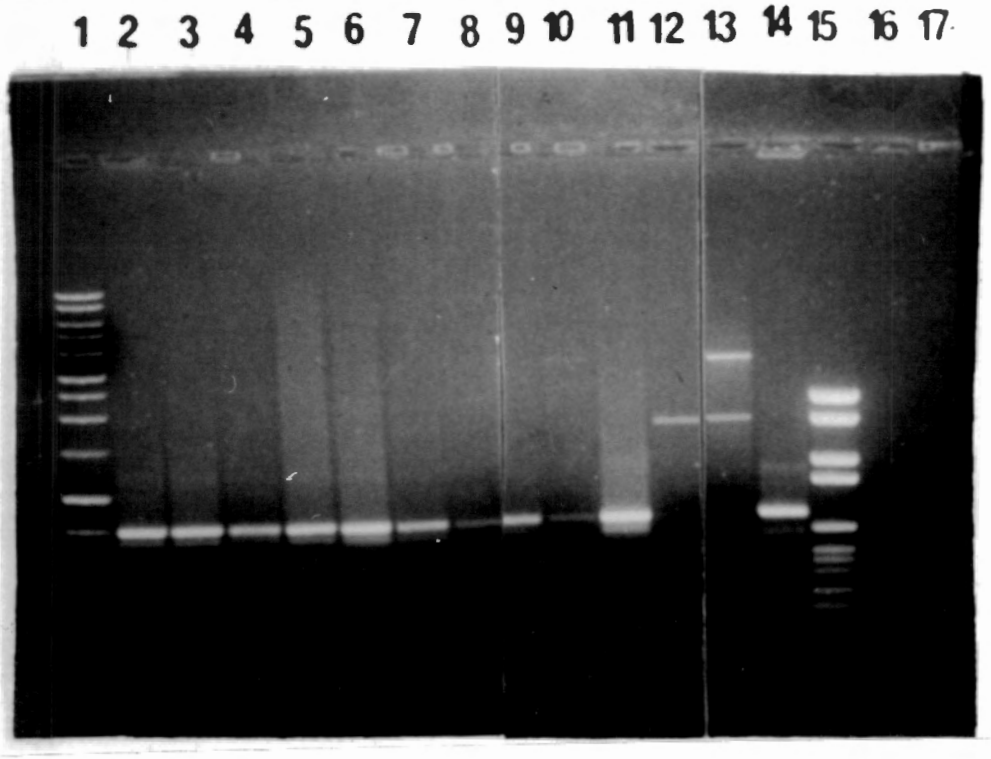
The next 602 bases (position 1308 to 1909; fig.4.7.) are identical to *dfrVII* and its 59-be (nucleotides 594 to 1194; Sundström *et al.*, 1988). Following the 59-be are 193 bases of sequence which are identical to the region upstream of the *qacEΔ1* gene (position 1910 to 2011; fig.4.7.) and to the first 90 bases of the *qacEΔ1* gene itself (position 2012 to 2102; fig.4.7.) described by Sundström *et al.* (1988; nucleotides 1194 to 1387).

4.C.3. Characterisation of the remaining 0.7kb amplification products

To determine whether any of the other 0.7 amplicons obtained from the acinetobacters are related to *dfrVII* sequences, hybridisation studies were performed using the *dfrVII* probe. Towner and Carter (1990), showed that the *Eco*RV fragment is specific for *dfr* type VII genes. A signal was obtained from the amplicon obtained from strain CAR, but not with that from strain CW20,

indicating that the 0.7kb product from this strain is not *dfrVII*-related. Seward and Towner have not identified the structural gene from the integron of strain CW20 (personal communication). All (22) of the 0.7kb amplicons hybridised to the probe suggesting that, like strain CAR, these isolates contain a trimethoprim resistance gene. Fig.4.8. shows the result of the hybridisation.

A.1.



A.	Lane	Sample
	1	1kb ladder
	2	G1
	3	G2
	4	G3
	5	G4
	6	G5
	7	G6
	8	G12
	9	G15
	10	G20
	11	G21
	12	G37
	13	G39
	14	CW20
	15	MW VI
	16	DH5α
	17	dH_2O

A.2.

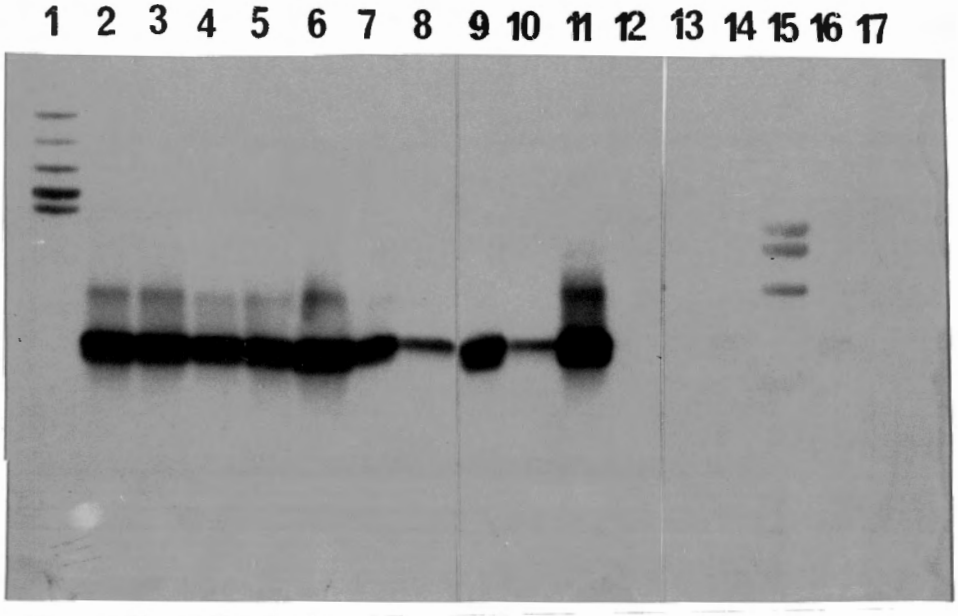


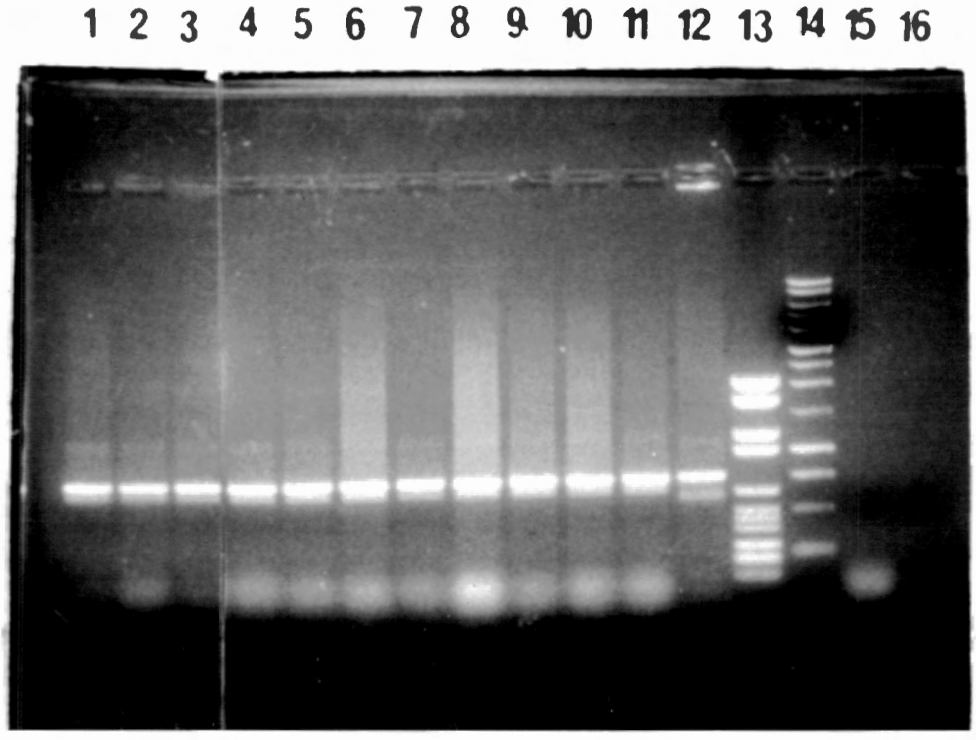
Fig. 4.8.A.: PCR products obtained from the *A.baumannii* clinical isolates, using the 5`-CS and 3`-CS primers, hybridised to the *dfrVII* probe.

(A.1.) PCR products obtained from the GSH isolates. PCR was performed in 50µl volumes containing 5pmol of each primer, 2.25mM $MgCl_2$, 0.2mM dNTPs, 1U *Taq* DNA polymerase, 1X polymerase buffer, and 500ng genomic DNA. The thermocycle profile consisted of 35 cycles denaturation at 94^{0}C for 1min, annealing at 60^{0}C for 1min, and extension at 72^{0}C for 1min. Products were electrophoresed on a 1% agarose gel at 80V for 1.5 hours. **(A.2.)** The autoradiograph of the blot of the PCR products shown in (A.1.) and (B.1.) hybridised to the *dfrVII* probe. Autoradiography was for 40min. The sizes of the bands of the molecular weight markers can be seen in Appendix B.

Fig. 4.8.B: PCR products obtained from the *A.baumannii* clinical isolates, using the 5`-CS and 3`-CS primers, hybridised to the *dfrVII* probe.

(B.1.) PCR products obtained from the UH isolates. PCR was performed in 50µl volumes containing 5pmol of each primer, 2.25mM $MgCl_2$, 0.2mM dNTPs, 1U *Taq* DNA polymerase, 1X polymerase buffer, and 500ng genomic DNA. The thermocycle profile consisted of 35 cycles denaturation at 94^{0}C for 1min, annealing at 60^{0}C for 1min, and extension at 72^{0}C for 1min. Products were electrophoresed on a 1% agarose gel at 80V for 1.5 hours. **(B.2.)** The autoradiograph of the blot of the PCR products shown in (A.1.) and (B.1.) hybridised to the *dfrVII* probe. Autoradiography was for 40min. The sizes of the bands of the molecular weight markers can be seen in Appendix B.

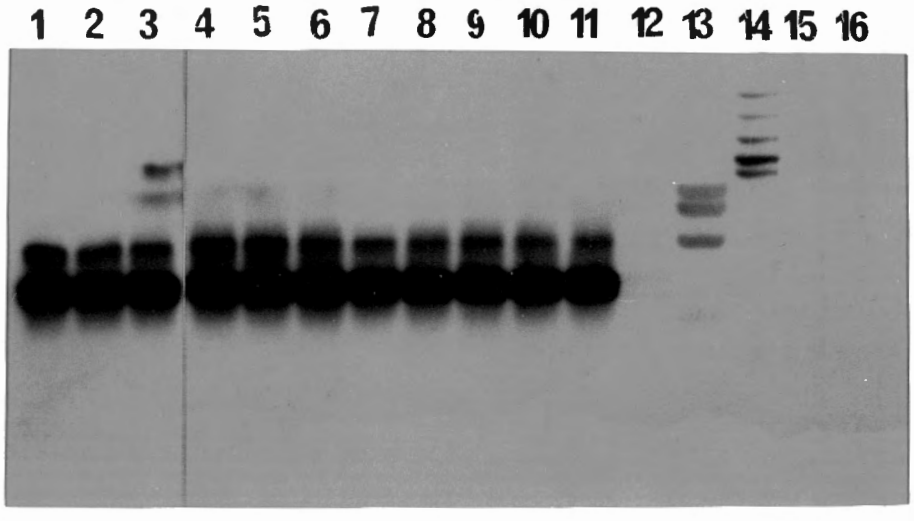
B.1.



B.

Lane	Sample
1	B1
2	B2
3	B3
4	B4
5	B6
6	B8
7	B9
8	B10
9	B11
10	B12
11	B13
12	B14
13	MW VI
14	1kb ladder
15	DH5α
16	dH_2O

B.2.



4.C.4. Location of the *dfrVII* gene

To determine whether the integron *dfrVII* genes are located on plasmids, plasmid DNA was extracted from the 22 clinical isolates and hybridised with the *dfrVII* probe. A signal was obtained from pDS100, the source of the probe, but no signal was obtained with the plasmid DNA from any of the isolates, suggesting that the integron-associated trimethoprim resistance gene is in the chromosomes of the clinical isolates (data not shown).

4.D. DISCUSSION

DNA sequencing of the 0.7kb amplicon from *A.baumannii* strain CAR showed that it contained an integron *dfrVII*. The *dfrVII* gene encodes dihydrofolate reductase type VII which mediates resistance to trimethoprim. Hybridisation studies showed that all the 0.7kb amplicons obtained from the acinetobacters contained *dhfrVII* sequences, suggesting that 22/57 isolates carry integron-associated trimethoprim resistance.

Trimethoprim is structurally analogous to DHFR and acts as a competitive inhibitor of bacterial DHFR, preventing the formation of the essential cofactor, tetrahydrofolate. Dihydrofolate reductase (DHFR) is an enzyme which catalyses the reduction of dihydrofolate to tetrahydrofolate. Trimethoprim resistance in Gram negative bacteria is mediated by the synthesis of additional DHFR enzymes insusceptible to the antibiotic. The genes encoding these DHFRs are plasmid-mediated and have mutations which give rise to the resistance. Thus far, 16 different types of DHFRs have been described. Two major families, which encode high-level trimethoprim resistance, have been identified based on amino acid sequence identity. Family 1 consists of types Ia, Ib, V, VI, and VII; while family 2 is made up of types IIa, IIb, and IIc. The remainder –types IIIa, IIIb, IIIc, VIII, IX, X, and XII- have not been classified into either family because they have a much lower amino acid sequence identity, to each other and to the other types.

There are two reports of plasmid-mediated high-level trimethoprim resistance in *Acinetobacter* (Goldstein *et al.*, 1983; Chirnside *et al.*, 1985). The biochemical mechanism of resistance in the *Acinetobacter* described by Goldstein *et al.* (1983) was not determined, but hybridisation studies showed that it was not a *dfrIa* gene, nor was it Tn*7*-related. In the other study (Chirnside *et al.*, 1985) the gene was not characterised at all. In France (Amyes and Young, 1996) and Chile (Amyes and Young, 1996) high-level trimethoprim resistance is common in *Acinetobacter* spp. responsible for nosocomial infections.

The most widespread DHFR is type Ia (Steen and Skold, 1985). This is mainly attributed to its location on Tn*7*. Tn*7* is able to insert into a wide range of different plasmids at a low frequency and into a specific site on the chromosomes of a range of bacteria at a high frequency. Interestingly, in a survey of trimethoprim resistance in South African isolates of Gram-negative aerobic bacteria, Wylie and Koornhof (1989) showed a low prevalence of DHFR type Ia. A more recent study showed that 74.2% of Gram-negative, aerobic faecal flora, isolated from rural and urban populations in South Africa, was resistant to trimethoprim (Shanahan *et al.*, 1993). Further studies on those isolates showed that the resistance was carried on self-transmissible plasmids in 54.9% of the strains (Adrian *et al.*, 1995a). The majority of plasmids (30%) encoded type Ib resistance and only 3% carried type VII. An investigation of the non-transferable trimethoprim resistance showed the DHFR type VII was the most prevalent in this group (17.4%) (Adrian *et al.*, 1995b). Furthermore, as described in our study, hybridisation experiments showed that the *dfrVII* genes have chromosomal loci, and similar studies demonstrated the presence of class 1-type integrase in these strains. The authors concluded that the *dfrVII* genes are probably located on integrons.

Further analysis of the sequences flanking the *dfrVII* from strain CAR showed that the structural gene is part of a cassette bounded by an *att1* site (GTTAGCC) and a 59 base element/ *attC* site identical to that associated with *dfrVII* in Tn*5086* (Sundstrom *et al.*, 1993). Analysis of the sequence upstream of the structural gene identified the weak P_{ANT} promoter,

TGGACA(-35)N_{17}TAAGCT(-10) (Collis and Hall, 1995). However, the promoter (P2) TTGTTA(-35)N_{17}TACAGT(-10), usually associated with P_{ANT} is not present (Collis and Hall, 1995). The sequence from strain CAR lacks the three G-residues between nucleotides 1173 and 1174 (fig.4.7.), which alter the space between the hexamers of P2 to 17, creating an alternative promoter. In this respect, the integron identified in this study is similar to Tn*5086*, a Tn*21*-like integron (Sundstrom *et al.*, 1993). This integron also contains a *dfrVII* cassette which is regulated by a weak P_{ANT}.

A number of trimethoprim resistance determinants have been identified on gene cassettes associated with either Tn*7*- or Tn*21*-like integrons (Sundstrom and Skold, 1990). The genes encoding the enzymes in families 1 and 2, and *dfrXII*, have been identified on gene cassettes (Sundstrom and Skold, 1990). That these genes are present on mobile elements has undoubtedly contributed to the spread of trimethoprim resistance.

CHAPTER 5: CHARACTERISATION OF THE 2KB AMPLIFICATION PRODUCTS

5.A. INTRODUCTION

The DNA from two of the *Acinetobacter* isolates, strains G37 and G39, yielded amplification products of 2kb using the 5`- and 3`-CS primers [Chapter 3]. These amplicons were cloned and partially sequenced.

5.B. MATERIALS AND METHODS

5.B.1. Cloning experiments

Amplification products were purified as described [section 4.B.4.a] and cloned using the pGEM®-T Easy Vector System (Promega; Madison, USA). An alternative to removing the gratuitous dATP residue (added to the ends of PCR products by the template-independent terminal transferase activity of *Taq* polymerase) is to clone the product into a "T-tailed" vector, such as pGEM®-T Easy. This vector is prepared by digesting pGEM®-5Zf(+) (Promega; Madison, USA) with *Eco*RV and adding a 3`-dTTP residue to both ends (Appendix D). PCR products can thus be ligated directly onto the vector without blunting. The ligation was performed following the manufacturer's protocol: 1μl T4 DNA ligase buffer, 50ng pGEM®-T Easy vector, 500-800ng PCR product, and 3U T4 DNA ligase were mixed in a microfuge tube in total volume of 10μl. The reaction was incubated at 4^0C overnight. The ligation reactions were introduced into competent *E.coli* DH5α cells as described [section 4.B.6.b]. Putative transformants were screened by blue-white colony selection [section 4.B.6.b]. Plasmid DNA was extracted from putative transformants [section 4.B.2.a], digested with *Eco*R1 (which cuts on either side of the cloning site; Appendix D), and electrophoresed on a 1% agarose gel [section 2.B.3.].

5.B.2. DNA sequencing

5.B.2.a. Template preparation

DNA sequencing template was prepared using the High Pure Plasmid Isolation Kit (Boehringer Mannheim; Mannheim, Germany) following the manufacturer's protocol. The transformants were cultured in 5ml 2XYT containing 50μg/ml of ampicillin (Ranbaxy; South Africa) at 37^{0}C overnight. The cells (1.5ml) were harvested by centrifugation in a microfuge (Eppendorf Centrifuge 5415C) at 14000rpm for 5 minutes and lysed according to the alkaline method [section 4.B.2.a] while simultaneously removing RNA with RNAse A. The cell pellet was resuspended in 250μl suspension buffer and lysed with 250μl lysis buffer for 5 minutes at RT. The lysate was neutralised with 350μl binding buffer on ice for 5 minutes. Chromosomal DNA and cellular debris were pelleted by centrifugation (Eppendorf Centrifuge 5415C) at 14000rpm for 10 minutes. The clear lysate was pipetted into the *High Pure* filter tube and centrifuged (Eppendorf Centrifuge 5415C) for 1 minute at 14000rpm. Plasmid DNA bound to the glass fibre fleece in the filter tube and the flow-through was discarded. Plasmid DNA was washed with 700μl wash buffer II by centrifugation as before and eluted with 100μl elution buffer.

5.B.2.b. DNA sequencing

DNA sequencing was performed using the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies; Wisconsin, USA)[section 4.B.7.a]. The universal M13 forward and reverse primers were used. DNA sequencing data were analysed as before [section 4.B.7.c] and using CLUSTALX version 1.74 (Thompson *et. al.,* 1997).

5.C. RESULTS

5.C.1. *A.baumannii* strain G37

The PCR product from strain G37 was cloned into pGEM®-T and the recombinant plasmid was designated pG37C. The nucleotide sequence obtained from the two primers is shown in fig 5.1.

A.

```
GGCATCCAAG CAGCAAGCGC GTTACGCCGT GGGTCGATGT TTGATGTTAT GGAGCAGCAA   60
  5`-CS PRIMER

CGATGTTACG CAGCAGCAAC GATGTTACGC AGCAGGGCAG TCGCCCTAAA ACAAAGTTAG  120

                          A          A    A
GCCGCATGGA CACAACGCAG GTCGCATTGA TACTCCAATT TCTAGCTGCG GCAGATGAGC  180
     → ant(2``)-Ia start

GAAATCTGCC GTCTGGATCG GTGGGGGCTG GGCGATCGAT GCACGGCTAG GGCGTGTA    238
```

B.

```
AAGCAGACTT GACCTGATAG TTTGGCTGTG AGCAATTATG TGCTTAGTGC ATCTAACGCA   60
3`-CS PRIMER                                          inverse core

TAGTTGAGCG GCGGGCGCAG CCTCCACTTC AACGCGGAGT TAGGCATCAA GTGCCTGGGC  120
                                          core

GCCGGTCGAT GCACTTTCGC ACGTCGTGCT CAACGCAAGA TTCTCTAATC GTTGCTTTGG  180

CATATCGAAC GAACGAACGC AGCCGGTCTC TTCGACGCGG CATTGCTAGG TCGTCGTCCT  240

CGTACCAGGT ACGC                                                    254
```

Fig. 5.1.: DNA sequence analysis of the pG37C insert generated with the M13 universal forward and reverse primers

(A) Forward sequence. The sequence from position 1 to 115 were identical to sequences related to the 5`-conserved region of class 1-type integrons (Bissonnette and Roy, 1992), except for the overlapping direct repeat of bases 58 to 76 (underlined and in bold). The 5`-CS primer is indicated in bold. The remaining 123 bases had 97.6% homology to the corresponding portion of the *ant(2``)-Ia* described by Cameron *et al.* (1986) and 98.4% homology to the *ant(2``)-Ia* described by Segal and Elisha (1997). The nucleotide changes are indicated above the sequence. The sequence from Cameron *et al.* (1990) contains an A at positions 144, 154 and 159, while the sequence from Segal and Elisha (1997) contains an A at positions 154 and 159. The start codon of the *ant(2``)-Ia* is shown in bold.

(B) Reverse sequence. The first 53 bases were identical to sequences related to the 3`-conserved region class 1-type integrons (Bissonnette and Roy, 1992).The 3`-CS primer is indicated in bold. Nucleotides 51 to 104 constitute a putative, atypical 59-be. The core and inverse core sites are underlined. The remaining 201 bases showed no significant homology to any sequences in the GenBank DNA database.

DNA sequencing using the universal M13 forward primer yielded 238 bp of data (fig 5.1.A.). The first 115 nucleotides were identical to sequences related to 5`-conserved region of class 1-type integrons (nucleotides 1190 to 1285; Bissonnette and Roy, 1992), except for an overlapping direct repeat of bases 53-76 from position 72-95 (fig. 5.1.A.). The remaining 123 nucleotides have 97.6% homology to the published *ant(2``)-Ia* sequence (nucleotides 1288 to 1412; Cameron *et al.*, 1986), which includes the recombination/*attI* site upstream (position 123 to 129) of the start codon. A transition from A→G at position 144 and transversions from A→T at positions 154 and 159 result in the incorporation of alanine instead of threonine, leucine instead of histidine and phenylalanine instead of isoleucine, respectively. The A→G transition and the A→T transversion at position 159 result in the preferred GCA for alanine and TTT for phenylalanine in *Acinetobacter*, respectively (White *et al.*, 1991). The A→T transversion at position 154 results in a CTC for leucine which is recognised in *Acinetobacter* (White *et al.*, 1991). Comparison of the 123 bases with the *ant(2``)-Ia* sequence from another *Acinetobacter* (*A.baumannii* strain SUN) yielded an homology of 98.4% (nucleotides 638 to 761; Segal and Elisha, 1997). This region also includes the upstream *attI* site. The A→T transversions at positions 154 and 159 account for the difference in homology in this case.

The universal reverse primer yielded 254 nucleotides (fig. 5.1.B.). The first 53 bases were identical to integron-related sequences (nucleotides 1342 to 1290; Bissonnette and Roy, 1992) from the 3`-conserved region of class 1-type integrons and included the 3`-CS primer sequence. Although a putative 59 base element/ *attC* site is present (nucleotides 51 to 104; fig. 5.1.B.), a comparison of the remaining 201 bases with the sequences in the GenBank DNA and protein database yielded no significant homologies. Alignment of the putative 59 base element (59-be) from pG37C with the 59-bes from 40 other gene cassettes and with the 59-be consensus shows that it has little homology to any of them (fig. 5.2.). The 59-be from strain G37 was most similar to the 59-be contiguous with an open reading frame, *orfA* (Recchia and Hall, 1995) and to the putative 59-be from pG39SC [section 5.C.2.].

The 59-bes are the cassette-associated recombination sites [section 2.A.]. Although they are not highly conserved and vary in length, they are identified by their location (downstream of gene cassettes) and the relationship of over 20 bp at their outer ends to consensus sequences that are imperfect inverted repeats of one another (Hall *et al.*, 1991; Collis and Hall, 1992b). The most conserved features are the 7bp core site at the right hand end of the element and the inverse core site at the left hand end (Hall *et al.*, 1991; Collis and Hall, 1992b). The core site has the consensus sequence GTTAGGC or GTTRRRY and the inverse core site has the consensus sequence GCCTAAC or RYYYAAC (Hall *et al.*, 1991; Collis and Hall, 1992b). Recombination occurs between the G and the first T of the 7bp core site (Hall *et al.*, 1991). The putative 59-be from pG37C has a core site homologous to the consensus core. However, the inverse core site is not entirely complementary to the core. The imperfect inverted repeats at the ends of the element do not complement each other as well as those described for other 59-bes. The sequence is also shorter (54 bp) than previously identified 59-bes which range in size from 57 to 141bp (Recchia and Hall, 1995).

	1L →	← 2L		2R →	← 1R
consensus	GYCTAACAATTCGNTCAAGCCGACSSCNM			NNSSCGCCGCTTANCTCNGGCGTTAGCG	
orfA	GCCTAACCGGTCGTTCGAGCGGACTGCCC	12		GGTCAGCCGCTCAACTTCAACGTTAGGC	
G37	ATCTAACGCAT-AGTTGAGCGGCGGGCGC	0		AGCCTCCACTTCAACGCGGA-GTTAGCG	
G39	ATCTAACGCTTGAGTTAAGCCGCGCCGGC	0		AACGTCCG-TTGAACGAATT-GTTAGAC	
cmlA	GCCCAACAAATCGCTGCAGCCGACCCAAA	13		TTCGGTCGGCTGAGCTCAGGCGTTGGGC	
catB2	GCCTAACAATACGCTACACACGGACAAAT	16		AATTTGCCGGTGAGCGT-GGCGTTAGGC	
bla_{IMP}	TTCTAACAAGTCGTTGCAGCACGCCACTA	71		AAACTGCCGCTGAACTT-AGCGTTAGAA	
qacE	ATCTAACAAGTCGTTGCAGCACCGCTCCA	85		AAACTGCCGCTGAACTC-GGCGTTAGAT	
dfrA7	GGCTAACAAGTCGTTCCAGCACCAGTCGC	78		AAACCGCCGCTGAACTC-GGCGTTACGC	
dfrB1	GCCCAACTTGTCGCTCCAGCGGACGGC--	3		-GCCGCCCGCTGAGCTAATTCGTTGGGC	
dfrB3	GCCCAACTGGTCGCTCCAGCGGACGGC--	3		-GCCGCCCGCTGAGCTAGAGCGTTGGGC	
dfrB2	GCCTAACAATTCGCTCCAGCGGACGGC--	3		-GCCGCCCGCTGAGCTTTATCGTTAGGC	
oxa7	GGCTAACAATTCGCTGCAGGCGCGACGGC	7		GGCCGCGGCCTGAGCTCAAACGTTAGCC	
aac(6`)-IIb	GCCTAACTTTTCGCTCCAACGGACGCTTG	40		CAACCGCCGCTGACCTCCGGAGTTAGGC	
orfD	CTCTAACATTTCGGTCAAGCCGACCCGCA	3		TGCGGTCGGCTTACCTCGCCCGTTAGTA	
orfF	TTCTAACATTTCGGTCAAGCCGACCCGCA	3		TGCGGTCGGCTTACCTCGCCCGTTAGCA	
orfE	GTCTAACTCTGCGGTCAAGCGGACCCGCA	3		TGCGGGCCGCTTACCTTGGCCGTTAGTC	
aac(6`)-Ia	GCCTAACCCTTCCATCGAGGGGATGCCCA	15		TGTGCACCCCTCATGTCAAACGTTAGGC	
aac(6`)-Ib	GCCTAACCCTTCCATCGAGGGGGACGTCC	13		TTGGCCGCCCTCATGTCAAACGTTAGGC	
dfrA12	GGCTAACCATTCCGTCAACGGGACGCCAA	33		CCGGCTCTCGTTACGTCCAACGTTAGCC	
oxa2	GCCCAACCCGGCAGTCAACTCGGACGCTG	14		GCAGCGCCGGTTACTTC-AACGTTGGGC	
oxa3	GCCCAACCCGGCAGTCAACTCGGACGCAG	14		GACAGCCCGGTTACTTC-TACGTTGGGC	
oxa1	GCCCAACCCTTCAATCAAGTCGGGACGGC	33		TCGGCGCCCCTTATTTCAAACGTTGGGC	
oxa9	GCATAACCCTGCGCTCGAGCGGACCTCGC	12		GCGAGTCCGCTCACCTTGAACGTTATGC	
catB3	GTCTAACAATTCAATCAAGCCGATGCCGC	3		GCGGCACGGCTTATTTCAGGCGTTAGAC	
catB5	GTCTAACAATTCATTCAAGCCGACGCCGC	3		GCGGCACGGCTTAATTCTGGCGTTAGAC	
aac(6`)-IIa	GCCTAACAACTCATTCAAGCCGACGCCGC	3		GCGGCGCGGCTTAATTCAGGCGTTAGGC	
sat	GCCTAACAATTCATTCAAGCCGACACCGC	3		GCGGCGCGGCTTAATTCAGGAGTTAGGC	
ant(2``)-Ia	GCCTAACAATTCGTCCAAGCCGACGCCGC	3		GCGGCGCGGCTTAACTCAGGTGTTAGGC	
ant(3``)-Ia	GTCTAACAATTCGTTCAAGCCGACGCCGC	3		GCGGCGCGGCTTAACTCAAGCGTTAAAC	
ant(3``)-Ib	GTCTAACAATTCGTTCAAGCCGACGCCGC	3		GCGGCGCGGCTTACCTTGGCCGTTAAAC	
ant(3``)-II	GTCTAACAATTCGTTCAAGCCGACCGCGC	3		GCGCGGCGGCTTAACTCCGGCGTTAGAC	
ant(3``)-VII	GCCTAACAATTCGTTCAAGCCGAACTTGC	55		GCAAGTCGGCTTAACTCAGGCGTTAGGC	
aac(3)-Ia	ACCTAACAATTCGTTCAAGCCGAGATCGG	52		GCGCTCCGGCTTAACTCAGGCGTTAGGT	
blaP1	GGCTAACAAGGCCATCAAGTTGACGGCTT	55		AAGCCGCAACTTATGGC-GGCGTTAGCC	
blaP2	GGCTAACAAGGCCATCAAGTTGACGGCTT	55		AAGCCGCAACTTATGGC-GGCGTTAGCC	
oxa10	GGCTAACAAGTCGCTCAAGGTCGCTCCCT	54		TGCCTGCCCCTTAGCTCCAACGTTAGCC	
oxa5	GGCTAACAAGGCGCTCAAGGCTCGGTTCG	50		TAGCCGCCCCTTAGCTT-TGCGTTAGCC	
dfrA5	GGTTAACAAAGCTATGCAATTGACGGTAA	31		TTACCGCAATTGATAAC-GGCGTTAACC	
dfrA14	GGTTAACAAAGCTATGCAATCGACGGCAA	30		TTTCCGCGATTGATAGC-GACGTTAACC	
orfC	CCTTAACAAGTTTCGGCAAGGGACGCTCC	4		GTCGCGCCCCTGCTAAA-AGCGTTAAGG	
dfrA1	GGTTAACAAGTGGCAGCAACGGATTCGCA	39		TGCGATCCGCTGTGCCA-GGCGTTAACC	
	1L →	← 2L		2R →	← 1R
consensus	GYCTAACAATTCGNTCAAGCCGACSSCNM			NNSSCGCCGCTTANCTCNGGCGTTAGCG	

Fig. 5.2. Alignment of 59 base elements.
The sequences of the outer ends from 41 59-bp elements are aligned. Putative Int1 binding domains at the LH (1L and 2L) and RH (1R and 2R) ends are indicated by arrows and are underlined. Numbers indicate the number of bases in the central region of each element. The 59-bp element from pG37C is second in the list. Adapted from Stokes *et al.* (1997). Sources of the cassettes can be found in Recchia and Hall (1995). R, purine; Y, pyrimidine, M, A or C; S, .C or G; N, A, C, G, T.

5.C.2. *A.baumannii* strain G39

The 2kb amplicon from strain G39 was also cloned into pGEM®-T. The recombinant plasmid was designated pG39SC. The nucleotide sequence obtained from the two primers are shown in fig.5.3.

A.

```
AAGCAGACTT GACCTGATAG TTTGGCTGTG AGCAATTATG TGCTTAGTGC ATCTAACGCT   60
 3`-CS PRIMER                                          inverse core

TGAGTTAAGC CGCGCCGCGA AGCGGCGTCG GCTTGAACGA ATTGTTAGAC ATTATTTGCC  120
                                              core  ant(3``)-Ia stop

GACTACCTTG GTGATCTCGC CTTTCACGTA GTGAACAAAT TCTTCCAACT GATCTGCGCG  180

CGAGGCCAAG CGATCTTCTT CTTGTCCAAG ATAAGCCTGT CTAGCTTCAA GTATGACGGG  240

CTGATACTGG GCCGGCAGGC GCTCCATTGC CCAGTCGG                        278
```

B.

```
GAATTCACTA GTGATTAAAA CCGATATGAA CGTACGCACT TGCACTGAAT CTGACGTCGC   60
 inverted repeat          → orfX start

CTCTATCGCA GTCGTATTTA CTGAGTCTAT TCATGTACTT GGAGCGTCTC ACTATGAGGC  120

TTCGCAAAGG AATGCGTGGG CACCGCGTCC CGCAGATATA GAGGCTTGGT CAGCTCGCTT  180

ATCTGGCCTA CAGACTCTTC TAGCAATTGA GGGAGATGCG GTTATCGGGT TCATCTCTTA  240

CGAGCTTAGC GGCCACATCG AGTTTCTTTA CAC                             273
```

Fig. 5.3.: DNA sequence analysis of the pG39SC insert generated with the M13 universal forward and reverse primers

(A) Forward sequence. The sequence from position 1 to 53 was identical to 3`-conserved region of class 1-type integrons (Bissonnette and Roy, 1992). The 3`-CS primer is indicated in bold. Nucleotides 51 to 105 constitute a putative, atypical 59-be. The core and inverse core sites are underlined. The remaining 147 nucleotides (105 to 252) are identical to the 3`-end of the *ant(3``)-Ia* described by Hollingshead and Vapnek (1985), including the first base after the stop codon. The stop codon is indicated in bold.
(B) Reverse sequence. The first 16 bases of the sequence contained a 14bp inverted repeat (underlined). The 5`-CS primer was absent and there was no sequence homologous to the 5`-conserved region of class 1-type integrons as expected. Adjacent to this region (position 17 to 273) was a portion of a putative gene cassette, *orfX*, including the 9 bases upstream of the cassette start site. The *orfX* start codon is indicated in bold.

DNA sequencing using the universal forward primer yielded 252 bp of data (fig 5.3.A). In this case, it was the first 53 nucleotides of the forward sequence which were identical to sequences from the 3`-conserved region (nucleotides 1342 to 1290; Bissonnette and Roy, 1992) of class 1-type integrons; including the 3`-CS primer sequence. Nucleotides 51 to 105 (fig. 5.3.A.) constitute a putative 59-be. The remaining 147 nucleotides (position 106 to 252) are

identical to the last 147 bases of the published *ant(3``)-Ia* (nucleotides 1022 to 1191; Hollingshead and Vapnek; 1985), including the first base after the stop codon.

The putative 59-be is not homologous to the 59-bes previously described for the *ant(3``)-Ia* (fig 5.2.). It was most homologous to the putative 59-be from pG37C [section 5.C.1]. It too has the core site, and an inverse core site which is not entirely complementary to the core. The imperfect IRs at the ends are also less complementary than those described for other 59-bes. It is 57 bases long.

The 273bp of data (fig.5.3.B) generated with the universal reverse primer did not contain the 5`-CS primer nor any portion homologous to the 5`-conserved region of class 1-type integrons. The first 16 nucleotides of sequence contained, instead, an inverted repeat sequence, TCACTAGTGA (bases 5 to 14). This region was adjacent to sequence identical to a portion of a putative gene cassette, *orfX*, including the nine bases upstream of the cassette start codon (nucleotides 1504 to 1760; Tosini *et al.,* 1998). Like the *orfX* from pG39SC, the original *orfX* was upstream of an *ant(3``)-Ia* gene cassette. The original gene, however, was third in array of 4 cassettes, the first two being *aac(6`)-Ib* and *aac(3)-Ia*.

5.D. DISCUSSION

The hybridisation studies and PCR experiments [Chapter 2 and 3, respectively], together with the DNA sequencing data in this chapter, suggest that *A.baumannii* strain G37 carries an *ant(2``)-Ia* gene cassette and a putative cassette in the variable region of a class 1-type integron. The *ant(2``)-Ia* was most similar to the *ant(2``)-Ia* from another *Acinetobacter* (strain SUN). Based on the results of this study it is not possible to make any conclusions as to the probable function, if any, of the putative cassette.

The sequenced portion of the 5`-conserved region of this integron is unlike the corresponding region in other class 1-type integrons in that it has 2 overlapping direct repeat sequences. This region is downstream of the integrase gene in an area where repeat sequences are not uncommon (Mercier, 1990).

The origin and function of the repeat sequence are unknown but, interestingly, the equivalent region of the original integron carrying the *orfX* gene cassette (Tosini *et al.*, 1998) has the same overlapping repeats. These unpublished results are in the form of a GenBank database report (accession no. AJ009820) and describe an integron isolated from a plasmid in *Salmonella typhimurium.* The DNA sequencing data for a portion of the integrase gene and for the 4 gene cassettes in the variable region (*aac(6`)-Ib*, *aac(3)-Ia, orfX,* and *ant(3``)-Ia*) are presented. The portion of the integrase gene which was sequenced was 96.7% and 73% homologous to the same portion of the *int1* gene at the DNA and amino acid level, respectively (Mercier *et al.*, 1990).
It is possible that the integron in *A.baumannii* strain G37 has a similar integrase gene and was introduced via a plasmid from *Salmonella.*

The *ant(2``)-Ia* encodes ANT(2``), an aminoglycoside adenyltransferase, which mediates resistance to gentamicin, tobramycin, dibekacin, sisomicin, and kanamycin. ANT(2``) is widespread among all Gram-negative bacteria (Shaw *et al.*, 1993). The distribution of aminoglycoside modifying enzymes in clinical isolates of *Acinetobacter* spp. was investigated by Shaw *et al.* (1991; 1993). In the first survey, there was only a 1.4% incidence of ANT(2``) in the isolates tested. However, this increased to 15.5% in 1993. Murray and Moellering (1980) were the first to identify ANT(2``) in *Acinetobacter* and their results suggested that the gene for the enzyme was plasmid-mediated. Elisha and Steyn (1991a) identified the gene encoding ANT(2``) on a plasmid in a clinical strain of *Acinetobacter* by hybridisation, but Northern blot analysis of RNA transcripts showed that the gene was not expressed. The most recent report of ANT(2``) activity in *Acinetobacter* was by Segal and Elisha (1997). They describe an *ant(2``)-Ia* gene cassette integrated at a secondary site on a plasmid in a clinical isolate of *Acinetobacter.* The only other report of an *ant(2``)-Ia* gene cassette on an integron in *Acinetobacter* is by Vila *et al.* (1997). The *ant(2``)-Ia* is the first cassette in the variable region, followed by a gene encoding an OXA-derived β-lactamase.

The putative cassette from strain G37 had an atypical 59-be. It had a core site homologous to the consensus core. The inverse core site, however, was not fully complementary to the core. The imperfect inverted repeats at the ends were less complementary than expected and the element was shorter than previously described 59-bes. Whether this 59-be is active in recombination cannot be determined based on these results.

The hybridisation, PCR and DNA sequencing studies performed on *A.baumannii* strain G39 suggest that it carries a class 1-type integron with two gene cassettes in its variable region. The 3`-end of the variable region has an *ant(3``)-Ia* gene cassette which, like strain G37, has an atypical 59-be.

The *ant(3``)-Ia* encodes ANT(3``), another aminoglycoside adenyltransferase, which mediates resistance to streptomycin and spectinomycin (Hollingshead and Vapnek, 1985). The *ant(3``)-Ia* has been identified in association with several transposons and is widespread among Gram-negative bacteria (Shaw *et al.,* 1993). Various investigators have reported the presence of the enzyme in *Acinetobacter* (Shannon *et al.*, 1978; Murray and Moellering, 1980; Devaud *et al.*, 1982; Goldstein *et al.*, 1983; and Vila *et al.*, 1993). In the study by Devaud *et al.* (1982), the enzyme was found to be transposon-mediated.

The 5`-end of the variable region of pG39SC has a putative gene cassette, *orfX*. In the original description of this cassette (Tosini *et al.*, 1998), it was also on an integron and was associated with an *ant(3``)-Ia* gene cassette. The sequence upstream of *orfX* in pG39SC did not contain integrase-related sequences, nor the sequence of the 5`-CS primer as was expected. Instead, it had a 14bp inverted repeat sequence. The amplification product cloned into pG39SC must have been generated from only the 3`-CS primer or the product was somehow altered during the cloning or sequencing procedure so that the expected sequence on the 5`-end was deleted. There is no adequate explanation for how either of these events may have occurred. If the 2kb product from strain G39 is not the full amplicon, the variable region may

contain further cassettes, however, the region upstream of *orfX* should then have contained a 59-be.

Further analysis, including sequencing the inserts in pG37C and pG39SC on both strands to avoid errors due to compressions or secondary structure, is necessary to determine the full structure of the integrons in strains G37 and G39, respectively.

CHAPTER 6: PARTIAL CHARACTERIZATION OF INTEGRASE-RELATED SEQUENCES FROM A MULTI-RESISTANT CLINICAL ISOLATE OF *A.baumannii*, STRAIN PAU

6.A. INTRODUCTION

Hybridisation studies performed previously in our laboratory demonstrated that a multi-resistant clinical isolate of *A.baumannii* designated strain PAU, carries a 6.5kb *Hin*dIII fragment with homology to the *int1* integrase gene. The 6.5kb fragment was characterized further.

6.B. MATERIALS AND METHODS

6.B.1. Bacterial isolates and plasmids

A.baumannii strain PAU was isolated from the leg stump of an amputee in 1992 by the Clinical Microbiology Laboratory, GSH. The isolate was shown to be resistant to amoxicillin, trimethoprim, gentamicin, cotrimoxazole, piperacillin, and kanamycin [section 2.B.2.]

6.B.2. Cloning experiments

6.B.2.a. Cloning the 6.5kb *Hin*dIII fragment from *A.baumannii* strain PAU

Approximately 10μg of genomic DNA from strain PAU (extracted as in section 2.B.4.) and 1μg of pUC19 DNA were digested with 20U of *Hin*dIII (Boehringer Mannheim; Mannheim, Germany) in 1X buffer B (Boehringer Mannheim; Mannheim, Germany) at 37^{0}C for 2 hours. The DNA in both digests was purified by extraction with phenol and precipitation with 4M LiCl and 100% ethanol [section 2.B.5.b]. Ligation [section 4.B.5.] and transformation [section 4.B.6.] have been described. The transformations were plated on selective media containing 5μg/ml of gentamicin (Intramed; Port Elizabeth, South Africa) since it was proposed that the observed gentamicin resistance of strain PAU could be linked to the integrase-associated sequence on the 6.5kb *Hin*dIII fragment. Plates were incubated at 37^{0}C overnight.

Putative transformants, capable of growth on the gentamicin-containing agar, were screened as in section 4.B.6.b. The recombinant plasmid containing the 6.5kb *Hin*dIII fragment was designated pSKIF100.

6.B.2.b. Subcloning the 6.5kb *Hin*dIII fragment

Digests (*Bam*HI/*Hin*dIII, *Bam*HI/*Sph*I, *Sph*I, *Pst*I, and *Xba*I) of pSKIF100 were electrophoresed on 1% agarose and transferred to Hybond^{TM}-N^{+} membrane as in section 4.B.3.b. The *int1* probe [section 2.B.5.b] was then hybridised to this DNA [section 2.B.5.c.] A 1.3kb *Sph*I fragment which hybridised to the probe was cloned into the *Sph*I site of the pUC19 multiple cloning site as described in sections 4.B.5 and 4.B.6.

pSKIF100 was digested with *Sal*I and a 2kb fragment was cloned into pUC19 as described for the 6.5kb *Hin*dIII fragment [section 6.B.2.a]. Transformants were selected on 2X YT agar containing 5μg/ml of gentamicin (Intramed).

6.B.3. DNA sequencing

DNA sequencing data were generated by the dideoxy chain termination method (Sanger *et al.*, 1977) using either a ^{T7}Sequencing Kit (Pharmacia Biochemicals; Uppsala, Sweden), the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies; Wisconsin, USA), or rapid automated fluorescence in an automated DNA sequencer (373A, Applied Biosystems; Perkin Elmer; California, USA). The latter was performed in the Department of Chemical Pathology, UCT. The protocols for the sequencing reactions were the same as in sections 4.B.7.

6.C. RESULTS

6.C.1. Cloning experiments

The 6.5kb *Hin*dIII fragment carrying integrase-related sequences from *A.baumannii* strain PAU was cloned into the *Hin*dIII site of pUC19 and conferred gentamicin resistance on *E.coli* DH5α. The recombinant plasmid was designated pSKIF100.

The two fragments which were subcloned from pSKIF100 were:
1) a 1.3kb *Sph*I fragment carrying integron-related sequences and
2) a 2kb *Sal*I fragment conferring gentamicin-resistance on its host *E.coli*.
The recombinant plasmids were designated pOINT100 and pGmR100, respectively.

6.C.2. DNA sequencing

The sequencing strategy employed to obtain the sequence of the 6.5kb pSKIF insert is illustrated in Fig. 6.1. DNA sequencing data was generated for 6059 nucleotides of the 6.5kb insert. Fig. 6.2. shows the sequence of the insert with all relevant feature indicated. The sequence was confirmed on both strands.

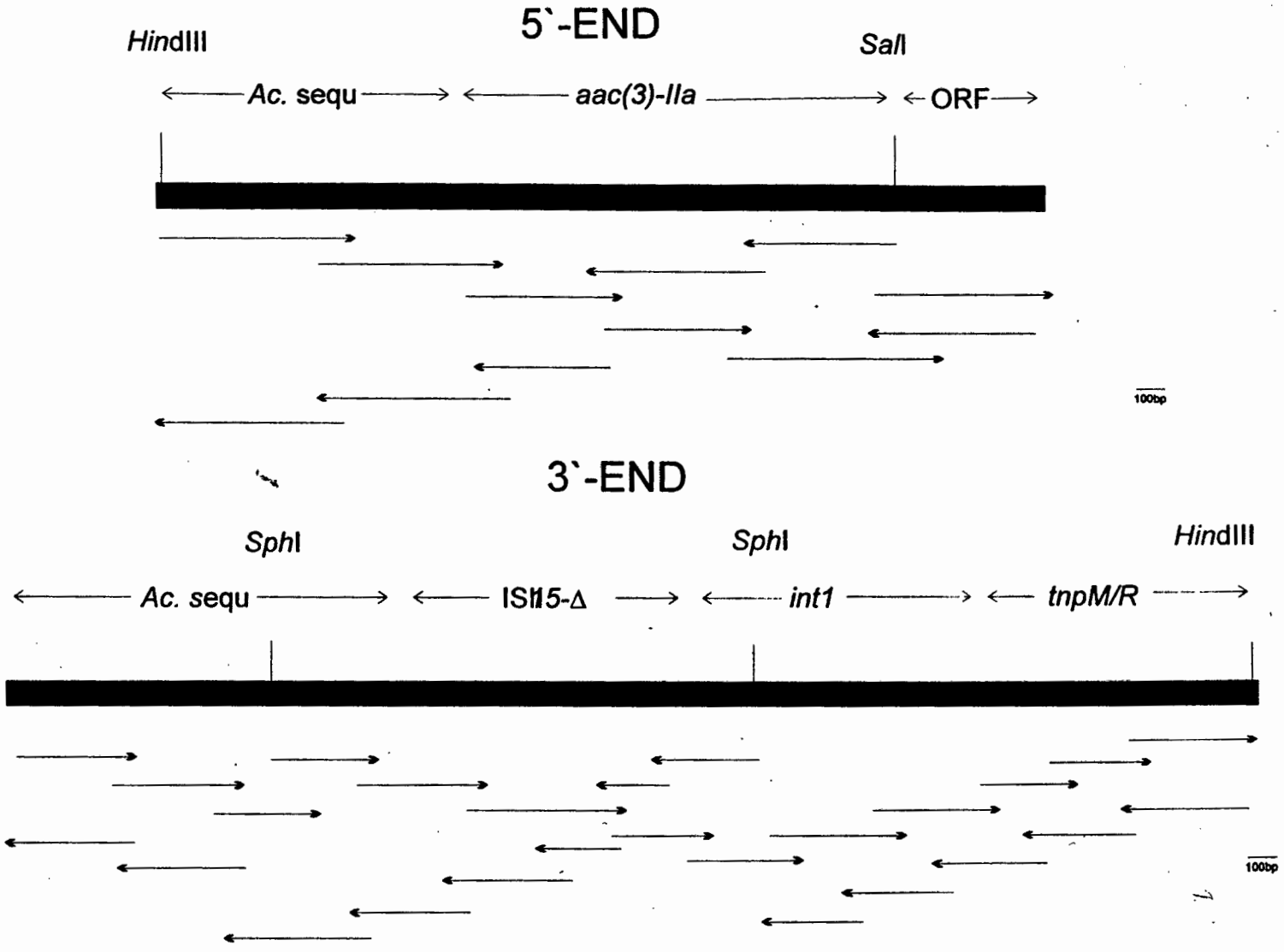


Fig. 6.1.: DNA sequencing strategy for the 6.5kb *Hin*dIII pSKIF100 insert derived from *A.baumannii* strain PAU
The arrows below the bar indicate the direction and extent of the sequenced regions. The location of the different regions of the fragment are indicated above the bar.

HindIII C

1 **AAGCTT**ATAG CTAACACCGC AATCAATTTT TTCACTCGTC TAGGGTCTGT CAAGCGCGTA
61 TTTTCAAGAT TAAACCCGCG TCCTTTGAGA CAACTGAATA AGGTTTCAAT TTCCCAGCGT
121 AATGCATAAT CCTGAATAGC ATTGGCATTA AACTGAGGAG AAACGACGAG TAAAAGCTCT
181 CCATTTTCTA ACTGTAGTGC ACTTATATAT AGTTTCACCC GACCAACCAA AATCCGTCGT
241 TTACGACATT CAATTTGACC AACTTTAAGA TGGCGAAATA AATCACTAAT TTTATGATTC
301 TTTCCTAAAT GATTGGTGAC AATGAAGTTT TTTTAACACG AATGCAGAAG TTGATGTCTT
361 GGTTCAATTA ACCATGTAAA CCACTGCTCA CCGATAAACT CTCTGTCTGC GAACACATTC

TT A

421 ACAATACGGT CTTTACCAAA AATGGCTATA AAGCG-GGAAT CAAAGCAATG CGCTCTTTCG
481 TATCTGAATT TCCACGTTTA TTAAGCAATG TCCAAAGGAT AGGTATCGCT ATTCCACGAT
541 AAACGATTGC GAGCATCAGG ATATTAATAT TTCGTTTTCC CCATTTCCAA TTGGTTCTAT
601 CTAAAGTCAG TTGCACTTGG TCGAATGAAA ACATATTGAA AATCAACTGA GAAATTTGAC
661 GATAATCAAA ATACTGACCT GCAAAGAAGC GCTGCATACG TCGATAAAAT GATTGTGGTA
721 AGCACTTGAT GGGCAAGGCT TTAGATGCAG AAGAAAGATT ACATGTTTGC TTTAAAATAA
781 TCACAAGCAT GATGAGCGCA AAGCACTTTA AATGTGACTT GTTCCATTTT AGAGATTTGT
841 TTAAGATAAG ATATAACTCA TTGAGATGTG TCATAGTATT CGTCGTTAGA AAACAATTAT
901 TATGACATTA TTTCAATGAG TTATCTATTT TTGTCGTGTA CAGAGAAATG GCTTTTATAA

← →

RA3849 homology **IS*1133* homology**

961 TCCACGCCGA AGACATTCAA CACTCGGCTG GAAATCGCCG GTGGCATTTG AGAAAAAAGC

T

1021 CGCTTAAAAT GAGAGATAGA CCGGAACACA ACCGGTGCAA GACCAAAACG CTTGGCCAAA

IS*1133* inverted repeat

1081 AAGCCACCAA CGGCGACGGT CCTCCACCTC GGTCAAGCAG CGAGAGGTAG AGTGATATCT

CAP

1141 CGATAGGTAT AGTGTTTTGC AGTTTAG**AGG AG**ATATCGCG **ATG**CATACGC GGAAGGCAAT

-10 **-RBS--** **→ *aac(3)-IIa* start**

1201 AACGGAGGCA ATTCGAAAAC TCGGAGTCCA AACCGGTGAC CTGTTGATGG TGCATGCCTC
1261 ACTTAAAGCG ATTGGTCCGG TCGAAGGAGG AGCGGAGACG GTCGTTGCCG CGTTACGCTC
1321 CGCGGTTGGG CCGACTGGCA CTGTGATGGG ATACGCGTCG TGGGACCGAT CACCCTACGA
1381 GGAGACTCTG AATGGCGCTC GGTTGGATGA CAAAGCCCGC CGTACCTGGC CGCCGTTCGA
1441 TCCCGCAACG GCCGGGACTT ACCGTGGGTT CGGCCTGCTG AATCAATTTC TGGTTCAAGC
1501 CCCCGGCGCG CGGCGCAGCG CGCACCCCGA TGCATCGATG GTCGCGGTTG GTCCGCTAGC

G

1561 TGAAACGCTG ACGGAGCCTC ACGAACTCGG TCACGCCTTG GGGAAAGGGT CGCCCGTCGA
1621 GCGGTTCGTC CGCCTTGGCG GGAAGGCCCT GCTGTTGGGT GCGCCGCTAA ACTCCGTTAC
1681 CGCATTGCAC TACGCCGAGG CGGTTGCGGA TATCCCCAAC AAACGATGGG TGACGTATGA
1741 GATGCCGATG CTTGGAAGAA ACGGTGAAGT CGCCTGGAAA ACGGCATCAG AATACGATTC
1801 AAACGGCATT CTCGATTGCT TTGCTATCGA AGGAAAGCCG GATGCGGTCG AAACTATAGC
1861 AAATGCTTAC GTGAAGCTCG GTCGCCATCG AGAAGGTGTC GTGGGCTTTG CTCAGTGCTA
1921 CCTGTTCGAC GCGCAGGACA TCGTGACGTT CGGCGTCACC TATCTTGAGA AGCACTTCGG
1981 AGCCACTCCG ATCGTGCCAG CACACGAAGC CGCCCAGCGC TCTTGCGAGC CTTCCGGT**TA**

***aac(3)-IIa* stop**

2041 **G**AGGCC**GTCG ACAATG**ATAA TCTGGATCAA CGGACCTTTC GGCGCCGGAA AGACGACTCT

SalI →

ORF start

2101 CGCTGAGCGG CTGCGCGATC GGCGTCCGAA ATCGCTGATC TTTGACCCTG AGGAAATCGG
2161 GTTCGTGGTG AAAGAAACGG TCCCCATACC AGCGAGCGGA GACTATCAGG ATCTCCCCTT
2221 GTGGAGGGGA CTTACGATCG CTGCGGTCAG CGAGATTCGC AGGAATTACT CGCAGGACAT
2281 CATCATCCCA ATGACGCTCG TGTACCCGGA CTATCAGAGG TGGCTCGGGA A

Fig.6.2.A. DNA sequence analysis of the 5`-end of pSKIF100 (2331 bases)

The first 929 nucleotides are 99.6% homologous to intergenic *Acinetobacter* sequence from *Acinetobacter* strain RA3849. The difference in homology was due to transversions at positions 44 and 456, a transition at position 470 and a deletion at position 455 (indicated in bold above the sequence). The sequence from position 946 to 1065 is 99.2% homologous to the right hand side of IS*1133*. A transversion of T→A at position 1062 within the 27-bp inverted repeat accounts for the difference in homology. The transversion is indicated in bold above the sequence and the 27-bp IR is underlined. The *aac(3)-IIa* structural gene (position 1181 to 2041) is identical to the *aac(3)-IIa* described by Elisha (1991) and Elisha and Steyn (1991). This sequence differs from the original *aac(3)-IIa* (Vliegenthart *et al.*, 1989) by a transition of G→A (position 1604; indicated in bold above the sequence).The upstream regulatory region of the *aac(3)-IIa* includes a ribosomal binding site (RBS; in bold), a –10 promoter sequence (underlined) and a CAP protein binding site (underlined). There is no sequence corresponding to the –35 consensus. Downstream of the *aac(3)-IIa* is a region encoding an open reading frame (ORF) of unknown function (start codon indicated in bold). This ORF was described by Allmansberger *et al.* (1985) on an R-plasmid, pWP116a. The ORF does not have a promoter but it is likely that a polycistronic messenger is transcribed from the *aacC2* promoter (Allmansberger *et al.*, 1985). The *Hin*dIII and *Sal*I sites are shown in bold.

The 5`-end of pSKIF100 was sequenced for 2331 bases (fig.6.2.A.), then there is a break in data, and the sequence continues to the 3`-end for 3728 bases (fig.6.2.B.). The first 929 nucleotides on the 5`-end (fig.6.2.A.) are 99.6% homologous to sequence generated for *Acinetobacter* sp. strain RA3849 (Schembri *et al.*, 1995). Transversions of C→G and T→G at nucleotides 44 and 456, respectively, a transition of A→G at nucleotide 470, and a deletion of a T at nucleotide 455 account for the difference in homology. The sequence from RA3849 is intergenic DNA flanking an acetoacetyl-CoA reductase gene (*phaB*).

The DNA sequence from position 930 to 2052 (fig.6.2.A.) is identical to sequence of an *aac(3)-IIa* and its upstream flanking region from an isolate of *A.baumannii,* strain SAK (Elisha, 1991; Elisha and Steyn, 1991a). The sequence from position 1181 to 2041 (fig.6.2.A.) in pSKIF100 contains the *aac(3)-IIa* structural gene (nucleotides 252 to 1110; Elisha, 1991). The sequence from strain SAK showed a 99.9% homology with the sequence for the *aac(3)-IIa* originally described by Vliegenthart *et al.* (1989), the only difference being a transition of G→A at position 1604, which results in the incorporation of lysine rather than glutamic acid. Nucleotide sequence comparisons showed that the *aac(3)-IIa* was equivalent to the *aac(3)-IIa*

described by Allmansberger *et al.* (1985). As with the *aac(3)-IIa* from strain SAK, the region upstream (position 1100 to 1180; fig.6.2.A.) of the *aac(3)-IIa* from pSKIF100 contains the regulatory region. This region contains a ribosomal binding site (AGGAG) and there is a -10 region (TATAGT) but no sequence corresponding to the *E.coli* -35 consensus sequence, TTGACAT. However, 12 bp from the -10 region, there is a sequence (AGTGA) similar to the consensus sequence described for cAMP/CAP controlled promoters (De Crombrugghe *et al.*, 1984; Elisha, 1991; Elisha and Steyn, 1991a). It has been suggested (McClure, 1985) that an activator protein often controls promoters with poor homology to the consensus sequence in the -35 region. CAP is one such protein, which binds to cAMP to form an active complex. Upstream of the pSKIF100 regulatory region (position 946 to 1065; fig.6.2.A.) is a region which is 99.2% homologous to portion of an insertion sequence, IS*1133* (nucleotides 4772 to 4891; Chiou and Jones, 1993). IS*1133* was identified as part of Tn*5393*, a Tn*3*-type transposon (Chiou and Jones, 1993). It is a 1.2kb insertion element with 27bp inverted repeats (IRs) and 3bp duplicates and its nucleotide sequence shared a 45% identity with IS*3* (Chiou and Jones, 1993). The area of homology is in the last 120 nucleotides of IS*1133* and includes the right-hand IR (fig.6.2.A.1.). The difference in homology is due to a transversion of T→A (position 1062; fig.6.2.A; fig.6.2.A.1.), which is within the IR. The 3bp duplicate (TAG) is absent in pSKIF100. The regions flanking the partial IS element (positions 1 to 946 and 1066 to 1099; fig.6.2.A.) in pSKIF100 are not homologous to any portion of Tn*5393.* Interestingly, Elisha (1991) showed that the sequence corresponding to position 930 to 1024 (fig.6.2.A.) in pSKIF100 (nucleotides 1 to 155; Elisha, 1991) had a 54.8% identity with the right end of IS*981* (an element isolated from *Lactococcus lactis* and related to IS*2* and IS*3*) and felt that this may imply that the *aac(3)-IIa* was carried on a transposon in strain SAK. The homology to IS*1133* in strain PAU may imply the same about its *aac(3)-IIa*.

```
Sequence 1   IS1133 from pSKIF100 (position 946 to 1065; fig.6.2.A.)
Sequence 2   IS1133 from Chiou and Jones (1993)

Sequence 2  TGGACTTGCCTCGGTTTCGTTCCGGTCTTTTTGAGAGCATTATTTGTTATCAAGGAGACC   60
Sequence 2  ATATATGTCATTAAAGCATAGTGATGAATTTAAGCGTGATGCAGTTCGCATAGCACTCAC  120
Sequence 2  TAGTGGCTTAACACGCCGTCAAGTTGCGTCAGATTTAAGTATTGGGCTTTCCACGCTTGG  180
Sequence 2  GAAATGGATCGCATCAATTTCCGATGAAACTAAAATTCCTACCCAAGACACTGATCTTCT  240
Sequence 2  GCGTGAGAATGAACGTTTACGCAAAGAGAACCGTATCCTTCGGGAGGAGAGGGAGATATT  300
Sequence 2  AAAAAAGGCAGCAATATTTTTCGCAGTACAAAAGCTGTGAGATTTCAGTTTATTACGGAT  360
Sequence 2  TACCGTGGCTCTCTCTCACGTTCACGCATATGTCGTTTGATGGGCGTAACAGATCGTGGT  420
Sequence 2  TTACGTGCATGGAAACGCCGTCCTCCATCACTGCGCCAGCGTCGTGATCTTATACTTCTA  480
Sequence 2  GCGCATATACGTGAGCAGCATCGGTTGTGTTTGGGGAGCTATGGTAGGCCGCGTATGACA  540
Sequence 2  GAAGAGTTGAAAGCGCTGGGCCTGCAGGTTGGGCAGCGTCGGGTTGGACGTTTGATGCGC  600
Sequence 2  CAGAATAACATTACAGTTGTTCGAACGCGTAAATTCAAACGGACAACGGATAGTCATCAT  660
Sequence 2  ACCTTCAACATTGCACCGAACCTATTAAAACAAGACTTTAGCGCAAGCGCACCCAACCAG  720
Sequence 2  AAATGGGCAGGCGATATCACTTATGTTTGGACCAGAGAAGGATGGGTCTATCTTGCTGTT  780
Sequence 2  ATCCTTGACCTGTATTCCCGTCGCGTGATTGGCTGGGCAACAGGTGATCGATTAAAGCAG  840
Sequence 2  GATCTTGCATTAAGGGCACTGAATATGGCGTTGGCTTTACGCAAACCACCACCGGGTTGT  900
Sequence 2  ATTCAACACACAGACCGTGGGAGCCAATATTGCGCTCATGAATATCAAAAGCTACTGCTC  960
Sequence 2  AAACATCAATTGCTGCCGTCCATGAGCGGGAAAGGCAATTGTTTTGATAACTCCGCAGTA 1020
Sequence 2  GAAAGCTTCTTTAAATCATTAAAGGCTGAGTTGATTTGGCGCAGACACTGGCAAACAAGG 1080
Sequence 1                                  AAATGGCTTTTATAATCCACGCCGAAGA  973
                                            ****************************
Sequence 2  CGAGATATTGAGATTGCAATCTTCGAATATATAAATGGCTTTTATAATCCACGCCGAAGA 1140
Sequence 1  CATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAG 1033
            ************************************************************
Sequence 2  CATTCAACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAG 1200
Sequence 1  AGATAGACCGGAACACAACCGGTGCAAGACCA                              1065
            ***************************** ***
Sequence 2  AGATAGACCGGAACACAACCGGTGCAAGTCCA                              1232
```

Fig.6.2.A.1. Comparison of the IS*1133* region of pSKIF100 with the published IS*1133* sequence

The sequence from position 946 to 1065 (fig.6.2.A.1.) was compared to the full IS*1133* sequence (Chiou and Jones, 1993). The homology is 99.2% and is at the right hand end of the IS*1133* sequence. The T→A transversion accounts for the difference in homology. An asterisk (*) indicates an identical base. The 27-bp inverted repeats are underlined.

The remaining 279 bases of the sequencing data from pSKIF100 (position 2053 to 2331; fig.6.2.A.) are identical to an open reading frame (nucleotides 1059 to 1336; Allmansberger *et al.,* 1985) which resides on three related R-plasmids, pWP14a, pWP116a and pWP113a, downstream of an *aac(3)-IIa*. These plasmids were isolated from various strains of *Serratia marcescens* and *Klebsiella pneumonia*. As in the three R-plasmids, the open reading

frame (ORF) on pSKIF100 started at the 12th bp downstream from the stop codon of the *aac(3)-IIa* and a suitable ribosomal binding site was found 9 bp upstream from the start codon. The ORF is not preceded by a promoter and, as in the three R-plasmids, there is no stop codon within the ORF. The homology between pWP116a and the two other R-plasmids ended inside the ORF (nucleotide 1321; Allmansberger *et al.*, 1985). The corresponding region in pSKIF100 (position 2316 to 2331; fig.6.2.A.) is homologous to pWP116a and not pWP14a or pWP113a (fig.6.2.A.2.). The authors felt that although no attempts had been made to test for the expression of a protein from it, it was likely that a polycistronic messenger transcribed from the *aac(3)-IIa* promoter was translated from the ORF. The function of a possible gene product expressed from this ORF was not determined (Allmansberger *et al.,* 1985). Also of note is that pWP14a and pWP116a, but not pWP113a, have an IS*140* element upstream of their *aac(3)-IIa* genes (Allmansberger *et al.,* 1985). In pWP116a, the element is inverted relative to its orientation in pWP14a. IS*140* bears palindromic –35 sequences at both ends, inside its 14bp IRs. It is, however, an IS26- and IS*15*-related element and bears no similarity to IS*1133* or IS*3* (Allmansberger *et al.,* 1985).

Sequence 1 **pSKIF100 ORF** (position 2053 to 2331; fig.6.2.A)
Sequence 2 **pWP116a ORF** (position 1047 to 1336; Allmansberger *et al.*, 1985)

```
Sequence 1                      2053  AGGCCGTCGACAATGATAATCTGGATCAACGGAC  2086
                                      **********************************
Sequence 2                      1047  AGGCCGTCGACAATGATAATCTGGATCAACGGAC  1080
                                                    → ORF start

Sequence 1  CTTTCGGCGCCGGAAAGACGACTCTCGCTGAGCGGCTGCGCGATCGGCGTCCGAAATCGC  2146
            ************************************************************
Sequence 2  CTTTCGGCGCCGGAAAGACGACTCTCGCTGAGCGGCTGCGCGATCGGCGTCCGAAATCGC  1140

Sequence 1  TGATCTTTGACCCTGAGGAAATCGGGTTCGTGGTGAAAGAAACGGTCCCCATACCAGCGA  2206
            ************************************************************
Sequence 2  TGATCTTTGACCCTGAGGAAATCGGGTTCGTGGTGAAAGAAACGGTCCCCATACCAGCGA  1200

Sequence 1  GCGGAGACTATCAGGATCTCCCCTTGTGGAGGGGACTTACGATCGCTGCGGTCAGCGAGA  2266
            ************************************************************
Sequence 2  GCGGAGACTATCAGGATCTCCCCTTGTGGAGGGGACTTACGATCGCTGCGGTCAGCGAGA  1260

Sequence 1  TTCGCAGGAATTACTCGCAGGACATCATCATCCCAATGACGCTCGTGTACCCGGACTATC  2326
            ************************************************************
Sequence 2  TTCGCAGGAATTACTCGCAGGACATCATCATCCCAATGACGCTCGTGTACCCGGACTATC  1320

Sequence 1  AGAGGTGGCTCGGGAA                                              2331
            ****************
Sequence 2  AGAGGTGGCTCGGGAA                                              1336
```

Fig.6.2.A.2. Comparison of the ORF from pSKIF100 with the ORF from pWP116a

The sequence from position 2053 to 2331 (fig.6.2.A.) of pSKIF100 was compared to the ORF from pWP116a (Allmansberger *et al.*, 1985). The sequence from pSKIF100 was identical to the published sequence. The start codon of the ORF is shown in bold.

***Ac.* sequence**

→

```
   1 GGGTCATCCC TGAGCTGAGT CCACAGCGGC TAGCTGAGAT TAGGTGAATC
  51 TCGAGACCAT CCAAATACTA GGAGCAGTCA CCCTCTTTTG GAGCTGACCA
 101 AAGCCGTGAG CTGCTGTGAC AATTGGCAGC TCATAGAGTC AGTCTATCAG
 151 GCCAGGAGCT CGCAGTAACC GTACGTACTA CAGGAGACTC AAAGACGGTT
 201 AGAGGACTGG CGTAGACTAG CATTCAGGGG AGAGTAGTTC CCATCAAGGA
 251 CTATCAGAGG CGAGCGACCA TAGCCCTGGC AAAGAAAGTG GATGCTTGGG
 301 CTGAAAGGAA GTCGTAAGCC GTACCGCCTG ACGGTCGGTT TACGTGACGC
 351 GAATGGCGAC TTCCGTTTCG GAAGAAGCAC CACCGCGGCA TAGAGACGCC
 401 AGCTGCCGAA GCCTCCGCCT TGAAGGGACA CGAACCGTCG ACCCGCAAGT
 451 TGGTGTACTA AGTGATTGAA ATCCAGACGC TCAGGCAAGC GGGGTGTCGG
 501 CTGTAGCGTG ACGTTAAATT ATAGAACCTA AAGCTCCATA TGCTAAATGG
 551 TATCAAGGGG CCAAATAGCG GCGGCTACAG TCGCCGCCTT TAACGGATAA
 601 CAACCCGCAA CGACTTTCGA GCCTGATTGG CTTCGCACGG CCTAGACACC
 651 AGGGTTGCAC ATATTTCCAG CATACAATTA CACTATAGCT GGATCAAGCC
 701 CAAGGTGCAC GCCGTACTAC GACGCACTTG ATCTAAAGTT GGCCCTTATC
 751 TCATTGAGAC GGATAAAGAA ACTCCTCGCA AAGCTCGATT TGTGGCTTCA
 801 GCATCGCGGT TCGGCGGTGG ACCGCCGTGG TCATCGGCTT CATCGGAGTG
 851 CTTCTAGTGG TCAAGCCGGG CTATCTGCCG TTCCAATTGG GTCATTTGGC
 901 TGCCATCGGC ACCGGGCTGG CCACGGACGC GGCAATCATC ATCCTCAGGC
 951 GCGTCGGCGG AAAGGTGAAG ACCACCAGCA TGCTGGGCAT CAGTGCCGGC
```

***Sph*I**

```
1001 GCTTGCAGCG CGCTGAGGCA CGACATATGA AGGCGTAGCT GGAAACCCCG
1051 TCAGCCAAAA TTGGCGACAG GGTCTAGATC CCTTATTTTA AAGCGCGGAC
1101 CGACCCATAG TCGCTATTGG AGTGTCTCAA GCCGCAGTGG TAGAAACGGC
1151 CGAGGCTCCT CAAATGATGG AGATGTCAGC AACAGATGGT CACGCTTTCC
1201 TTATCGCTGG CAGATTTTAT GGACCACCTC ACCAACACGG TGGAAGTTTT
1251 GCCAGCAGAT CGCTTGTGCA AAACATAGCA ATCTTGTCTA TGCGGTGATT
1301 GGGCAGCCGG TGCGAAGATC TCATCAACGA AATCCGGGAT CTGATCTTTA
1351 CGCATTTCAT CCGGCACTGT TGCAAATAGT CGGTGGTGAT AAACTTATCA
```

← ***IS15-Δ*II IR**

***Ac.* sequence**

```
1401 TCCCCTTTTG CTGATGGAGC TGCACATGAA CCCATTCAAA GGCCGGCATT
1451 TTCAGCGTGA CATCATTCTG TGGGCCGTAC GCTGGTACTG CAAATACGGC
1501 ATCAGTTACC GTGAGCTGCA GGAGATGCTG GCTGAACGCG GAGTGAATGT
1551 CGATCACTCC ACGATTTACC GCTGGGTTCA GCGTTATGCG CCTGAAATGG
1601 AAAAACGGCT GCGCTGGTAC TGGCGTAACC CTTCCGATCT TTGCCCGTGG
1651 CACATGGATG AAACCTACGT GAAGGTCAAT GGCCGCTGGG CGTATCTGTA
1701 CCGGGCCGTC GACAGCCGGG GCCGCACTGT CGATTTTTAT CTCTCCTCCC
1751 GTCGTAACAG CAAAGCTGCA TACCGGTTTC TGGGTAAAAT CCTCAACAAC
1801 GTGAAGAAGT GGCAGATCCC GCGATTCATC AACACGGATA AAGCGCCCGC
1851 CTATGGTCGC GCGCTTGCTC TGCTCAAACG CGAAGGCCGG TGCCCGTCTG
1901 ACGTTGAACA CCGACAGATT AAGTACCGGA ACAACGTGAT TGAATGCGAT
1951 CATGGCAAAC TGAAACGGAT AATCGGCGCC ACGCTGGGAT TTAAATCCAT
2001 GAAGACGGCT TACGCCACCA TCAAAGGTAT TGAGGTGATG CGTGCACTAC
2051 GCAAAGGCCA GGCCTCAGCA TTTTATTATG GTGATCCCCT GGGCGAAATG
2101 CGCCTGGTAA GCAGAGTTTT TGAAAGGTAA GGCCTTTGAA TAAGACAAAA
2151 GGCTGCCTCA TCGCTAACTT TGCAACAGTG CCCTTCAGGA GATCGGAAGA
```

***IS15-Δ*II IR** →

truncated *int1*

```
2201 CCTCGGCCGT CGCGGCGCTT GCCGGTGGTG CTGACCCCGG ATGAAGTGGT
2251 TCGCATCCTC GGTTTTCTGG AAGGCGAGCA TCGTTTGTTC GCCCAGCTTC
2301 TGTATGGAAC GGGCATGCGG ATCAGTGAGG GTTTGCAACT GCGGGTCAAG
```

***Sph*I**

```
2351 GATCTGGATT TCGATCACGG CACGATCATC GTGCGGGAGG GCAAGGGCTC
2401 CAAGGATCGG GCCTTGATGT TACCCGAGAG CTTGGCACCC AGCCTGCGCG
2451 AGCACGTGTC GCGTGCACGG GCATGGTGGC TGAAGGACCA GGCCGAGGGC
2501 CGCAGCGGCG TTGCGCTTCC CGACGCCCTT GAGCGGAAGT ATCCGCGCGC
2551 CGGGCATTCC TGGCCGTGGT TCTGGGTTTT TGCGCAGCAC ACGCATTCGA
2601 CCGATCCACG GAGCGGTGTC GTGCGTCGCC ATCACATGTA TGACCAGACC
2651 TTTCAGCGCG CCTTCAAACG TGCCGTAGAA CAAGCAGGCA TCACGAAGCC
```

```
2701 CGCCACACCG CACACCCTCC GCCACTCGTT CGCGACGGCC TTGCTCCGCA
2751 GCGGTTACGA CATTCGAACC GTGCAGGATC TGCTCGGCCA TTCCGACGTC
2801 TCTACGACGA TGATTTACAC GCATGTGCTG AAAGTTGGCG GTGCCGGAGT
2851 GCGCTCACCG CTTGATGCGC TGCCGCCCCT CACTAGTGAG AGGTAGGGCA
                                              intI stop
2901 GCGCAAGTCA ATCCTGGCGG ATTCACTACC CCTGCGCGAA GGCCATCGGT
2951 GCCGCATCGA ACGGCCGGTT GCGGAAAGTC CTCCCTGCGT CCGCTGATGG
3001 CCGGCAGCAG CCCGTCGTTG CCTGATGGGG ATCCAACCCC TCCGCTGCTA
3051 TAGTGCAGTC GGCTTCTGAC GTTCAGTGCA GCCGTCTTCT GAAAACGACA
                                    25-bp Brown's repeat
3101 ATGGAGGTGG TAGCCGAGGG TGTGGAAACA CCCGACTGCC TTGCGTGGTT
     →
  tnpM start
3151 GCGGCAGGCG GGTTGCGACA CGGTGCAGGG TTTCCTGTTC GCCAGGCCGA
3201 TGCCGGCGGC GGCCTTCGTC GGCTTCGTCA ACCAATGGAG GAACACCACC
3251 ATGAACGCCA ATGAACCGAG CACCAGTTGC TGCGTGTGCT GCAAGGAAAT
3301 CCCGCTCGAT GCCGCCTTCA CGCCGGAAGG GGCCGAGTAC GTGGAGCATT
3351 TCTGCGGGCT GGAGTGCTAT CAGCGCTTCC AGGCGCGGGC CAGCACTGCG
3401 ACCGAAACCA GCGTCAAACC GGACGCTTGT GATTCGCCGC CGTCAGGTTG
                                                tnpM stop
3451 AGGCATACCC TAACCTGATG TCAGATGCCA TGTGTAAATT GCGTCAGGAT
3501 AGGATTGAAT TTTGAATTTA TTGACATATC TCGTTGAAGG TCATAGAGTC
3551 TTCCCTGACA TTTTGCAGGG AATTCCATGA CTGGACAGCG CATTGGGTAT
                                 →
                              tnpR start
3601 ATCAGGGTCA GCACCTTCGA CCAGAACCCG GAACGGCAAC TGGAAGGCGT
3651 CAAGGTTGAT CGCGCTTTTA GCGACAAGGC ATCCGGCAAG GATGTCAAGC
3701 GTCCGCAACT GGAAGCGCTG ATAAGCTT
                              HindIII
```

Fig.6.2.B. DNA sequence analysis of the 3`-end of pSKIF100 (3728 bases)

The first 1362 bases (between the arrows) of data have no significant homology to any sequences in the GenBank DNA and protein database. This region is most likely *Acinetobacter* intergenic sequence. Position 1363 to 2182 encompass an insertion sequence, IS*15-Δ*II. The 14-bp inverted repeats marking the boundary of the IS are underlined. Downstream of IS*15-Δ*II is a truncated *int1*. In addition to truncating the *int1*, IS*15-Δ*II has deleted the variable region and 3`-conserved region of the integron. The 5`-outer boundary of the *int1*, the 25-bp Brown's repeat (underlined) and the *int1* stop codon (in bold) are indicated. The remaining sequence consists of a the Tn*21*-related *tnpM* gene (start and stop codon in bold) and the first 152 bases of the *tnpR* gene (start codon in bold). The *Sph*I and *Hind*III sites are shown in bold.

The 3`-end of pSKIF100 consists of 3728 bases of data (fig.6.2.B.) and begins with 1362 nucleotides which have no significant homology to any sequences in GenBank (DNA and protein). This sequence is most likely *Acinetobacter* intergenic sequence.

The intergenic region is followed by 820 bases (position 1363 to 2182; fig.6.2.B.) which are identical to an insertion sequence, IS*15-Δ*II (Trieu-Cuot and Courvalin, 1984). The insertion of the IS*15-Δ*II element has truncated a Tn*21*/*int1* integrase gene and deleted the region which presumably contained the variable region and 3`-conserved segment of Tn*21*-like integrons [section 2.A.]. IS*15-Δ*II is one of the genetic variants of IS*15-Δ* (Labigne-Roussel and

Courvalin, 1983). IS*15* is formed by the insertion of IS*15*-Δ I into IS*15*-Δ II, an event which is accompanied by the duplication of 8bp of the target sequence. The next 741 nucleotides (position 2183 to 2923; fig.6.2.B.) are 100% homologous to the last 715 nucleotides of the *int1* and the 13 bases downstream of the gene (nucleotides 850 to 111; Bissonnette and Roy, 1992).

The region downstream of the *int1* contains the 5`-outer boundary of the integron, the 25-bp Brown's repeat (position 3076 to 3100; fig.6.2.B.). The remaining 628 bases are identical to the *tnpM* gene (position 3101 to 3451; fig.6.2.B.) -encoding a Tn*21* modulator protein- and its downstream flanking sequence (position 3451 to 3576; fig.6.2.B.), and the first 152 nucleotides of the *tnpR* gene (position 3577 to 3728; fig.6.2.B.)–encoding a Tn*21* resolvase protein (nucleotides 178 to 653 and 654 to 805, respectively; Hyde and Tu, 1985).

6.D. DISCUSSION

The pSKIF100 insert cloned from *A.baumannii* strain PAU has an interesting genetic organisation. Hybridisation studies showed that the fragment carried sequences homologous to the integrase gene and it was hypothesised that the fragment carried an integron. Partial DNA sequence analysis revealed that this was not the case. The insert did carry an integrase gene, but a truncated one. The gene is interrupted at its 5`-end by an insertion sequence, IS*15*-Δ II. The IS*15*-Δ II not only truncated the *int1,* it also deleted the variable region and 3`-CS of the integron. The truncated integrase gene is upstream of the genes encoding the Tn*21* modulator protein (*tnpM*) and resolvase protein (*tnpR*). Upstream of the insertion sequence and Tn*21*-related genes, on the 5` side of pSKIF100, is an *aac(3)-IIa* encoding an AAC(3)-II aminoglycoside resistance gene. This gene is flanked by a portion of an insertion sequence, IS*1133* (on the 5`-end) and an ORF, the product of which has not been identified (on the 3`-end).

Aminoglycoside 3-acetyltransferase (AAC(3)-II) is characterised by resistance to gentamicin, tobramycin, dibekacin, netilmicin, 2`-*N*-ethylnetilmicin, 6`-*N*-ethylnetilmicin, and sisomicin (Shaw *et al.*, 1993). It has been previously designated AAC(3)-III, but DNA sequence analysis showed that the genes encoding AAC(3)-II and AAC(3)-III were identical (Allmansberger *et al.,* 1985; Vleigenthart *et al.*, 1989). Although common in Gram-negative bacteria, AAC(3)-II occurs with varying frequency in different genera. In *Acinetobacter* it occurs with a 21.3% frequency (Shaw *et al.*, 1993). The *aac(3)-IIa* –also designated *aacC2, aacC3, aacC5,* and *aac(3)-Vb* – is one of 3 genes found to encode AAC(3)-II (Vliegenthart *et al.,* 1989) and is the most common, being present in 84.8% of isolates expressing the enzyme (Shaw *et al.*, 1993).The DNA sequences of the other genes, *aac(3)-IIb* and *aac(3)-IIc*, are 72% and 97% identical to that of the *aac(3)-IIa* (Shaw *et al.,* 1993).

The *aac(3)-IIa* and its upstream flanking region described in this study are identical to the *aac(3)-IIa* described by Elisha (1991). The transition at nucleotide 1604 of a G to an A results in the incorporation of lysine rather than glutamic acid. The region immediately upstream of the *aac(3)-IIa* encompasses the regulatory region, which includes a -10 region, but there is no sequence corresponding to the *E.coli* -35 consensus sequence. However, as with strain SAK (Elisha, 1991; Elisha and Steyn, 1991a), there is a region similar to the consensus sequence for cAMP/CAP controlled promoters (De Crombrugghe *et al.*, 1984). Studies to prove conclusively that the *aac(3)-IIa* from strain SAK had a catabolite sensitive promoter were not performed and the necessity for control associated with the availability of energy sources was not explained (Elisha, 1991; Elisha and Steyn (1991a). Elisha (1991) hypothesised that the reason may be related to the origins of *Acinetobacter* as a common soil organism in an ecological niche where special metabolic demands are placed on it. The *aac(3)-IIa* on pSKIF100 is expressed but whether it is affected by catabolite repression when in strain PAU was not investigated.

On the 5`-end of the regulatory region of the *aac(3)-IIa* is a portion of IS*1133*, encompassing the right hand IR of the element. This insertion sequence was originally identified as part of Tn*5393*, a Tn*3*-type transposon, isolated in the Gram-negative plant bacterium, *Erwinia amylovora*. Tn*5393* carries two streptomycin resistance genes, *strA* and *strB*.

There is an ORF on the 3`-end of the *aac(3)-IIa* from strain PAU. In the original description of this ORF, it was shown to be downstream of an *aac(3)-IIa* (Allmansberger *et al.*, 1985). The function of a possible gene product from this ORF has not been elucidated (Allmansberger *et al.*, 1985). The ORF lacks a promoter but it does have a suitable ribosomal binding site and the authors postulate that a possible gene product may be translated from a polycistronic messenger transcribed from the *aac(3)-IIa* promoter. Two of the plasmids on which the ORF was originally identified, pWP14a and pWP116a, had an IS*140* element upstream of their *aac(3)-IIa*. IS*140,* however, is not related to IS*1133*.

Because the insert was only partially sequenced, downstream of the ORF there is a gap in the data. The sequence resumes with more than a kilobase of data which has no significant homology to any sequences in the GenBank DNA or protein database, nor any ORFs, and is more than likely *Acinetobacter* intergenic sequence of unknown function.

Inserted between this intergenic sequence and a truncated integrase gene is a second insertion sequence, IS*15-Δ*II. Transposable elements –insertion sequences and transposons – are discrete segments of DNA which are able to insert into various sites of prokaryotic and eukaryotic genomes, in the absence of sequence homology (Kleckner, 1981). The integration event is usually accompanied by the duplication of a short sequence of the target DNA (Kleckner, 1981) and the process of transposition probably results from the action of a Tn/IS-encoded transposase. Tn*1525* is a transposable element in which a kanamycin resistance determinant is flanked by two copies of an insertion sequence, IS*15,* in direct orientation (Labigne-Roussel *et al.*, 1983).

IS*15* is widely spread among enterobacteria (Labigne-Roussel and Courvalin, 1983) and is capable of independent transposition. It is 1648bp long with 14bp inverted repeats at its termini (Trieu-Cuot and Courvalin, 1984). It results from the transposition, in direct orientation, of a smaller insertion sequence, IS*15-Δ*, into itself. This event is accompanied by the duplication of 8bp in the target DNA (Trieu-Cuot and Courvalin, 1984). The parental IS*15-Δ* consists of two large overlapping ORFs located on opposite strands and, because of this, IS*15* possesses 4 large ORFs (Trieu-Cuot and Courvalin, 1984). The two 820bp IS elements which make up IS*15* have been designated IS*15-Δ*I and IS*15-Δ*II (Trieu-Cuot and Courvalin, 1984). They differ by only four point substitutions (at positions 57, 207, 208, and 362; Trieu-Cuot and Courvalin, 1984) and are considered to be two genetic variants of IS*15-Δ* (Labigne-Roussel and Courvalin, 1983). The transition from IS*15* to *IS15-Δ* was observed *in vivo* (Labigne-Roussel and Courvalin, 1983) and the reverse was also shown to be possible (Trieu-Cuot and Courvalin, 1984).
Trieu-Cuot and Courvalin (1984) showed that IS*15-Δ* is not related to IS1, IS*2*, IS*3*, IS*4*, IS*5*, IS*50*, and IS*903*, but is closely related, structurally, to IS*26* (Iida *et al.*, 1982; Mollet *et al.*, 1983), IS*140* (Bräu and Pierpersberg, 1983) and IS*46* (Trieu-Cuot and Courvalin, 1984). IS*26* is generated by homologous recombination between IS*15-Δ*I and IS*15-Δ*II (Trieu-Cuot and Courvalin, 1984). IS*15-Δ*I and -*Δ*II, IS*26*, IS*140*, and IS*46* are variants of the same genetic mobile element, IS*15-Δ* (Trieu-Cuot and Courvalin, 1984). IS*15* and IS*15-Δ* have been detected in a variety of plasmids of various incompatibility groups and have been described as the two interconvertible forms of a ubiquitous transposable module (Trieu-Cuot and Courvalin, 1984). The presence of IS*15-Δ* was revealed in a multi-resistant plasmid pIP1031 from a clinical isolate of *A.calcoaceticus* strain BM2500 (Goldstein *et al.*, 1983).

The remaining sequences are homologous to Tn*21*-related sequences including a portion of the gene encoding a Tn*21*-like integrase (*int1*), the gene encoding the modulator protein (TnpM), and a portion of the gene encoding the resolvase protein (TnpR).

In summary, partial sequence analysis of the 6.5kb *Hin*dIII fragment from strain PAU reveals an interesting "slice" of the genome. It consists of an aminoglycoside resistance gene, *aac(3)-IIa*, downstream of a partial insertion sequence, IS*1133*. Upstream of the *aac(3)-IIa* is an ORF, expressing a product of unknown function. On the other end of the fragment is another insertion sequence, IS*15-Δ*II, which has deleted all but a portion of a Tn*21*-like integron. The 3`-end of the integrase gene is all that remains. The truncated integrase gene is upstream of the Tn*21*-associated *tnpM* and *tnpR* genes, the products (modulator protein and resolvase, respectively) of which are involved in transposition. The sequence upstream of the IS*15-Δ*II element appears to be constitute a non-coding, intergenic region. It is that unlikely that the *aacC2* gene originally constituted a part of the variable region of the integron deleted by IS*15-Δ*II, since it is transcribed from the other strand. It is more likely that the *aac(3)-IIa* was acquired during a transposition event involving IS*1133* or the transposon on which it originally resided. Since IS*1133* was originally identified in a transposon from a plant bacterium, the acquisition could have occurred far back in the history of strain PAU. However, this hypothesis is questioned by the fact that the IS*1133* element is interrupted by *Acinetobacter* intergenic sequence. One would expect the reverse to occur: that the insertion of the IS element and its associated sequence would interrupt intergenic sequence. Another possibility is that the *aac(3)-IIa* was introduced into strain PAU during an infection by a plasmid similar to pWP116a.

The results presented in this chapter highlight the limitations of hybridisation experiments. In this case, hybridisation with the *int1* probe suggested that the fragment carried an integrase gene and it could have been assumed that strain PAU contained an integron. However, further studies showed that only a portion of the integrase gene was present.

CHAPTER 7: SUMMARY

The incidence of integrons in 57 clinical isolates of *Acinetobacter* from two South African hospitals, Groote Schuur Hospital (GSH), Cape Town and Universitas Hospital (UH), Bloemfontein, was investigated by hybridisation with a specific probe and PCR. Hybridisation studies with the *int1* probe indicate that 53.5% of the GSH isolates and 92.9% of the UH isolates (63.2% overall; 36/57) contain integrase related sequences. However, only 24 of the 36 (67%) isolates yielded amplification products using primers directed at the 5`- and 3`-conserved regions of class 1-type integrons. A single product of 0.7kb was obtained from 22/36 strains, suggesting that these acinetobacters contain integrons with one cassette in the variable region. One of these strains also yielded a 0.65kb product, suggesting that this strain contains 2 integrons. A product of 2.0kb was obtained from 1 strain, suggesting that the variable region of this integron consists of at least 2 cassettes. Two products (2.0kb and 3.5kb) were obtained from another strain, suggesting that this acinetobacter contains at least 2 integrons. No PCR products were obtained with the DNA from 12 acinetobacters which had hybridised to the *int1* probe. This may be due to various reasons: firstly, the isolates may contain an In0-like integron. In0 lacks a variable region, and the 5`-conserved region is contiguous with the 3`-conserved region in this integron (Bissonnette and Roy, 1992). A second and more likely possibility is that the variable regions of these integrons contain a number of cassettes, resulting in variable regions too large to be amplified using the PCR assay described in this study. A final reason could be that the isolates contain only a portion of the integrase gene - hence the positive hybridisation signal with the *int1* probe - but lack other conserved integron-related sequences [see Chapter 6].

The 0.7kb amplicon from *A.baumannii* strain CAR contained an integron *dfrVII* which encodes dihydrofolate reductase type VII (mediates resistance to trimethoprim). Hybridisation studies showed that all the 0.7kb amplicons obtained from the acinetobacters contained *dfrVII* sequences, suggesting that 22/57 isolates carry integron-associated trimethoprim resistance.

Further analysis of the sequences flanking the *dfrVII* from strain CAR showed that the structural gene is part of a cassette bounded by an *att1* site and a 59 be/ *attC* site identical to that associated with *dfrVII* in Tn*5086* (Sundstrom *et al.*, 1993). The *dfrVII* is transcribed from the weak P_{ANT} promoter, TGGACA(-35)N_{17}TAAGCT(-10) (Collis and Hall, 1995). The promoter (P2) TTGTTA(-35)N_{17}TACAGT(-10), usually associated with P_{ANT} is not present (Collis and Hall, 1995) since strain CAR lacks the three G-residues which alter the space between the hexamers of P2 to 17, creating an alternative promoter. The integron in this study is similar to Tn*5086*, a Tn*21*-like integron (Sundstrom *et al.*, 1993).

The 2kb amplicons from *A.baumannii* strain G37 and *A.baumannii* strain G39 were cloned and partially sequenced. DNA sequencing data suggest that *A.baumannii* strain G37 carries an *ant(2``)-Ia* gene cassette and a putative cassette in the variable region of a class 1-type integron. The *ant(2``)-Ia* was most similar to the *ant(2``)-Ia* from another *Acinetobacter* (strain SUN). Based on the results of this study it is not possible to make any conclusions as to the probable function, if any, of the putative cassette. The 5`-conserved region of this integron is unlike the corresponding region in other class 1-type integrons in that it has 2 overlapping direct repeat sequences. This region is downstream of the integrase gene in an area where repeat sequences are not uncommon (Mercier, 1990). While the origin and function of the repeat sequence are unknown it is interesting to note that the equivalent region of the original integron carrying the *orfX* gene cassette has the same overlapping repeats (Tosini *et al.*, 1998). The putative cassette from strain G37 has an atypical 59-be. Although the core site is homologous to the consensus core, the inverse core site is not fully complementary to the core. In addition, the imperfect inverted repeats at the ends were less complementary than expected and the element was shorter than previously described 59-bes. Whether this 59-be is active in recombination cannot be determined based on these results.

The hybridisation, PCR and DNA sequencing data for *A.baumannii* strain G39 suggest that it carries a class 1-type integron with two gene cassettes in its

variable region. The 3`-end of the variable region has an *ant(3``)-Ia* gene cassette with an atypical 59-be (core site, non-complementary inverse core site, poorly complementary imperfect inverted repeats at the end). The 5`-end of the variable region has a putative gene cassette, *orfX*. In the original description of this cassette (Tosini *et al.*, 1998), it was also on an integron and was associated with an *ant(3``)-Ia* gene cassette. The integrase-related sequences and the sequence of the 5`-CS primer were not identified upstream of *orfX* in strain G39. Instead, there was a 14bp inverted repeat sequence. This suggests that the amplification product cloned into pG39SC must have been generated from the 3`-CS primer alone or that the product was altered during the cloning or sequencing procedure so that the sequence on the 5`-end was deleted. There is no adequate explanation for how either of these events may have occurred. If the 2kb product from strain G39 is not the full amplicon, the variable region may contain further cassettes. The region upstream of *orfX* should then have contained a 59-be. Further analysis is necessary to determine the full structure of the integron in strain G39.

A 6.5kb *Hin*dIII fragment from *A.baumannii* strain PAU hybridised to the *int1* probe. This fragment was cloned into pUC19 to give pSKIF100 and the insert was partially sequenced. Since the hybridisation studies showed that the fragment carried sequences homologous to the integrase gene, it was hypothesised that the fragment carried an integron. Partial DNA sequence analysis revealed that this hypothesis was incorrect. On the 3`-end of pSKIF100, the insert did have an integrase gene, but it was truncated due to the insertion of IS*15-Δ*II. The IS*15-Δ*II not only truncated the *int1* gene, it also deleted the variable region and 3`-CS of the integron. The truncated integrase gene is upstream of the genes encoding the Tn*21* modulator protein (*tnpM*) and resolvase protein (*tnpR*). The 5` side of pSKIF100, carries an *aac(3)-IIa* encoding an AAC(3)-II aminoglycoside resistance gene which is flanked by a portion of IS*1133* (on the 5`-end) and an ORF, the product of which has not been identified (on the 3`-end).

This study has demonstrated that integrons are prevalent in the *Acinetobacter* isolates from the two hospitals, particularly those from Universitas Hospital. The integrons no doubt play a role in the dissemination and acquisition of resistance genes within the bacterial population in the hospitals.

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APPENDIX A: MEDIA, BUFFERS, and SOLUTIONS

A.1. Media

Note: volumes were adjusted with distilled water unless otherwise indicated.

2X YT

Broth (1l): 16g Tryptone
10g Yeast extract
5g NaCl
Autoclave

Agar: 100ml 2X YT broth
1.5g Agarose
Autoclave

A.2. Buffers and Solutions

Note: all volumes were adjusted with distilled water unless otherwise indicated and all buffers/solutions were made up volumetrically.

50% Acrylamide, 2.5% Bis (500ml)
250g Acrylamide
12.5g Bis
100ml Distilled water
Filter and store at 4^0C in an amber bottle.

10% APS (10ml)
1g APS
Aliquot and store at -20^0C.

1M $CaCl_2$ (100ml)
14.7g $CaCl_2.2H_2O$
Autoclave and store at 4^0C.

Chloroform-Isoamylalcohol
Mix in a ratio of 24:1.

CTAB/NaCl
Dissolve 4.1g NaCl in 80ml dH_2O and slowly add 10g CTAB. Heat at 65^0C to dissolve and adjust the volume to 100ml.
Store at RT for no longer than 6 months.

Denaturation solution (1l)
300ml 5M NaCl
50ml 10N NaOH

ECL hybridisation solution (100ml)
100ml ECL hybridisation buffer
2.9g NaCl
5g blocking agent
Mix at RT for 1hour on a magnetic stirrer and then heat to 42^{0}C for 1hour.
Store aliquots at –20^{0}C.

ECL wash buffer (1l)
360g urea
4g SDS
5ml 20X SSC
Store at 4^{0}C for up to 3 months.

0.5M EDTA (500ml)
Dissolve 93.05g EDTA in 400ml dH_2O by stirring vigorously. Adjust the pH to 8.0 with NaOH pellets (~10g).
Autoclave.

Ethidium Bromide solution (10ml)
0.1g Ethidium bromide

Gel tracking dye (10ml)
25mg Bromophenol blue
4g Sucrose
0.4ml 0.5M EDTA (pH 8.0).

1M HCl (1l)
86.2ml HCl

200mg/ml IPTG (10ml)
2g IPTG
Freeze 150µl aliquots at –20^{0}C.

Isopropanol, salt saturated

Mix isopropanol with ¼ volume 5M NaCl. A white precipitate forms and two phases appear. Use the top phase.

4M LiCl (100ml)

16.96g LiCl
Autoclave.

Neutralization solution (1l)
500ml 1M Tris (pH 7.2)
300ml 5M NaCl
2ml 0.5M EDTA

10X NNB (1l)
162g Tris base
27.5g Boric acid
9.3g EDTA

Phenol

To 500g of commercial crystallized phenol, add the following:

0.6g 8-Hydroxyquinolone
7.5ml 2M NaOH
130ml dH_2O
6ml 1M Tris (pH 7.6)
Leave overnight to liquefy or at 40^0C until the solution is clear.
Aliquot and store at -20^0C.

Phenol-chloroform-isoamylalcohol

Mix the 3 components in a ratio of 25:24:1.

5M Potassium acetate (100ml)
49.1g Potassium acetate
Store at 4^0C.

10% SDS (100ml)
10g SDS.
Heat to about 80^0C to dissolve. Add a few drops of HCl to adjust pH to 7.2

3M Sodium acetate (500ml)
204.05g sodium acetate in 400ml dH_2O. Dissolve and adjust to desired pH with glacial acetic acid. Make up to 500ml.

5M Sodium Chloride/NaCl (100ml)
29.22g NaCl
Autoclave.

10N Sodium Hydroxide/NaOH (100ml)
40g NaOH
Store in a plastic container.

Solution I (100ml)

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

Solution II (100ml)
2ml 10N NaOH
5ml 20% w/v SDS
Made fresh weekly.

Solution III (100ml)
60ml 5M Potassium acetate
11.5ml Glacial acetic acid
Store at 4^0C.

20X SSC (1l)

175.3g NaCl
88.2g Sodium citrate
Adjust pH to 7.0 with a few drops of 10N NaOH.
Autoclave.

50X Tris-Acetate-EDTA/TAE Buffer (1l)
242g Tris
57.1ml Glacial acetic acid
100ml 0.5M EDTA
Autoclave.

1M Tris-Cl (100ml)
121.1g Tris
Adjust pH to required level with concentrated HCl.
Autoclave.

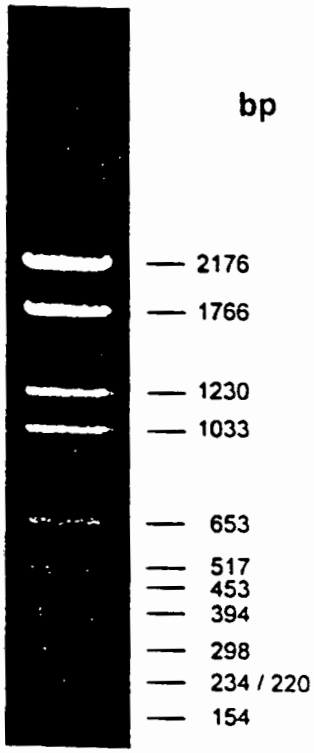
Tris-EDTA/TE Buffer

10mM Tris-Cl (pH 8.0)
1mM EDTA (pH 8.0)
Autoclave.

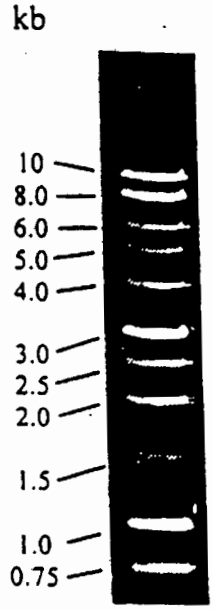
40mg/ml X-gal
Dissolve 0.4g X-gal in 10ml dimethyl sulfoxide (DMSO) and add 1 volume dH_2O.
Store at -20^0C in an amber bottle.

APPENDIX B: Molecular weight markers used

Molecular weight marker VI
(Boehringer Mannheim)



1kb ladder
(Promega)



APPENDIX C: pUC19

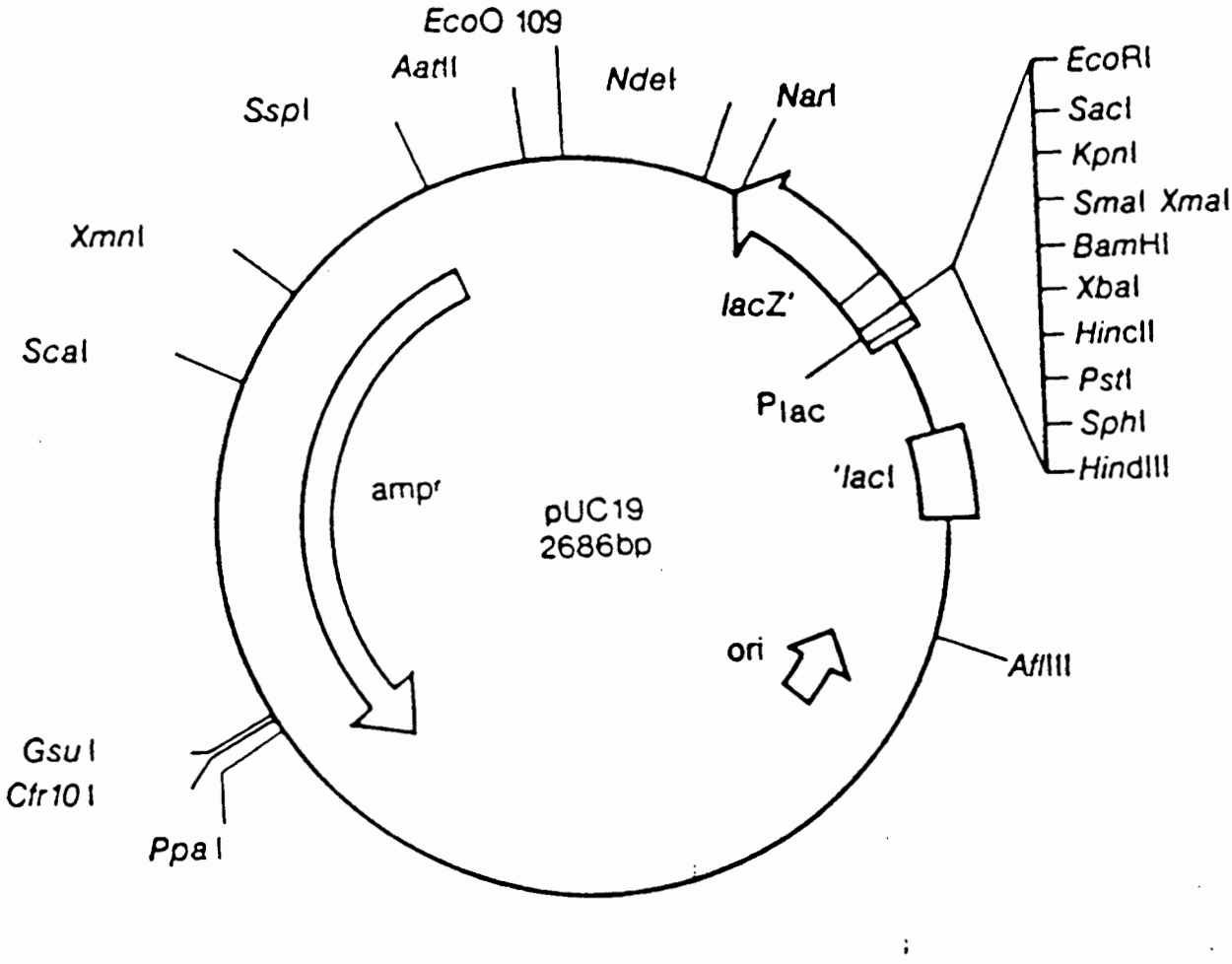


Fig.C.1.: Schematic diagram of the plasmid, pUC19

(taken from Ausubel *et al.*, 1987)

APPENDIX D: pGEM-T

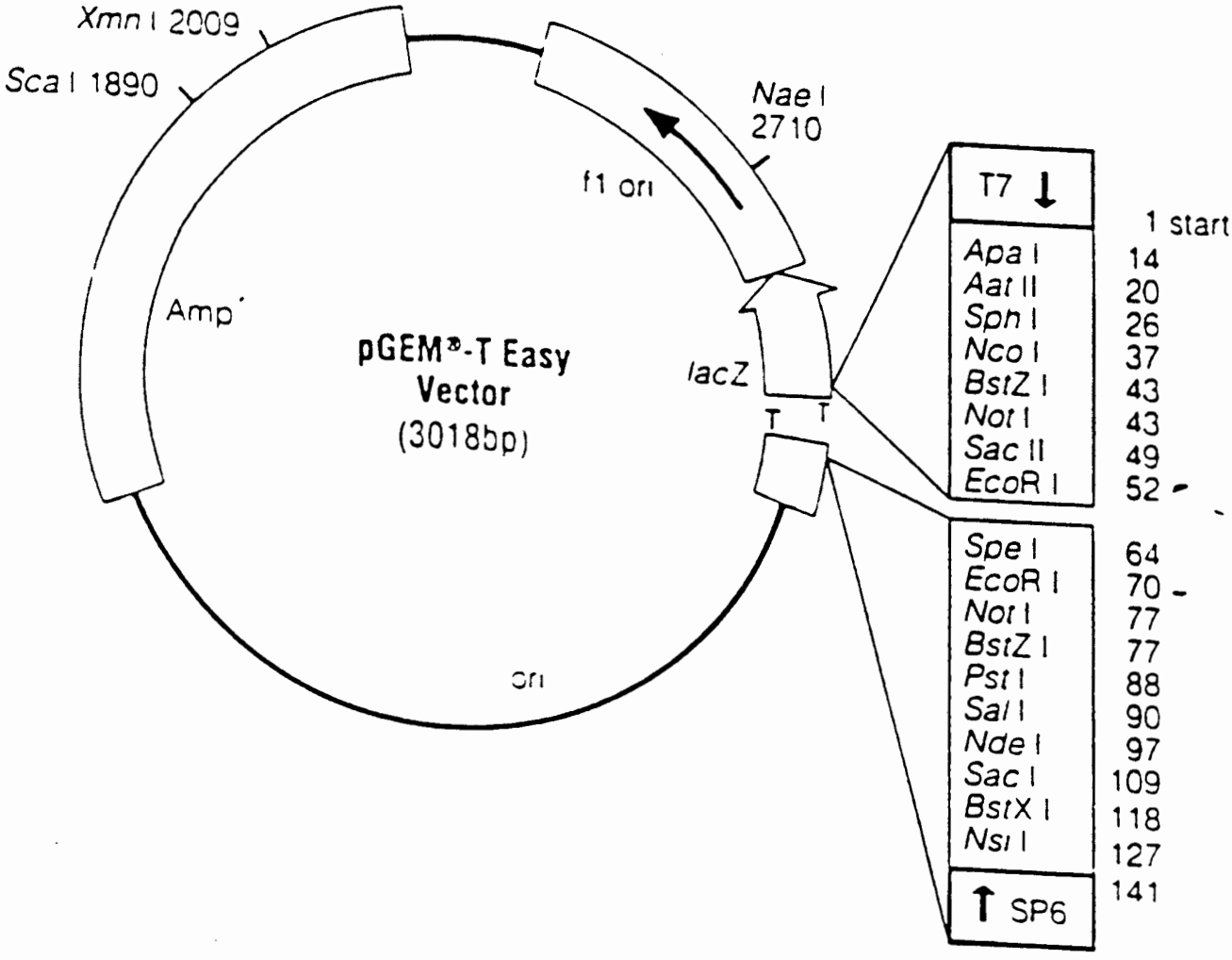


Fig.D.1.: Schematic diagram of the plasmid, pGEM-T

(taken from the pGEM-T Easy Vector manual)