THE GENETIC LOCATION AND REGULATION OF AMINOGLYCOSIDE RESISTANCE GENES IN *ACINETOBACTER*

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For my parents,

CONTENTS

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ABBREVIATIONS

μ	micro
σ	sigma
°C	degrees Celsius
59-be	59-base elements
A.	*Acinetobacter*
AAC	acetyltransferase
AAD	adenylyltransferase
AME	aminoglycoside-modifying enzyme
Amik	amikacin
Amp	ampicillin
APH	phosphotransferase
ATP	adenosine triphosphate
bp(s)	base pair(s)
cDNA	complimentary DNA
Cm	chloramphenicol
Co-A	coenzyme A
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylenediaminetetra-acetic acid
g	gram
Gm	gentamicin
ICU	intensive care unit
IPTG	isopropyl-β-D-thio-galactopyranoside
IR	inverted repeat
kb	kilobase
Km	kanamycin
l	litre
m	milli

M	Molar
MIC	minimum inhibitory concentration
(m)/(r)RNA	(messenger)/ (ribosomal) ribonucleic acid
n	nano
Nal	nalidixic acid
Net	netilmicin
OD	optical density
OM	outer membrane
ORF	open reading frame
p	pico
P.	*Pseudomonas*
PEG	polyethylene glycol
R	resistance
rpm	revolutions per minute
RT	room temperature
S	sensitive
SDS	sodium dodecyl sulphate
Ser	Serine
spp.	species
TE	Tris-EDTA
Tet	tetracycline
Tm	trimethoprim
Tn	transposon
Tob	tobramycin
U	Units
UCT	University of Cape Town
UV	ultra-violet
v/v	volume per volume
var.	variety
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YT	yeast-tryptone

FIGURES

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ABSTRACT

The genetic basis of aminoglycoside resistance in clinical isolates of *Acinetobacter* was investigated. The *aadB* genes cloned from two clinical isolates, strain CHA and strain SUN, were sequenced. Analysis of the sequencing data indicated that both genes were contained on gene cassettes, which had integrated at secondary sites on a plasmid isolated in each strain.

Gene cassettes are usually associated with integrons, and cassettes recombined at secondary sites are thought to be stably integrated. However, conduction assays indicate that the *aadB* gene cassette described in this study is potentially mobile.

Outside of an integron, transcription of the structural gene on a cassette is dependent on insertion of the cassette downstream of correctly aligned promoter sequences. A number of putative promoters were identified upstream of the *aadB* structural gene. Primer extension studies were carried out to study the regulation of *aadB*. These experiments showed that in *Acinetobacter* the *aadB* gene is regulated by a promoter consisting of a -10 hexamer only. Similar experiments showed that, in *Escherichia coli*, the same gene is transcribed from a different promoter, which is typical of those recognized by the major RNA polymerase in this organism. Thus, the transcription signals recognized in *Escherichia coli* were different from those recognized in *Acinetobacter*.

Naturally occurring plasmids in clinical isolates of *Acinetobacter* have not been fully characterized: pRAY was characterized by sequencing. An analysis of the sequencing data identified features consistent with an origin of replication. Moreover, this analysis suggests that pRAY may be mobilizable.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 EMERGENCE OF *ACINETOBACTER* AS A NOSOCOMIAL PATHOGEN

The use of new antimicrobial agents and the increased use of broad-spectrum antibiotics in the treatment of hospital patients has resulted in both an increase in the isolation of resistant organisms, and broadened the spectrum of nosocomial isolates posing clinical problems (Finland, 1970, 1979; Levy 1982; McGowan, 1983; Seifert *et al.*, 1993). Amongst the opportunistic pathogens associated with the changed character of nosocomial infections are *Serratia*, *Pseudomonas* and *Acinetobacter* spp. (Daschner and Nopper, 1980; McGowan, 1983; Mayer and Zinner, 1985; Bergogne-Bérézin and Towner, 1996). The common predisposing factors leading to the emergence of these organisms as nosocomial pathogens include, extended hospitalization of patients, the use of invasive and mechanical devices, and prior antibiotic therapy (Buxton *et al.*, 1978; Cunha *et al.*, 1980; Ghoneim and Halaka, 1980; Holton, 1982; McGowan, 1983; Mayer and Zinner, 1985; Buisson *et al.*, 1990; Bergogne-Bérézin and Joly-Guillou, 1991; Lortholary *et al.*, 1995). In part, the intrinsic resistance of acinetobacters and pseudomonads to multiple antibiotics, due to their relatively impermeable outer membranes (Sato and Nakae, 1991), has contributed to the success of these opportunistic pathogens in hospitals where antibiotics are extensively used.

The emergence of *Acinetobacter* spp. as nosocomial pathogens (Bergogne-Bérézin and Joly-Guillou, 1985, 1991; Peacock *et al.*, 1988) necessitated clarification of the genus *Acinetobacter* (*A.*). Although only one species, *A. calcoaceticus,* was recorded in Bergey's Manual of Systematic Bacteriology (Juni, 1984), clinical microbiologists continued to recognize two varieties, *A. calcoaceticus* var. *anitratus* and var. *lwoffii.* Largely due to the work of Bouvet and Grimont (1986), the heterogeneity of this genus is now well recognized. Based on DNA-DNA hybridization studies, the genus *Acinetobacter* comprises 21 genospecies (Ehrenstein *et al.*, 1996). Most clinical isolates belong to genospecies 2 or *A. baumannii* (Glew *et al.*, 1977; Vieu *et al.*, 1979, cited in Towner *et al.*, 1991; Bouvet and Grimont, 1986, 1987; Godineau-Gauthey *et al.*, 1988, cited in Towner *et al.*, 1991); Bergogne-Bérézin and Joly-Guillou, 1991). Although *A. baumannii* is the most important nosocomial pathogen, *Acinetobacter* species 3, *A. johnsonii* and *A. lwoffii* have also been associated with nosocomial infections (Seifert *et al.*, 1993). The success of *A. baumannii* in the hospital setting, where antibiotics are extensively prescribed, could be attributed to it being more resistant to antibiotics than other *Acinetobacter* species (Seifert *et al.*, 1993).

Patients receiving antibiotic therapy, particularly the immunocompromised and those in ICU, are most at risk of infection by *Acinetobacter* (Ramphal and Kluge, 1979; Peacock *et al.*, 1988; Bergogne-Bérézin and Towner, 1996). The most common *Acinetobacter*–associated nosocomial infections include bacteraemia, pneumonia, urinary tract infections, abscesses, meningitis and wound infections (Levi and Rubinstein, 1996), although rare, community-acquired infections have been described (Rudin *et al.*, 1979; Hoffmann *et al.*, 1982).

1.2 MECHANISMS AND GENETIC BASIS OF ANTIBIOTIC RESISTANCE IN *ACINETOBACTER*

The ability of *Acinetobacter* to rapidly become resistant to antibiotics (Towner, 1991), combined with its inherent resistance, has posed clinical problems (French *et al.*, 1980; Larson, 1984; Bergogne-Bérézin and Joly-Guillou, 1985; Buisson *et al.*, 1990; Struelens *et al.*, 1993). Treatment of infections associated with this organism is complicated by the multiple antibiotic resistance of many clinical isolates of *Acinetobacter* (Garcia *et al.*, 1983; Obana *et al.*, 1985). Although this dissertation focuses on the characterization and regulation of an aminoglycoside resistance gene, for the sake of completeness, I shall briefly describe the mechanisms and genetic basis of resistance in *Acinetobacter* to other groups of antibiotics.

1.2.1 Role of outer membrane in resistance

There is increasing evidence implicating the outer membrane (OM) of bacteria, in conjunction with other mechanisms, in antibiotic resistance (Nikaido, 1989). As most antibiotics must cross the bacterial membrane to access their targets, the OM acts as a selective barrier between the bacteria and its external environment. In this context, it is interesting to note that multiresistant *Acinetobacter* contains a low proportion of porins in its OM. Porins, which serve as non-specific channels for the passage of substances across the bacterial membrane, comprise less than 5 % of the total OM proteins of *Acinetobacter*, compared to 60 % in *Escherichia* (*E.*) *coli* (Sato and Nakae, 1991). While certain antibiotics, including β-lactams, tetracyclines, chloramphenicol and aminoglycosides, cross the OM through these narrow protein channels, larger molecules are excluded and porins thereby act as an effective barrier against larger, hydrophobic antibiotics, including macrolides, novobiocin, and the more hydrophobic β-lactams (Nikaido, 1989). The porin-deficient nature of *Acinetobacter's* OM probably plays a significant role in inhibiting entry of antibiotics, resulting in the broad spectrum of antibiotic resistance observed in this genus.

A further OM-mediated mechanism of resistance involves the active efflux of antibiotics (Levy, 1992; Nikaido, 1996). Tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, and trimethoprim, as well as β-lactamase inhibitors (Li *et al.*, 1998), are amongst the substrates of multidrug efflux pumps, contributing to antibiotic resistance in *Pseudomonas* (*P.*) *aeruginosa* (Masuda *et al.*, 1995; Srikumar *et al.*, 1997). Analogous efflux systems have been described in *E. coli, Erwinia chrysanthemum* and *Bordetella pertusis* (Nikaido, 1996), but no efflux pumps have yet been described in *Acinetobacter*.

1.2.2 Alteration of target site

Darwin's theory of survival of the fittest manifests itself in bacterial populations exposed to the selective pressures of antibiotic treatment. A certain subset of mutants may contain an altered target site which precludes antibiotic binding, resulting in resistance and hence survival of these mutants. As suggested by Spratt (1994), resistance to antibiotics due to an altered target site occurs most rapidly for antibiotics that inactivate a single target.

1.2.2.1 Resistance to quinolones

Although reduced permeability of the *Acinetobacter* OM has been suggested to contribute to resistance to the quinolones (Pascual *et al.*, 1997; Vila *et al.*, 1997), it is mutations in DNA gyrase, the antibiotic target site, which are primarily associated with quinolone resistance in *Acinetobacter*. Mutations in the *E. coli gyrA* and *gyrB*, which encode the DNA gyrase, confer resistance to quinolones (Nakamura *et al.*, 1989). The predominant mutation in *gyrA*, resulting in quinolone resistance, was mapped to the Ser83 residue of GyrA in *E. coli* (Cullen *et al.*, 1989). The mutation at Ser83 was also identified in the *Acinetobacter* GyrA, and alters the quinolone target binding site in *Acinetobacter,* resulting in resistance (Vila *et al.*, 1995). A number of *Acinetobacter* strains carrying this mutation, had ciprofloxacin MICs of ≤16 mg/L, whereas others had ciprofloxacin MICs of ≥32 mg/L, suggesting that an additional mutation was responsible for the higher level of resistance observed. Further analysis indicated that ParC, encoding a topoisomerase IV subunit, is a secondary target for quinolones (Vila *et al.*, 1997) which, together with the primary *gyrA* mutation, results in high levels of resistance to quinolones in *Acinetobacter* (Vila *et al.*, 1997). High-level quinolone resistance in *E. coli* is also due to a primary mutation in *gyrA*, followed by a secondary mutation in *parC* (Vila *et al.*, 1996). Interestingly, in *Staphylococcus aureus* and *Streptoccocus pneumoniae*, the primary mutation occurs in *parC*, followed by a secondary mutation in *gyrA* (Muñoz and de la Campa, 1996; Ng *et al.*, 1996).

1.2.3 Chromosomal- and plasmid-encoded enzymes

Perhaps the most common mechanism of resistance to antibiotics in bacteria is through the production of an enzyme that modifies or inactivates the antibiotic, rendering it ineffective.

1.2.3.1 Resistance to β-lactam antibiotics

Acinetobacter is resistant to many β-lactam antibiotics and extensive studies have been carried out on β-lactamases in this organism (Amyes and Young, 1996). The first report of a β-lactamase in *Acinetobacter* was of a plasmid-encoded class A TEM-1 in strain BM2500 (Goldstein *et al.*, 1983). This gene, in addition to other resistance determinants, was associated with a transposable element, IS15, previously identified on resistance (R) plasmids from a variety of Gram-negative bacteria. Identification of these transposon-encoded r-determinants on a conjugative plasmid illustrates the important interactive role of plasmids and transposons in the transfer of resistance. Antibiotic resistance genes "hunt in packs" (Salyers and Amábile-Cuevas, 1997).

As TEM-1 and TEM-2 have been identified in *Acinetobacter* (Amyes and Young, 1996), it is perhaps surprising that plasmid-encoded extended spectrum β-lactamases (ESBLs), derived from these enzymes, have not been described in this organism. Indeed, ESBLs have not played a significant role in β-lactam resistance in *Acinetobacter* (Amyes and Young, 1996), and is in stark contrast to their role in resistance to these antibiotics in *Enterobacteriaceae* (Sanders and Sanders, 1992). A number of chromosomal β-lactamases (ACE) have been described in *Acinetobacter* (Hood, 1991, cited in Amyes and Young, 1996; Hood and Amyes, 1991). There have been conflicting reports with respect to the expression of these enzymes; some authors have classified all of the enzymes as inducible, while others found no evidence for inducibility (Amyes and Young, 1996). As has been noted (Amyes and Young, 1996), the jury is still out on whether the *Acinetobacter* chromosomal enzymes are inducible. It may be that some are inducible, whereas others are constitutive (Amyes and Young, 1996). As suggested by Amyes and Young (1996), enhanced resistance to the β-lactams is probably due to a combination of mechanisms including, β-lactamase production, reduced permeability, and altered penicillin-binding proteins.

Until recently, imipenem, a carbapenem β-lactam, remained the most active drug against *Acinetobacter* spp. (Seifert *et al.*, 1993; Vila *et al.*, 1993). Resistance to this antibiotic has emerged and there is evidence to suggest a link between the overuse of imipenem and resistance to the drug (Go *et al.*, 1994; Tankovic *et al.*, 1994). Recently, reduced susceptibility of *Acinetobacter* to carbapenems, by an unknown mechanism, was noted in a UK burns unit (Weinbren *et al.*, 1998). Low-level resistance has been detected in strains from various locations worldwide (Afzal-Shah and Livermore, 1998). Cause for concern is the identification of two plasmid-encoded carbapenemases (ARI-1 and ARI-2) in clinical isolates of *Acinetobacter* (Paton *et al.*, 1993, cited in Amyes and Young, 1996; Brown *et al.*, 1998). Unlike most carbapenemases, these enzymes do not require the presence of zinc for activity and belong to the rare subclass of class A carbapenemases. ARI-1 was identified in isolates from Edinburgh and the plasmid encoding this enzyme was transferred by conjugation to *A. junii* but not to *E. coli* or *P. aeruginosa* (Scaife *et al.*, 1995). ARI-2 was identified in isolates from Argentina and plasmid curing experiments suggested that the gene encoding this enzyme is also on a plasmid.

1.2.3.2 Resistance to trimethoprim

Gram-negative bacteria mediate resistance to trimethoprim by synthesizing a plasmid- or transposon-encoded dihydrofolate reductase (DHFR), which is less sensitive to inhibition by trimethoprim (Smith and Amyes, 1984). Numerous types of plasmid-encoded DHFRs have been described. To date, 15 different types belonging to 10 major groups have been identified in *Enterobacteriaceae* (Adrian *et al.*, 1995). Of these, only *dhfrIa* has been identified in *Acinetobacter*. This gene is one of a number of antibiotic resistance gene cassettes identified on a Tn7-like transposon in a clinical isolate of *Acinetobacter* from Chile (Amyes and Young, 1996; Gonzalez *et al.*, 1998). In an earlier report, transferable high-level trimethoprim resistance, encoded by an uncharacterized gene on a plasmid in *Acinetobacter*, was described (Goldstein *et al.*, 1983).

1.2.3.4 Resistance to chloramphenicol

A further report of transposon-mediated resistance in *Acinetobacter* concerns chloramphenicol resistance in a multiresistant strain of *Acinetobacter*, strain SAK (Elisha and Steyn, 1991b,c). The gene, CAT1, encoding chloramphenicol acetyltransferase, is located in the chromosome and on a plasmid in this strain. An analysis of the nucleotide sequences flanking the structural gene, showed that the CAT1 gene is part of a transposon, which is similar to Tn*2670*. This was the first report of a transposon integrated into the chromosome of *Acinetobacter*.

1.2.3.5 Resistance to the aminoglycoside-aminocyclitol antibiotics

The aminoglycoside-aminocyclitol antibiotics comprise a large family of antimicrobial agents. They can be divided into two main groups on the basis of whether they contain streptidine or 2-deoxystreptamine (Davies and Smith, 1978; Foster, 1983).

Streptomycin is a member of the streptidine group, whereas the 2-deoxystreptamine group includes gentamicin, tobramycin, netilmicin, kanamycin, and amikacin. All of the aminoglycosides, irrespective of their structural classification, inhibit protein synthesis by binding to the ribosome (Tai and Davis, 1985).

As aminoglycosides have been used widely to treat *Acinetobacter* infections, it is not surprising that many reports have documented aminoglycoside resistance mechanisms in this organism.

Three mechanisms of aminoglycoside resistance have been described: (1) alteration of target site; (2) reduction in influx of aminoglycoside into cell; and (3) inactivation of the aminoglycoside by enzymatic modification (Davies, 1983). The latter is probably the most significant mechanism of resistance in clinical isolates. Three classes of aminoglycoside modifying enzymes (AMEs) have been described: acetyltransferases (AAC), adenylyltransferases (AAD), and phosphotransferases (APH) (Davis, 1987) (Fig. 1.1).

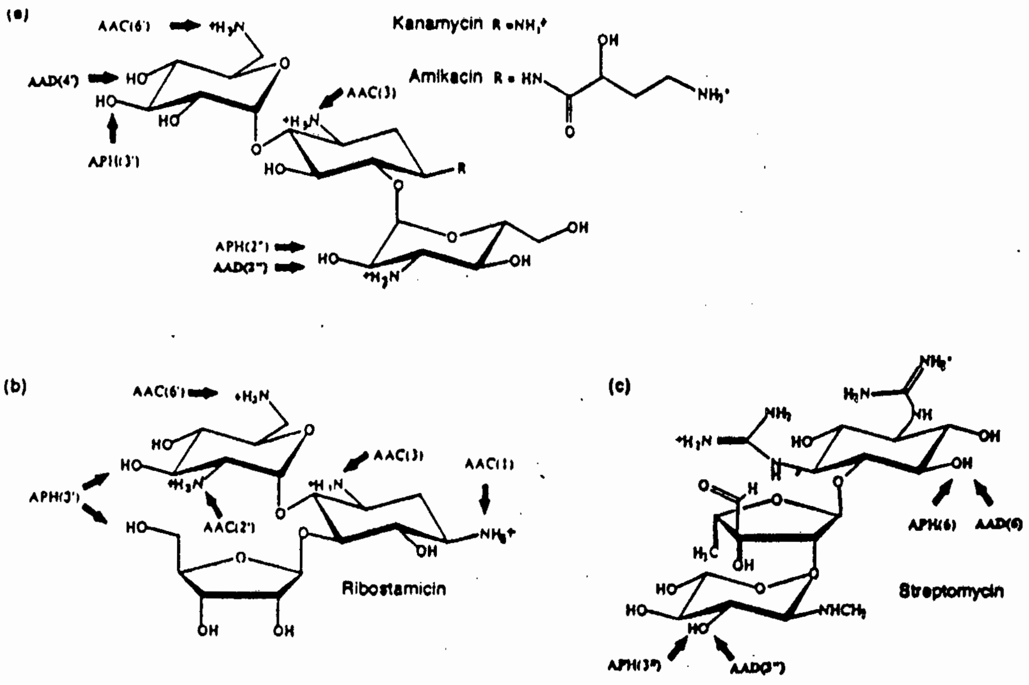


Figure 1.1 Structure of representative aminoglycoside antibiotics: **(a)** kanamycin/ amikacin, **(b)** ribostamicin and **(c)** streptomycin. The sites of modification of various enzymes are shown by arrows. *N*-acetyltransferases (AAC); *O*-adenylyltransferases (AAD); *O*-phosphotransferases (APH). (adapted from Davies and Wright, 1997).

AACs use Co-A as a substrate to catalyze the acetylation of the susceptible amino groups on the aminoglycoside. They comprise AAC(3), which acts at position 3 on the 2-deoxystreptamine ring, AAC(2') and AAC(6'), which act at the 2' and 6' positions on the aminoglycoside sugar, respectively (Davies and Smith, 1978). AADs use ATP as a co-substrate to catalyze the adenylylation of susceptible hydroxyl groups. These, too, are classified according to the site they modify on the aminoglycoside molecule. AAD(2") is the only member of the group that has activity against gentamicin. APHs use ATP as a co-substrate to modify hydroxyl groups on the aminoglycoside molecule.

With respect to the mechanism of aminoglycoside resistance, members of each of the 3 classes of AMEs have been identified in *Acinetobacter* (Table 1.1), however, little is known about the genetic basis of this resistance in *Acinetobacter*.

Table 1.1 Aminoglycoside-modifying enzymes identified in *Acinetobactor* spp.

Enzyme	Reference
Acetylases	
AAC(6')	Shannon *et al.* (1978); Murray and Moellering (1979); Kremery *et al.* (1985); Phillips *et al.* (1986); Lovering *et al.* (1988); Lambert *et al.* (1990; 1993); Rudant *et al.* (1994); Seward *et al.* (1998)
AAC(2')I	Dowding (1979)
AAC(3)I	Bergogne-Bérézin *et al.* (1980); Gomez-Lus *et al.* (1980); Devaud *et al.* (1982); Van de Klundert *et al.* (1984); Phillips *et al.* (1986); Lovering *et al.* (1988); Vila *et al.* (1993); Seward *et al.* (1998)
AAC(3)II	Murray and Moellering (1980); Elisha and Steyn (1991a,c); Shaw *et al.* (1993); Seward *et al.* (1998)
AAC(3)IV	Shaw *et al.* (1993)
Adenylylases	
AAD(3")(9)	Shannon *et al.* (1978); Gomez-Lus *et al.* (1980); Murray and Moellering (1980); Shimizu *et al.* (1981); Devaud *et al.* (1982); Goldstein *et al.* (1983); Vila *et al.* (1993); Seward *et al.* (1998)
AAD(2")	Murray and Moellering (1980); Elisha and Steyn (1991a,c); Shaw *et al.* (1993); Segal and Elisha (1997); Seward *et al.* (1998)

(continued)

Phosphorylases	
APH(3')I	Shannon *et al.* (1978); Bergogne-Bérézin *et al.* (1980); Gomez-Lus *et al.* (1980); Devaud *et al.* (1982); Goldstein *et al.* (1983); Divers *et al.* (1985); Shaw *et al.* (1993)
APH(3')II	Murray and Moellering (1979); Bergogne-Bérézin *et al.* (1980)
APH(3')III	Murray and Moellering (1979); Joly-Guillou *et al.* (1987)
APH(3')VI	Martin *et al.* (1988); Lambert *et al.* (1988; 1990); Shaw *et al.* (1993); Vila *et al.* (1993); Seward *et al.* (1998)
APH(3")I	Elisha and Steyn (1989)

The first report of a plasmid-encoded AME in *Acinetobacter* was by Murray and Moellering (1980). Using novobiocin, these authors cured an aminoglycoside-resistant *Acinetobacter* of a plasmid. The concomitant loss of AMEs [AAD(2") and APH(3')] suggested that the genes encoding these enzymes were on the plasmid that had been lost from the *Acinetobacter* strain. However, transfer of resistance by conjugation or transformation to *E. coli* and *Acinetobacter* was not demonstrated.

Other reports have shown unequivocally that genes encoding aminoglycoside resistance are located on plasmids and transposons (Devaud *et al.*, 1982; Krcméry *et al.*, 1985; Elisha, 1991; Goldstein *et al.*, 1983; Lambert *et al.*, 1988; 1990). The *aphA6*, encoding APH(3')-VIa activity that confers resistance to kanamycin and amikacin, was the first aminoglycoside resistance gene from *Acinetobacter* to be sequenced (Martin *et al.*, 1988). Dissemination of amikacin resistance was found to be due to *aphA6* which was associated with a transposable element, rather than due to a strain or a plasmid epidemic (Lambert *et al.*, 1990). Early reports of AMEs, including AAC(3)-I, APH(3')-I, and AAD(3"), conferring resistance to gentamicin, kanamycin, and streptomycin/spectinomycin, respectively, linked this resistance to a transposable element in a multiresistant clinical isolate of *Acinetobacter* (Devaud *et al.*, 1982). The APH(3')(5")-I gene identified on a plasmid, pIP1031, was associated with an IS15 element, suggesting that this gene was also part of a transposable element (Goldstein *et al.*, 1983). A later study by Divers *et al.* (1985) identified a plasmid, pAV5, encoding an APH(3')-I flanked by inverted repeats. The presence of inverted repeat sequences flanking the kanamycin resistance gene suggest that it may be located on a transposon, which was provisionally designated Tn*4411*. A further study identified an APH(3') gene encoding resistance to amikacin and kanamycin, on a plasmid, pIP1841, in *A. baumannii* (Lambert *et al.*, 1988). The plasmid was self-transferable to *A. baumannii, A. haemolyticus,* and *A. lwoffii*, but not to *E. coli.*

Integrons have been implicated in the spread of resistance genes amongst clinical isolates of bacteria (Stokes and Hall, 1989). Recently, aminoglycoside resistance genes associated with these elements have been described in *Acinetobacter.* In a study conducted by Seward *et al.* (1998), the *aac(3)-Ia*, which encodes an enzyme with activity against gentamicin, and *aad(3")-Ia*, which encodes activity against streptomycin and spectinomycin, were identified on integrons in *A. baumannii* and *Acinetobacter* sp. 3. Another aminoglycoside resistance gene, *aadB*, with activity against kanamycin, tobramycin and gentamicin, was also associated with an integron in *Acinetobacter*, strain SAK (Elisha, 1991). Curiously, the *aadB* gene was not expressed in strain SAK (Elisha and Steyn, 1991a,c).

A number of aminoglycoside resistance genes have been located in the *Acinetobacter* chromosome. The *aac(6')-Ig* gene, which encodes an AAC(6') that inactivates amikacin, was detected on the chromosome of a clinical isolate of *A. haemolyticus* (Lambert *et al.*, 1993; Ploy *et al.*, 1994). The absence of this gene in any other *Acinetobacter* species investigated, suggested that *aac(6')-Ig* is specific to *A. haemolyticus* (Lambert *et al.*, 1993) and that it could be used to identify this organism. Similarly, chromosomal genes (*aac(6')-Ij* and *Ik*) have been identified in other *Acinetobacter* species and these, too, are thought to be species-specific (Lambert *et al.*, 1994; Rudant *et al.*, 1994). Constitutively expressed aminoglycoside resistance genes have been identified in *Providencia stuartii* (Rather *et al.*, 1993), *Serratia marcescens* (Shaw *et al.*, 1992), *Enterococcus faecium* (Costa *et al.*, 1993) and mycobacterial species (Aínsa *et al.*, 1997). A role in cellular function has been proposed for some of the enzymes encoded by these genes. AAC(2')-Ia from *Providencia stuartii* is thought to play a role in peptidoglycan synthesis (Rather *et al.*, 1993).

1.3 REGULATION OF RESISTANCE GENES IN *ACINETOBACTER*

The majority of genes in *E. coli* and other *Enterobacteriaceae* are regulated by the major *E. coli* RNA polymerase associated with σ^{70}, $E\sigma^{70}$. $E\sigma^{70}$ recognizes promoter sequences with a consensus sequence, (-35) TTGACA $N_{17\pm1}$ (-10) TATAAT (Harley and Reynolds, 1987). However, little is known about transcription signals in *Acinetobacter*. For a number of reasons, it is possible to hypothesize that the signals recognized in *Acinetobacter* may be similar to, but different from, those recognized in *E. coli.*

Firstly, the *aadB* gene in strain SAK is part of an integron and is downstream of the integron-associated promoters, P_{ant} and P2, which are typical of those recognised by $E\sigma^{70}$ (Elisha, 1991). However, this gene is not expressed in the *Acinetobacter* strain SAK (Elisha and Steyn, 1991a,c).

Furthermore, atypical spacings of promoters identified in the regulatory regions of a number of chromosomally-located genes in *Acinetobacter* have been reported. The -35 (TTGAAA) and -10 (TAAGTT) hexamers of the *aac(6')-Ig* gene intrinsic to *A. haemolyticus* are separated by 21 nucleotides (Lambert *et al.,* 1993). Similarly, the hexamers of the proposed promoter of the *aac(6')-Ij* gene identified on the chromosome of *Acinetobacter* sp. 13 are separated by 15 nucleotides (Lambert *et al.*, 1994). Although the hexamers, - 35 (GTGAAC) and -10 (TTTACT), proposed to regulate *aph(3')VIa* are optimally spaced (Martin *et al.,* 1988), they do not show good homology to the corresponding *E. coli* consensus sequences. Elisha (1991) suggested an alternative promoter, TTGCTA (N_{13}) TAAGAT, which displays a greater degree of homology to the consensus sequences and has a 13-bp spacing.

This atypical spacing of promoter sequences observed in *Acinetobacter* is not limited to aminoglycoside resistance genes. Analysis of regulatory regions of *trpE* and the *trpFB* genes involved in tryptophan biosynthesis in *A. calcoaceticus* revealed hexamers with spacing between 16 and 19 nucleotides (Haspel *et al.,* 1991). Although the promoter of the *trpGDC* gene cluster was not identified by Kaplan *et al.* (1984), the sequence upstream of the cluster contains a -35 (TTGCCA) hexamer and -10

(TTAAAT) hexamer which are separated by 23 bps. Analysis of regulatory sequences upstream of esterase genes *estB* and *estR* identified TTGCGA (N_{16}) TAAGTT and TTGCCA (N_{19}) TACACT, respectively (Kok *et al.*, 1995). In addition, a polyhydroxyalkanoic acid (PHA) synthase gene cloned from *Acinetobacter* contains a -10 hexamer (TAATTT) but no -35 sequence (Schembri *et al.*, 1995). The synthase gene was shown to be positively regulated by phosphate and the authors identified a *pho*-box in the regulatory region.

Interestingly, no sigma factor corresponding to $E\sigma^{70}$ was detected in *Acinetobacter* when analyzing the ribonucleic acid polymerase of this genus (Kleppe and Kleppe, 1976). Possibly, an alternative σ factor, which recognizes atypically spaced promoters, is responsible for regulation of the majority of genes in *Acinetobacter.*

1.4 AIM OF STUDY

The aim of this study was two fold:

i) to characterize the molecular basis of aminoglycoside resistance in a clinical isolate of *Acinetobacter.*

ii) to gain an understanding of transcription signals recognized in *Acinetobacter*

CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF *aadB* GENES FROM *ACINETOBACTER*

2.1 INTRODUCTION

In an earlier study, two aminoglycoside resistance genes, *aadB* and *aacC2*, were cloned from a clinical isolate of *Acinetobacter* (strain SAK) and expressed in *E. coli* (Elisha and Steyn, 1991a). The *aadB* gene product [AAD(2")] has 2'-*O*-adenylyltransferase activity which confers resistance to gentamicin (Gm), tobramycin (Tob), and kanamycin (Km) (Davies, 1986), while 3-*N*-acetyltransferase [AAC(3)II] encoded by *aacC2* confers resistance to Gm, Tob and netilmicin (Net), but not Km (Davies, 1986; Shaw *et al.*, 1993). Although strain SAK contains an *aadB* gene, it was sensitive to Km, suggesting that the *aadB* gene is not expressed in this strain. This hypothesis was confirmed by DNA-RNA hybridization studies. No *aadB* transcripts were detected in total RNA prepared from strain SAK. On the other hand, *aacC2* transcripts were detected, indicating that AAC(3)II alone is responsible for the resistance phenotype (Gm^R, Tob^R, Net^R, Km^S) of strain SAK.

The *aadB* and *aacC2* structural genes, and their respective flanking regions, were cloned and sequenced (Elisha, 1991; Elisha and Steyn, 1991c). Both structural genes were intact. Analysis of the regulatory regions upstream of *aadB* and *aacC2* showed that the *aacC2* promoter comprised a -10 hexamer homologous to the consensus -10 promoter sequence recognized by $E\sigma^{70}$. However, no sequence relative to this -10 hexamer showed good homology to the -35 consensus hexamer. The *aadB* gene was downstream of integron-associated promoters, P_{ant} and P2. Studies have shown that, when present, both promoters, TTGACA (N_{17}) TAAACT (P_{ant}), and TGGACA (N_{17}) TAAGCT (P2), are used in the transcription of integron-associated gene cassettes (Lévesque *et al.*, 1994; Collis and Hall, 1995). A feature of these promoters is that they are typical of those recognized by $E\sigma^{70}$. It may be that these promoters are not the preferred regulatory sequences in *Acinetobacter*, which raised the question of transcriptional regulation in *Acinetobacter*.

To investigate further the regulation of *aadB* genes in *Acinetobacter*, clinical isolates were screened with an *aadB* probe for the presence of this gene.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Bacterial strains

Thirty-eight clinical isolates of *Acinetobacter* (Km^R, Gm^R, Tob^R) were collected in the Department of Medical Microbiology, Groote Schuur Hospital, Cape Town. *A. calcoaceticus* BD413 C91 (kindly provided by Dr A. Vivian, University of Western England, Bristol, UK), *E. coli* LK111 (Zabeau and Stanley, 1982) and DH5α (Hanahan, 1983) were used as recipients in transformation studies.

2.2.2 Antibiotic sensitivity testing

Antibiotic sensitivity testing was carried out by medical technologists using the Kirby Bauer disc diffusion test on Mueller-Hinton agar incubated aerobically at 37°C for 18 hours. The zone sizes to determine the susceptibility of the organism were those recommended by the National Committee for Clinical Laboratory Procedures. Oxoid discs with the following amounts were used: 10 μg Gm, 10 μg Tob, and 30 μg Km.

2.2.3 Isolation of DNA

2.2.3.1 Isolation of genomic DNA

Genomic DNA was prepared according to the method of Ausubel *et al.* (1987) which uses SDS and proteinase K to lyse the bacterial cell wall. Cetyltrimethylammonium bromide was added in the presence of 0.7 M NaCl to complex with, and precipitate, cell-wall debris, denatured proteins, and polysaccharides, leaving the nucleic acid in solution. Genomic DNA was precipitated using 0.6 volumes isopropanol, washed with 70 % ethanol, and resuspended in 50 - 100 μl TE buffer (Appendix A).

2.2.3.2 Isolation of plasmid DNA

Bacterial cells from overnight cultures (4 ml) grown in 2 x Yeast-Tryptone (YT) broth (Appendix A) plus appropriate antibiotics were harvested and plasmid DNA was isolated using the alkaline-lysis method described by Ish-Horowicz and Burke (1981). Isolation of plasmid DNA from *Acinetobacter* required the addition of 5 - 10 mg lysozyme, prior to the addition of NaOH/ SDS to facilitate disruption of the cell wall and subsequent release of DNA. Commercially available kits (Wizard Minipreps DNA Purifications Systems, Promega USA; pLASmix Minipreps, Talent, Italy; Nucleon MiP, Amersham, UK) or a silica-based matrix (Bio101, Inc) were also used to isolate plasmid DNA from *E. coli* strains.

Large-scale plasmid DNA was prepared from 100 - 200 ml cultures of *E. coli* and *A. calcoaceticus* cultures using a Nucleobond AX 100 PC-kit (Macherey-Nagel, Germany) or Wizard Maxiprep kit (Promega, USA). Alternatively, either the alkaline-lysis (Ish-Horowicz and Burke, 1981) or the method of Clewell and Helinski (1969) was used. Plasmid DNA was separated from chromosomal DNA by equilibrium centrifugation in a caesium chloride-ethidium bromide density gradient, collected, precipitated (Sambrook *et al.,* 1989), and resuspended in TE Buffer (Appendix A). The concentration of DNA was calculated by reading the absorbency at OD_{260} and using the relationship 1 unit = 50 µg/ml.

2.2.3.3 Isolation of single stranded DNA

DNA fragments were ligated to the replicative forms of M13mp18 and 19 and introduced into competent *E. coli* LK111 cells by transformation. After incubation on ice for 40 minutes the cells were heat shocked at 42 ° C for three minutes. IPTG (40 µl, 100 mM), X-gal (40 µl, 2 % in dimethylformamide), exponential phase *E. coli* cells (200 µl), and 3 ml "sloppy" LB agar (Appendix A) were added to the transformed cells, mixed, poured onto an agar base and incubated at 37 ° C overnight. An agar plug containing a single, white plaque was inoculated into 1.5 ml YT broth that had been inoculated with 10 µl of an overnight culture of *E. coli* LK111. The cultures were incubated at 37 ° C (with shaking) for 4.5 hours; afterwhich, the *E. coli* cells were pelleted by centrifugation and the supernatant containing the recombinant M13 phage was transferred to an Eppendorf tube. An overnight LK111 culture was diluted 1/50 with YT broth and incubated at 37 ° C until OD_{600} = 0.1 - 0.2; at which point, 100 µl phage stock was added. Incubation was allowed to continue for a further 4.5 hours.

LK111 cells were pelleted, 40 ml supernatant fluid was decanted into a flask and extruded phage was concentrated by adding 5.18 ml PEG (40 %) in the presence of 4 M NaCl (6.45 ml). The phage precipitate was harvested by centrifugation and resuspended in 500 µl 10 mM Tris-HCl,pH7.5, 10 mM NaCl, 0.5 mM EDTA. DNA was extracted by adding an equal volume of phenol, mixing, and placing the sample on ice for 10 minutes. The aqueous phase was re-extracted with phenol:chloroform isoamyl alcohol (X2) and the DNA precipitated with ethanol.

2.2.4 Analysis of DNA

2.2.4.1 Restriction endonuclease digestion

Restriction digests were carried out in 20 - 50 µl reactions at the temperatures and in the buffer reaction conditions specified by the manufacturers. Typically, 200 - 400 ng DNA was digested, using 2 U enzyme, for 1 - 2 hours when screening putative recombinant plasmids.

2.2.4.2 Agarose gel electrophoresis

Restricted DNA fragments were separated in horizontal gels of 0.8 – 1 % (w/v) agarose dissolved in 0.4 M Tris-acetate and 0.01 M EDTA. Gels were stained with ethidium bromide and the DNA was visualized by UV transillumination at 302 nm. Where necessary, DNA fragments were cut out of the agarose gel after electrophoresis, and extracted with phenol (Seth, 1984).

2.2.5 DNA ligation

Typically, 10 - 50 ng of DNA fragments to be cloned were ligated to 50 - 100 ng prepared vector, using a DNA ligation kit (Amersham International, UK). Ligation mixes were incubated at 16 °C for 30 minutes to 16 hours.

2.2.6 Transformation studies

E. coli LK111 and DH5α cells were made competent by the method of Dagert and Ehrlich (1979). Twenty (20) μl of ligation mix was added to 100 μl of competent cells and incubated on ice for 20 - 30 minutes. After heat shock at 42 °C for two minutes, 0.9 ml YT broth was added and the cells were incubated at 37 °C for one hour to allow for antibiotic gene expression. Cells were plated on YT agar containing the appropriate antibiotics for selection of transformants and incubated at 37 °C for 18 hours.

A. calcoaceticus has been shown to be maximally competent after 2 hours incubation at 30 °C (Palmen *et al.,* 1993). Approximately 2 μg plasmid DNA was added to 0.5 ml of maximally competent *A. calcoaceticus* BD413 C91 cells and incubated at 30 °C for one hour. Cells were plated on YT agar containing appropriate antibiotic for selection of transformants and incubated at 30 °C for 48 hours.

2.2.7 DNA-DNA hybridizations

2.2.7.1 Preparation of probe

Plasmid pGSH108 contains a *Bam*HI-*Hin*dIII insert of 1.9 kb which encodes integrase and AAD(2") (Elisha, 1991; Elisha and Steyn, 1991a). A 300 bp *Sac*I-*Sph*I fragment, internal to the *aadB* structural gene, was purified from pGSH108 by agarose gel electrophoresis, followed by extraction with phenol [2.2.4.2]. The DNA was labelled with either [^{32}P]dCTP using a nick translation kit (Amersham International, UK) or digoxigenin-dUTP (Boehringer Mannheim, Germany) by random primed DNA labelling. Both kits were used according to manufacturers' instructions. The labelled DNA fragments were used as probes in hybridization experiments.

2.2.7.2 Hybridization

The probes were hybridized to genomic and plasmid DNA which had been transferred by capillary action to Hybond-N^{+} membranes (Amersham International, UK) using the method of Southern (1975). Pre-hybridization (42 °C), hybridization (42 °C), and post-hybridization washes (68 ° C) were carried out according to the protocol supplied with the DIG Luminescent Detection Kit (Boehringer Mannheim, Germany). When using radio-labelled probes, the conditions for hybridization were as above, except for the post-hybridization washes, which were carried out at 55 ° C and not 68°C. Hybridization of probes to complimentary DNA on the membranes was detected by autoradiography.

2.2.8 Sequencing

2.2.8.1 DNA sequencing

DNA for sequencing was subcloned into either M13mp18 and 19, or pUC vectors. Either the dideoxy chain termination method (Sanger *et al.,* 1977) using Sequenase and [^{35}S]dATP or rapid automated laser fluorescence (Pharmacia Biotech AB, S-751 82 Uppsala, Sweden) was used to generate DNA sequencing data. The latter was carried out in the Department of Microbiology or the Department of Chemical Pathology, University of Cape Town. The sequencing reactions were electrophoresed through polyacrylamide-urea gels, and when necessary, polyacrylamide-urea/ formamide gels. Internal oligonucleotides were designed where necessary to ensure that both strands were sequenced.

2.2.8.2 Analysis of sequencing data

The DNA sequencing data was analyzed with Genpro 6.1 (Riverside Scientific) and Windows Dotplot Version 1.0 (R.C. Nakisa). Nucleotide and amino-acid sequences in GenBank databases were searched for homology to DNA sequences and deduced amino-acid sequences.

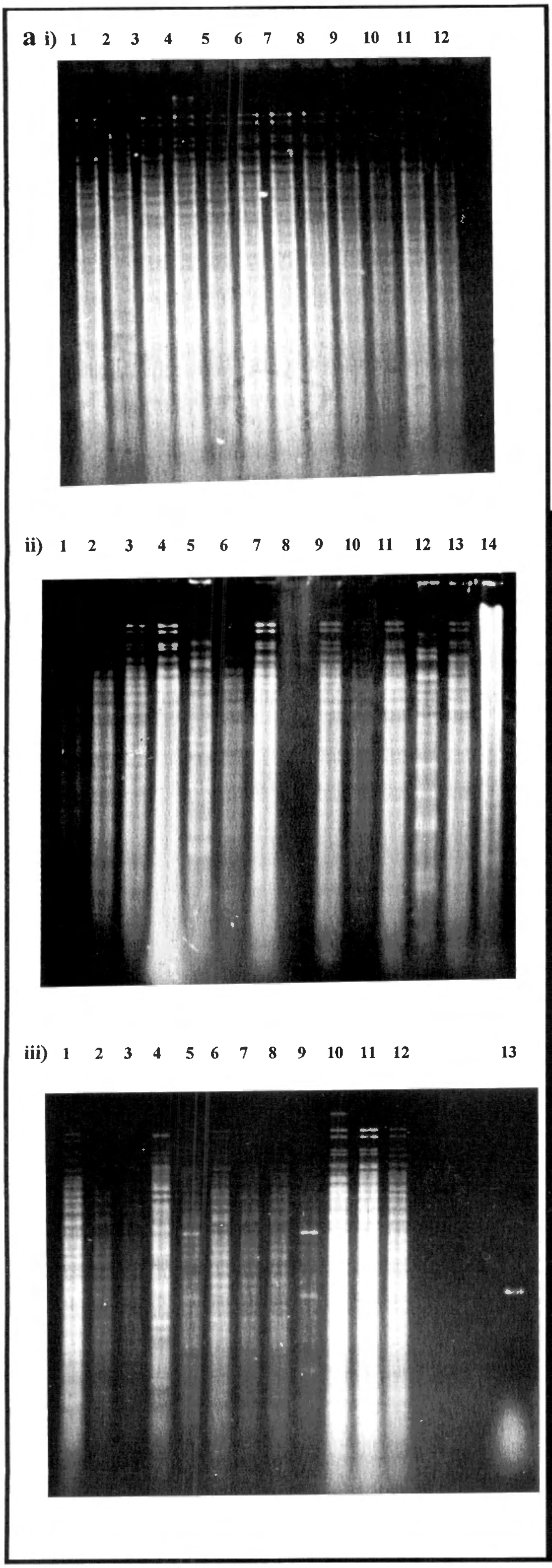
2.3 RESULTS

2.3.1 DNA-DNA hybridization studies with *aadB* probe

Genomic DNA from 38 clinical isolates of *Acinetobacter* (Gm^R, Tob^R, Km^R) was digested with *Bam*HI-*Hind*III. The fragments were separated on an agarose gel, transferred by capillary action to Hybond-N^+ membrane, and probed with an internal portion of the *aadB* structural gene [2.2.7]. The 300-bp *Sac*I-*Sph*I fragment hybridised to pGSH108, the source of the *aadB* probe, and to a fragment in the genomic DNA of two clinical isolates designated strain CHA and strain SUN (Fig. 2.1). The mechanism of aminoglycoside resistance in the remaining 36 isolates was not investigated further, but may be due to a combination of acetyltransferase and phosphotransferase activities.

2.3.2 Location of *aadB* gene in strain SUN and strain CHA

When *A. calcoaceticus* BD413 C91 was used as a recipient in transformation experiments, Gm^R transformants were obtained using genomic DNA from strain SUN and strain CHA. Disc sensitivity testing showed that the Gm^R transformants from both experiments were also resistant to Km and Tob. In similar experiments, using *E. coli* DH5α as a recipient, no transformants were obtained. Plasmid DNA analysis of four Gm^R *Acinetobacter* transformants (2 from strain SUN and 2 from strain CHA) revealed the presence of a plasmid of approximately 6.0 kb in each transformant. Representative plasmids were selected for further characterization. The plasmid from strain SUN was designated pRAY, and that from strain CHA, pCAS. The *aadB* probe [2.2.7] hybridized to a 1.7 kb *Bam*HI-*Hin*dIII fragment from pRAY and pCAS, confirming the presence of the gene on each of these plasmids (Fig. 2.2). A signal was obtained from a fragment of 2.3 kb, suggesting that either the plasmids were not completely digested or that they contained another copy of the *aadB* gene. DNA sequencing of pRAY showed that it contains one copy of the *aadB* gene (Chapter 5).



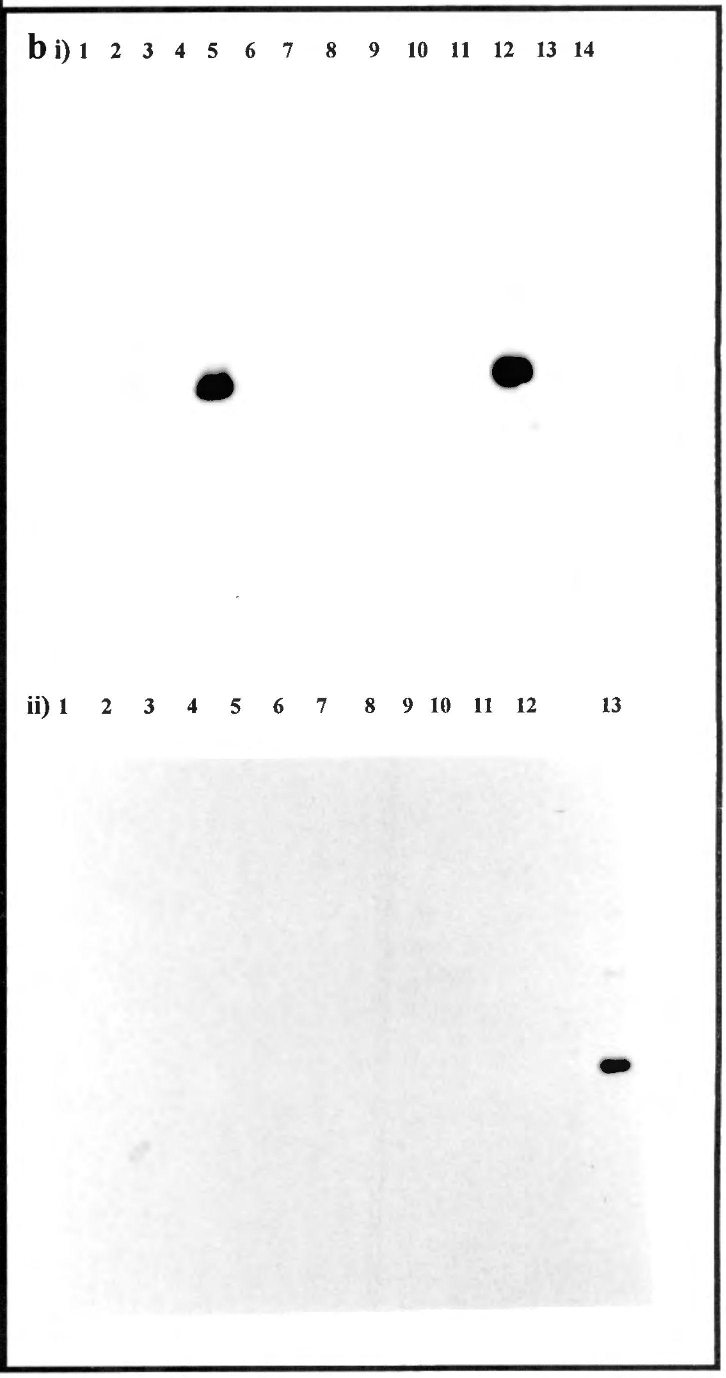


Figure 2.1 Genomic DNA from 38 clinical isolates of *Acinetobacter* hybridized to an *aadB*-specific gene probe. **(a)** *Bam*HI-*Hin*dIII digests of genomic DNA from 38 clinical isolates. **(i)** isolates 1 – 12 (**Lanes 1 – 12**); **(ii)** isolates 13 – 26 (**Lanes 1 – 14**); **(iii)** isolates 27 – 38 (**Lanes 1 – 12**); **Lane 13**: pGSH108 digested with *Bam*HI-*Hin*dIII. **(b)** Autoradiographs of DNA shown in **(aii)** and **(aiii)** hybridized with the *aadB* gene probe. A signal was obtained with the source of the probe (**Lane 13**) and with the DNA from two clinical *Acinetobacter* isolates (**Lanes 5 and 12**).

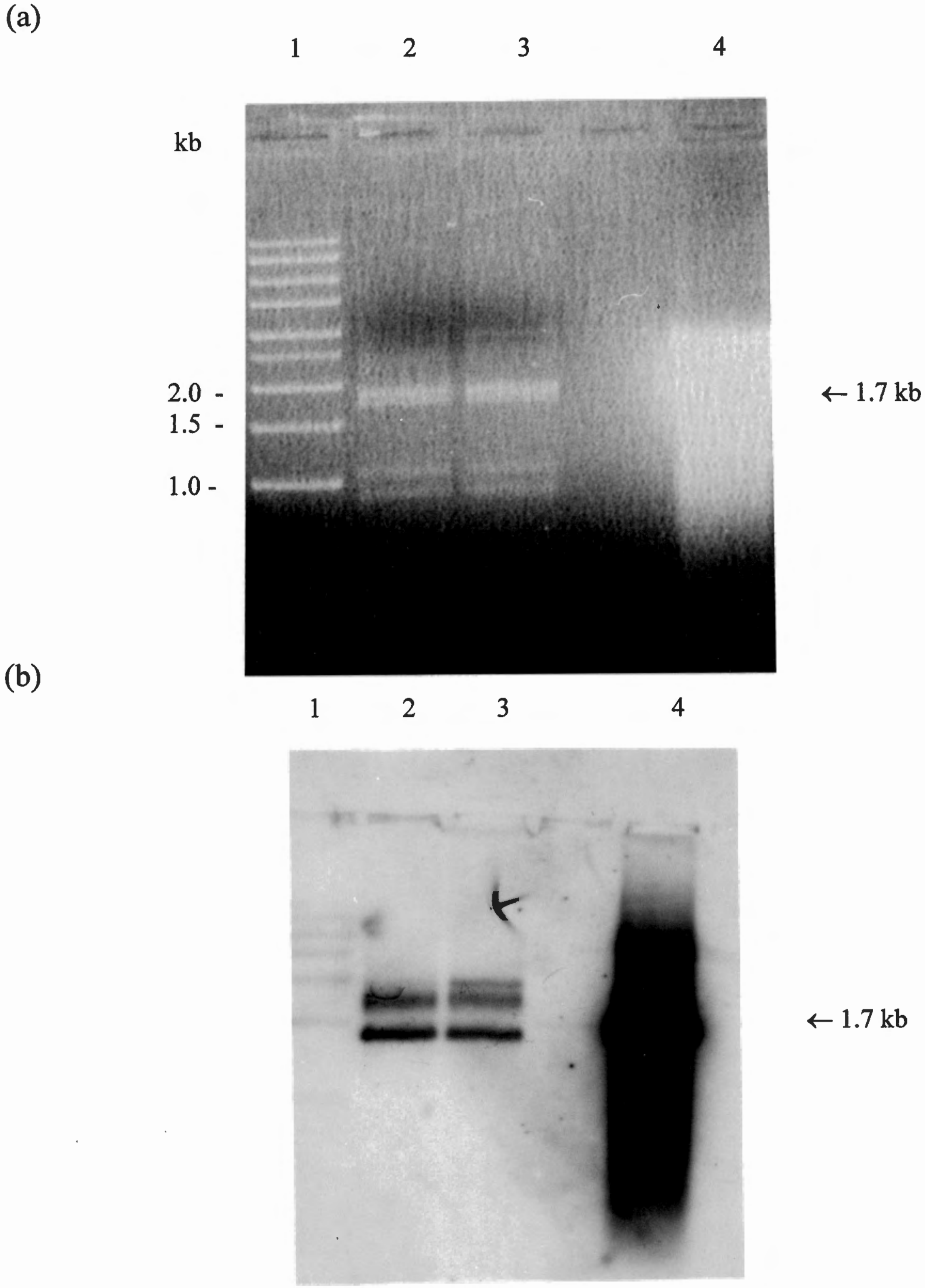


Figure 2.2 pRAY and pCAS hybridized to the *aadB* probe. **(a) Lane 1**: 10 kb BioMarker (Appendix D); **Lane 2**: pCAS digested with *Bam*HI-*Hin*dIII; **Lane 3**: pRAY digested with *Bam*HI-*Hin*dIII; **Lane 4**: pGSH108 digested with *Bam*HI-*Hin*dIII. **(b)** Autoradiograph of DNA shown in **(a)** hybridized with the *aadB* gene probe. Sizes of relevant fragments in BioMarker are indicated. The 1.7 kb fragments are indicated.

2.3.3 DNA sequence analysis of the 1.7 kb *Bam*HI-*Hin*dIII fragment from pRAY

The 1.7 kb *Bam*HI-*Hin*dIII fragment of pRAY, carrying the *aadB* gene from strain SUN, was cloned into pUC19, generating pHS100 (Appendix C), and sequenced. It was necessary to clone the same fragment into M13mp18 and 19 to complete the sequencing of both strands. The nucleotide sequence of the 1.7 kb *Bam*HI-*Hin*dIII fragment is shown in Figure 2.3.

Analysis of the sequencing data revealed an open reading frame (ORF) between ATG (648 - 650) and TAA (1179 - 1181) which showed 99.4 % homology to a published *aadB* gene sequence (Cameron *et al.*, 1986). The nucleotide changes observed at positions 791 and 986, where the structural *aadB* from strain SUN contains a T rather than a G and a C, respectively, probably reflects the preferred ACT for threonine, and GGT for glycine. This is in accordance with the data of White *et al.* (1991), who showed that more than 66 % of nucleotides in the third position of codons used in *Acinetobacter* are either an A or a T. Transitions A→G (666), T→C (853) and C→T (1107) result in the incorporation of alanine, threonine and phenylalanine instead of threonine, methionine and leucine, respectively. A glycine is substituted for a lysine due to a transversion (A→C) at nucleotide 678.

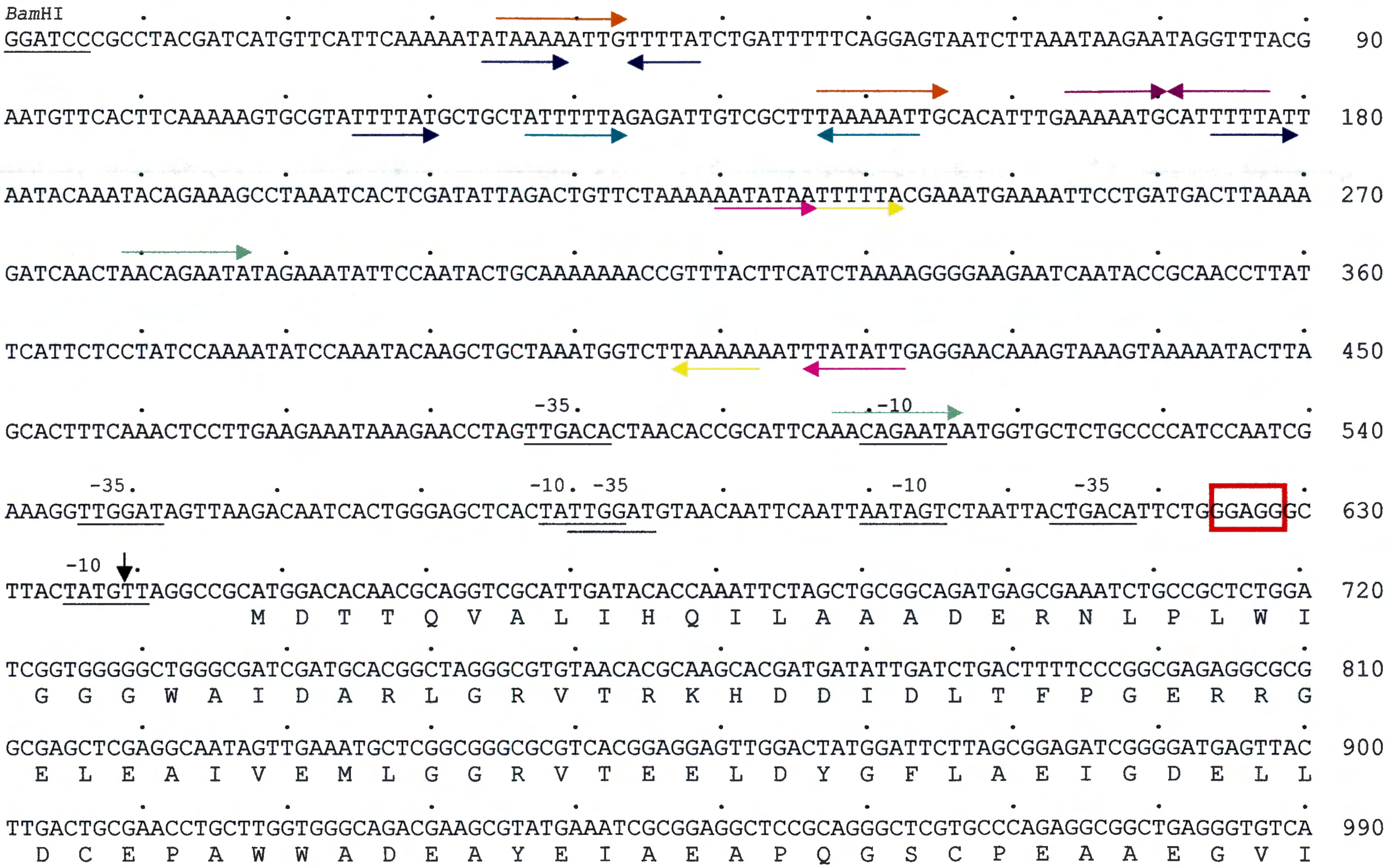
The *aadB* start codon is preceded by a ribosomal binding site-like sequence, GGAGG (624 - 628), complimentary to the 3'-OH terminal sequence of *E. coli* 16S rRNA (5'-GAUCA$\underline{\text{CCUCC}}$UUA-3') (underlined) (Shine and Dalgarno, 1974). A search for promoters in the region upstream of the start codon identified four potential promoter sequences: -35 (CTGACA) N_{16} -10 (TATGTT), -35 (TTGGAT) N_{14} -10 (AATAGT), -35 (TTGGAT) N_{26} -10 (TATTGG) and -35 (TTGACA) N_{17} -10 (CAGAAT).

The sequence 5' of the *aadB* gene (1 - 648) contains a number of direct and inverted repeat sequences (Fig. 2.3) ranging from 6 - 9 bp in length. This region also contains an ORF (1 - 439) which showed no homology to any sequences in GenBank. The sequence GTTAGGC (638 - 644), immediately upstream of the *aadB* structural gene, is consistent with that of *attI* sites (Recchia and Hall, 1995a) which are associated with gene cassettes, and recognized by integrase (Recchia *et al.*, 1994).

The sequence (1176 - 1235), contiguous with the 3' end of the *aadB* structural gene, is identical to 59-base elements (59-be) associated with *aadB* genes (Cameron *et al.*, 1986; Hall *et al.*, 1991). An ORF (1236 - 1746) downstream of the 59-be showed no homology to any sequences in GenBank. The presence of a 59-be is further evidence that the *aadB* gene is part of a cassette (Hall *et al.*, 1991).

2.3.4 DNA sequence analysis of the 1.7 kb *Bam*HI-*Hin*dIII fragment from pCAS

The 1.7 kb *Bam*HI-*Hin*dIII fragment of pCAS, from strain CHA, was cloned into pUC19, resulting in pHS200 (Appendix C), and sequenced. The sequencing data generated (1 - 783), was identical to the corresponding sequence obtained for the 1.7 kb insert from pRAY, and included the 5′ *attI* site (GTTAGGC) and the *aadB* structural gene. Similarly, the sequence 1000 – 1746, including the 59-be was identical to the corresponding sequence obtained from the *Bam*HI-*Hin*dIII fragment from pRAY.



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BamHI
GGATCCCGCCTACGATCATGTTCATTCAAAAATATAAAAATTGTTTTATCTGATTTTTCAGGAGTAATCTTAAATAAGAATAGGTTTACG   90

AATGTTCACTTCAAAAAGTGCGTATTTTATGCTGCTATTTTTAGAGATTGTCGCTTTAAAAATTGCACATTTGAAAAATGCATTTTTATT  180

AATACAAATACAGAAAGCCTAAATCACTCGATATTAGACTGTTCTAAAAAATATAATTTTTACGAAATGAAAATTCCTGATGACTTAAAA  270

GATCAACTAACAGAATATAGAAATATTCCAATACTGCAAAAAAACCGTTTACTTCATCTAAAAGGGGAAGAATCAATACCGCAACCTTAT  360

TCATTCTCCTATCCAAAATATCCAAATACAAGCTGCTAAATGGTCTTAAAAAAATTTATATTGAGGAACAAAGTAAAGTAAAAATACTTA  450

                                    -35.                    .-10
GCACTTTCAAACTCCTTGAAGAATAAAGAACCTAGTTGACACTAACACCGCATTCAAACAGAATAATGGTGCTCTGCCCCATCCAATCG  540

     -35.                           -10. -35                . -10        -35
AAAGGTTGGATAGTTAAGACAATCACTGGGAGCTCACTATTGGATGTAACAATTCAATTAATAGTCTAATTACTGACATTCTGGGAGGGC  630

    -10 ↓
TTACTATGTTAGGCCGCATGGACACAACGCAGGTCGCATTGATACACCAAATTCTAGCTGCGGCAGATGAGCGAAATCTGCCGCTCTGGA  720
                  M  D  T  T  Q  V  A  L  I  H  Q  I  L  A  A  A  D  E  R  N  L  P  L  W  I

TCGGTGGGGGCTGGGCGATCGATGCACGGCTAGGGCGTGTAACACGCAAGCACGATGATATTGATCTGACTTTTCCCGGCGAGAGGCGCG  810
   G  G  G  W  A  I  D  A  R  L  G  R  V  T  R  K  H  D  D  I  D  L  T  F  P  G  E  R  R  G

GCGAGCTCGAGGCAATAGTTGAAATGCTCGGCGGGCGCGTCACGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCGGGGATGAGTTAC  900
   E  L  E  A  I  V  E  M  L  G  G  R  V  T  E  E  L  D  Y  G  F  L  A  E  I  G  D  E  L  L

TTGACTGCGAACCTGCTTGGTGGGCAGACGAAGCGTATGAAATCGCGGAGGCTCCGCAGGGCTCGTGCCCAGAGGCGGCTGAGGGTGTCA  990
   D  C  E  P  A  W  W  A  D  E  A  Y  E  I  A  E  A  P  Q  G  S  C  P  E  A  A  E  G  V  I
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         .         .         .         .         .         .         .         .         .
TCGCCGGGCGGCCAGTCCGTTGTAACAGCTGGGAGGCGATCATCTGGGATTACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGC 1080
  A  G  R  P  V  R  C  N  S  W  E  A  I  I  W  D  Y  F  Y  Y  A  D  E  V  P  P  V  D  W  P
         .         .         .         .         .         .         .         .         .
CTACAAAGCACATAGAGTCCTACAGGTTCGCATGCACCTCACTCGGGGCGGAAAAGGTTGAGGTCTTGCGTGCCGCTTTCAGGTCGCGAT 1170
  T  K  H  I  E  S  Y  R  F  A  C  T  S  L  G  A  E  K  V  E  V  L  R  A  A  F  R  S  R  Y
         .         .         .         .         .         ↓         .         .         .
ATGCGGCCTAACAATTCGTCCAAGCCGACGCCGCTTTGCGGCGCGGCTTAACTCAGGTGTTAGGAAACACTGCTACCGTTATCGGATTTA 1260
  A  A  *
         .         .         .         .         .         .         .         .         .
CTGCTTTACTACTAACCTTGTTAATTCAACATTTGCAAAATAGTCTGACCTTTCGATTGTTTGGGGTGAAATTCTTATCTTAAAAATCTA 1350

         .         .         .         .         .         .         .         .         .
AATATTCTTTTAGATCTAATGCAGATTGCTCGATAAAATCGTATGTATTAATACAACGACAGTAAATAAATTTTGTTAAAAACCTCTAAA 1440

         .         .         .         .         .         .         .         .         .
TCATTTTCTAGATTACGGTTAAAAATAATCGGTTCATGATGAGCAATCCTGTTTCTTAATTCACGTAATTGATCTGTCCAATCGTAAAAA 1530

         .         .         .         .         .         .         .         .         .
ATTTGCCTGTTTTCAACTGTTGCATTAGGAAAAATAGAATTAAAACATCCCTGCCAAATACGTTCTTCATAATTAGCCATAAGCATCTTT 1620

         .         .         .         .         .         .         .         .         .
TGCCAGAATACAAATTTAAGCTCAGGGATCATTTTACTAGGCGAATCTGGGAACTTCTCTTTAGCACTATCAAACATACTTTTAGGGCTA 1710

         .         .         HindIII
TACCTCCCTCTACGGCTAAGGCTTCGAATAAAGCTT                                                       1746
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Figure 2.3 Nucleotide sequence of the 1746-bp *Bam*HI-*Hind*III fragment of pRAY from *Acinetobacter* strain SUN. Dots above the sequence correspond to every 1(nucleotides. The sequence between 648-1181 contains the *aadB* structural gene. The deduced amino acid sequence is shown below the DNA sequence. The terminatior codon is indicated with an asterisk (*). The putative -35 and -10 hexamers are underlined. The 59-be is indicated by two lines below the sequence. The structural gene i: preceded by a RBS-like sequence (boxed in red). Direct and inverted repeat sequences are indicated with arrows. The boundaries of the cassette are indicated by vertica arrows. The sequence has been deposited in GenBank under accession number BankIt 114846 AF003958.

2.4 DISCUSSION

The *aadB* genes from clinical isolates of *Acinetobacter*, strain SUN and strain CHA, were identified on 6.0 kb plasmids, designated pRAY and pCAS, respectively. Interestingly, no transformants were isolated when the plasmids were used in transformation experiments with *E. coli* recipients. This will be discussed further in Chapter 5.

Analysis of the sequencing data of the fragments cloned from pRAY and pCAS indicated that the *aadB* genes are contained on gene cassettes. Because of the identity observed between the DNA sequence of the *Bam*HI-*Hin*dIII fragments from pRAY and pCAS, the latter was not characterized further. The remainder of this dissertation will focus on *Acinetobacter* strain SUN (pRAY).

Gene cassettes are usually associated with larger mobile genetic elements, integrons, which function as expression systems for these cassettes. Four classes of integrons (Type I-IV) have been defined on the basis of their associated integrase gene sequence (Recchia and Hall, 1995a; Mazel *et al.*, 1998). Type I and type II integrons are frequently referred to as Tn*21*-like and Tn*7* transposons, respectively; the former type has been most commonly isolated from clinical isolates of enterobacter (Hall *et al.*, 1994). The "silent" *aadB* gene from *A. baumannii* strain SAK (Elisha and Steyn, 1991a,c) is part of a Tn*21*-like integron (Elisha, 1991). More recently, using DNA: DNA hybridization studies, Gonzalez *et al.* (1998) demonstrated the presence of type I and type II integrons in clinical isolates of *Acinetobacter* from Chile. Only one type III integron has been described (Arakawa *et al.*, 1995). This integron, identified in *Serratia marcescens*, carries a gene cassette encoding a metallo-β-lactamase, linked to an atypical 59-be containing a 67-bp inverted repeat. Integrase type IV, recently identified in *Vibrio cholarae*, is upstream of a large number of gene cassettes that do not encode resistance to antibiotics (Mazel *et al.*, 1998).

The structural features of type I integrons include a 5' conserved segment (CS), a variable region, and a 3' CS (Fig. 2.4) (Stokes and Hall, 1989).

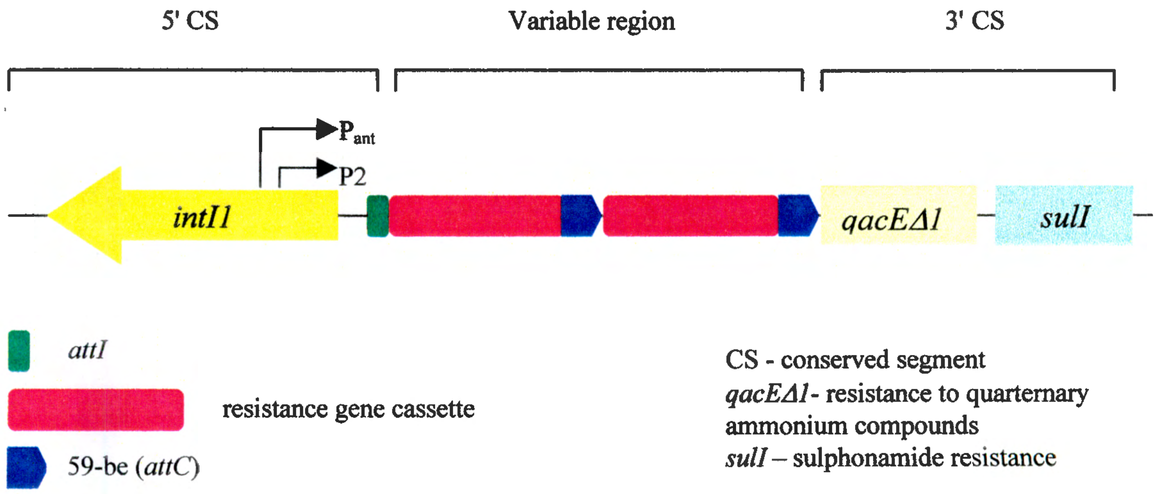


Figure 2.4 Organization of a type I integron containing two resistance gene cassettes. Direction of transcription of the integrase gene (*intI1*) and from the promoters, P_{ant} and P2, is indicated by arrows. Note that P2 is only present in some integrons (Collis and Hall, 1995).

The 5' CS encodes a site-specific recombinase, integrase, and contains promoters P_{ant} and P2, responsible for the expression of gene cassettes inserted within the variable region (Lévesque *et al.*, 1994; Collis and Hall, 1995). Alteration of the number and order of cassettes in this region is catalyzed by integrase (IntI1) which recognizes two recombination sites, *attI* and 59-be (Martinez and de la Cruz, 1988, 1990; Hall *et al.*, 1991; Recchia *et al.*, 1994). In a recent publication, Hansson *et al.* (1997) have referred to 59-be as *attC* sites. However, I shall continue to refer to these elements by their former name, 59-be. The 3' CS is contiguous with the 59-be of the promoter distal gene cassette (Stokes and Hall, 1989) and may encode resistance to sulphonamides (Sundström *et al.*, 1988).

The absence of integron-associated sequence upstream of the *aadB* gene cassette from strain SUN suggests that it is not associated with an integron. Furthermore, hybridization experiments (data not shown) indicated that an integrase type 1 gene (*intI1*) is not present in strain SUN. An *aadB* gene cassette outside of an integron has been identified on a plasmid, pIE723 (Recchia and Hall, 1995b). The *aadB* gene was presumed to be regulated by a promoter (P_4) on this plasmid. The site-specific insertion of the *aadB* gene cassette into RSF1010, generating pIE723, was assumed to have been catalyzed by integrase, since the boundaries of the cassette are identical to those generated following the activity of integrase. That secondary sites can take part in integrase-mediated recombination events has been clearly demonstrated in a number of studies (Francia *et al.*, 1993; Recchia *et al.*, 1994; Francia and García Lobo, 1996; Francia *et al.*, 1997; Hansson *et al.*, 1997).

The identification of the *aadB* gene cassette on pRAY raises two important questions:

i) How is the *aadB* structural gene regulated ?

ii) Which mechanism is responsible for the insertion of the *aadB* gene cassette at the secondary site on pRAY ?

To address the first question, the region upstream of the *aadB* gene was analyzed for promoter sequences. Based on the -35 (TTGACA) and the -10 (TATAAT) consensus sequences recognized by $E\sigma^{70}$ (Harley and Reynolds, 1987), four putative promoters were identified: CTGACA (N_{16}) TATGTT; TTGGAT (N_{14}) AATAGT; TTGGAT (N_{26}) TATTGG and TTGACA (N_{17}) CAGAAT. Although the hexamers of these promoters show good homology to the *E. coli* consensus sequences, only the promoter proximal to the gene cassette fulfils the spacer requirement (16 - 18 bp) of σ^{70} (Hawley and McClure, 1983). Thus, it may be that the *aadB* gene in strain SUN is regulated by an alternative σ factor with a different spacer requirement. Alternatively, none of the putative promoters may regulate the *aadB* gene. The regulation of the *aadB* gene in strain SUN is discussed in Chapter 4.

A possible explanation as to the mechanism of integration of the cassette on pRAY comes from an analysis of the sequences flanking the *aadB* gene cassette. Although the role of RecA in the recombination event cannot be ruled out, the boundaries of the *aadB* gene cassette on pRAY suggest that insertion of the cassette was precise and catalyzed by integrase. Integrase recognizes and cleaves between the G and T residues of the highly conserved GTT triplet (Hall and Collis, 1995; Stokes *et al.*, 1997) contained in both the 5' *attI*, and the 3' core site of the 59-be. Cleavage at these sites flanking the *aadB* gene cassette and subsequent excision of the cassette indicates that the insertion site on pRAY is GTTAGGA. This secondary site is very similar to the preferred *attI* site (GTTPuPuPuPy) recognized by integrase (Collis *et al.*, 1993). Further support for integrase-mediated integration of *aadB* is suggested by the sequence upstream of the secondary site. This sequence (198 - 606) contains motifs (CTAA-CAA-GTAA) thought to be involved in integrase recognition (Schmidt *et al.,* 1989). Moreover, the 12-mer ATGTAACAATTC (584 - 595) differs from the consensus sequence of the 5'-end of the 59-be by only 2 bases (underlined) (Cameron *et al.,* 1986).

Other features, including topology of DNA sequence, are also known to play a role in recombination (Davies and Hutchison III, 1995). In this respect, Francia *et al.* (1997) have shown that the efficiency of IntI1-recognition of a secondary site is increased by the presence of a stem-loop structure, involving the secondary site and a second inverted pentanucleotide. In that study it was shown that an inverted pentanucleotide 3 - 7 bps upstream of the secondary site increased the preference for recognition of the secondary site. Interestingly, the sequence upstream of the *aadB* gene cassette on pRAY contains several direct and inverted repeat sequences that could be involved in formation of secondary structures. Moreover, 3 bps upstream of the insertion site on pRAY (GTTAGGA) is an imperfect inverted pentanucleotide (CTTAC), which may have affected insertion of *aadB* at the secondary site on pRAY.

Assuming the role of integrase in the insertion of the *aadB* gene cassette on pRAY, one of two mechanisms could result in the transfer of the cassette to pRAY. Firstly, and analogous to the mechanism described for the transfer of the *aadB* cassette to pIE723 (Recchia and Hall, 1995b), the cassette may have been inserted into pRAY by a single, integrase-mediated event. Following integrase-mediated cleavage at the 5′ *attI* and 3′ core sites, gene cassettes are released from integrons as circular molecules (Collis and Hall, 1992a; Collis *et al.*, 1993). Recombination of a circular *aadB* gene cassette, excised from an integron located on a transient plasmid in strain SUN, at the insertion site (G↓TTAGGA) on pRAY, would result in the acquisition of the gene cassette by this plasmid (Fig. 2.5).

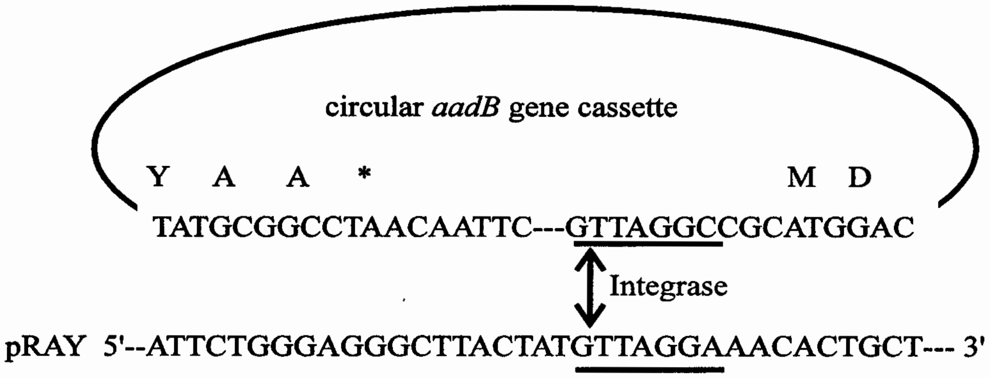


Figure 2.5 Model for direct insertion of the *aadB* gene cassette into pRAY. A free, circular cassette is generated by integrase-mediated excision of the cassette from an integron. The first two and last three amino acids of AAD(2"), encoded by *aadB*, are shown above the sequence. An asterisk (*) indicates the termination codon. The remaining sequence (---) constitutes the 59-be contiguous with the 3' core site, GTTAGGC (underlined). Integrase-mediated insertion of the *aadB* cassette occurs at GTTAGGA (underlined) in pRAY. A double-headed arrow indicates the cross-over point between the insertion site on pRAY and the 59-be 3' core site.

Alternatively, acquisition could have occurred as illustrated in Fig. 2.6. Integrase-mediated cleavage of the *attI* site (G↓TTAGGC) of the *aadB* gene cassette on the plasmid-located integron, and the secondary site (G↓TTAGGA) on pRAY, followed by recombination catalyzed by the same enzyme, would result in the formation of a cointegrate. Resolution of the cointegrate by integrase would be dependent on the regeneration of an *attI* site (Hall and Collis, 1995). It is possible to assume the regeneration of an *attI* site because the putative recombination site (GTTAGGA) contains the conserved GTT associated with these sites. Thus, using the regenerated *attI* site and the 59-be-associated recombination site, integrase could catalyze resolution of the cointegrate, resulting in the transfer of the cassette to pRAY.

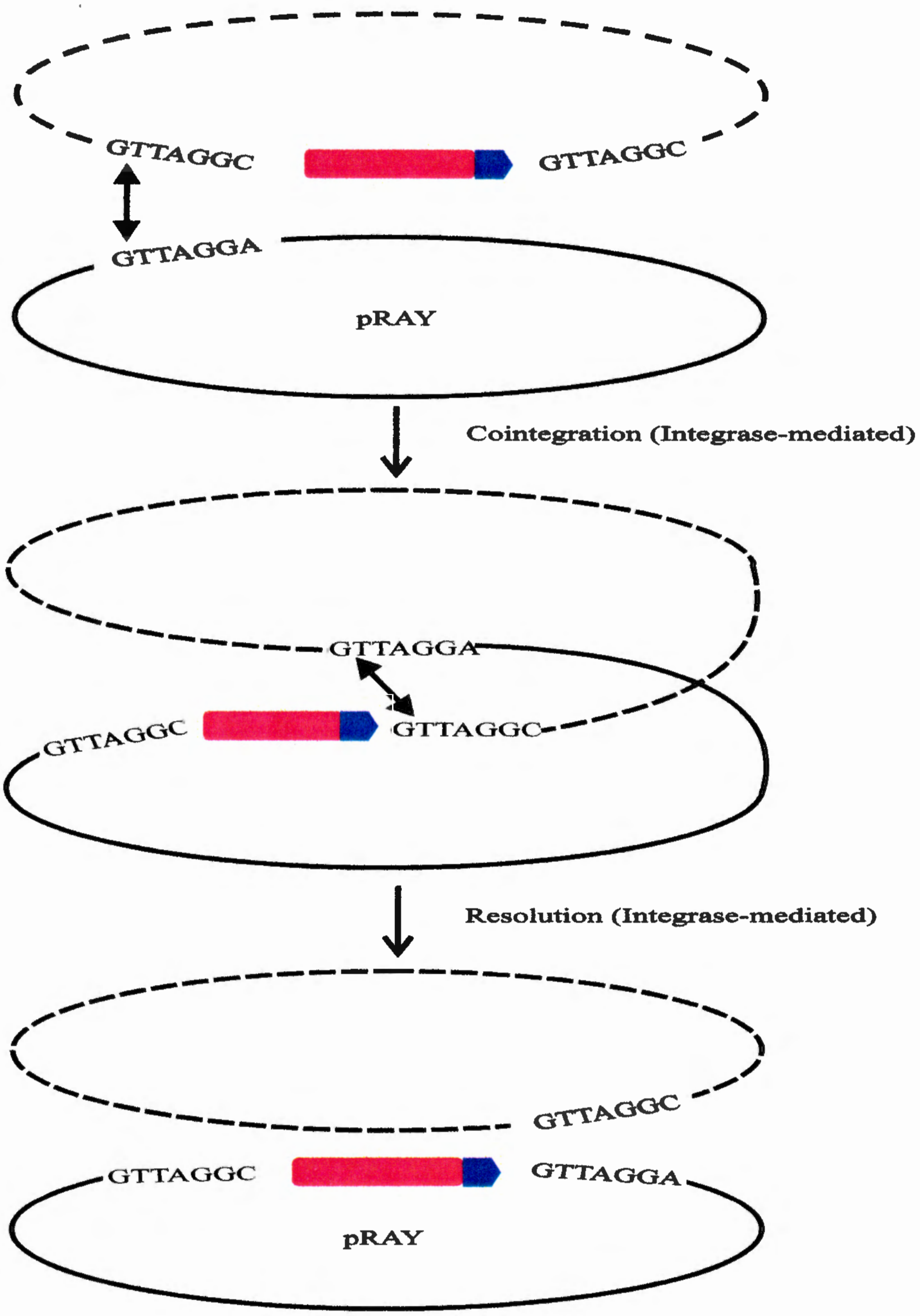


Figure 2.6 Acquisition of an *aadB* gene cassette by pRAY following integrase-mediated cointegrate formation. Integrase catalyzes the formation of a cointegrate between pRAY and an integron-associated *aadB* gene cassette on a second plasmid. The plasmid carrying the integron-associated *aadB* gene cassette, consisting of the *aadB* structural gene (🟥), *attI* site (GTTAGGC), 59-be (🟦), and 3' core site (GTTAGGC), is indicated by stippled lines. An integrase-mediated recombination event between *attI* (GTTAGGC) of the *aadB* gene cassette and the insertion site on pRAY (GTTAGGA) (indicated by double-headed arrow) would generate a cointegrate. Integrase-mediated resolution of the cointegrate (indicated by double-headed arrow), involving the regenerated *attI* site (GTTAGGA) and the 3' core site of the 59-be associated with *aadB* (GTTAGGC) would result in transfer of the *aadB* gene cassette to pRAY.

Hall and Collis (1995) suggest that of the two integrase-mediated recombination events, the one which proceeds via a cointegrate is the more common mechanism by which gene cassette transfer occurs. Regardless of which route was taken, the *aadB* gene cassette on pRAY would be flanked by an *attI* site (GTTAGGC) and a 59-be terminating in GTTAGGA, because the secondary site on pRAY is almost identical to the preferred site for integrase-recognition. The 3' core site (GTTAGGA) of the gene cassette on pRAY differs from the consensus (GTTPuPuPuPy) at only one position (underlined). Recombination assays used to monitor the effect of base changes in the core sequence on the recombination frequency showed that a transversion of the final base had no effect on the ability of the site to be recognized by integrase (Stokes *et al.*, 1997). Thus, the *aadB* gene cassette on pRAY may be flanked by two recombination sites and is therefore potentially mobile. The same is not true of the *aadB* cassette on pIE723. Following the insertion of the cassette on pIE723, the 59-be was disrupted and terminates in a composite 3′ core site, GATCAAA, which differs from the core site at one conserved and two consensus positions (underlined). This composite core site was not recombinationally active, resulting in the stable integration of the *aadB* cassette on pIE723 (Recchia and Hall, 1995b).

CHAPTER 3

IS THE *aadB* GENE CASSETTE ON pRAY MOBILE ?

3.1 INTRODUCTION

The data presented in the previous chapter strongly suggest that the *aadB* gene cassette on pRAY is potentially mobile. To test this hypothesis, IntI1-mediated gene cassette excision and cointegrate formation experiments were carried out. A number of studies have determined the frequency of integrase to recognize different sites, including 59-base elements (59-be), *attI* sites, and secondary sites, and in so doing, investigate the potential mobility of gene cassettes (Martinez and de la Cruz, 1988, 1990; Hall *et al.*, 1991; Collis and Hall, 1992a,b; Collis *et al.*, 1993; Francia *et al.*, 1993; Recchia *et al.*, 1994; Francia and García Lobo, 1996; Francia *et al.*, 1997; Hansson *et al.*, 1997).

3.1.1 Integrase-mediated cointegrate formation

3.1.1.1 Recombination between 59-be, and 59-be and attI

Gene cassettes comprise a 5' *attI* site, a structural gene (or an ORF), and a 3' 59-be (Fig. 2.4) (Hall *et al.*, 1991). The 59-be family of recombination sites (Recchia *et al.*, 1994) range in size from 57 to 141 bp (Recchia and Hall, 1995a) and contain a 3' core sequence (GTTRRRY) and an inverse core sequence (RYYYAAC) at its 5' end (Cameron *et al.*, 1986; Stokes and Hall, 1989; Recchia *et al.*, 1994; Hall and Collis, 1995). All 59-be contain an imperfect inverted repeat sequence, and mismatches in these repeat sequences are thought to favour integration of gene cassettes at the 3' core sequence rather than at the 5' inverse core sequence (Hall *et al.*, 1991; Collis *et al.*, 1993; Stokes *et al.*, 1997). Furthermore, these imperfect inverted repeat sequences play a role in the orientation of the cassettes, which are always inserted in the same direction, in integrons (Hall *et al.*, 1991; Collis and Hall, 1992a; Stokes *et al.*, 1997). The participation of 59-be in integrase-mediated recombination has been extensively studied (Martinez and de la Cruz, 1988, 1990; Hall *et al.*, 1991; Collis and Hall, 1992a,b; Collis *et al.*, 1993; Recchia *et al.*, 1994). Conduction assays carried out in these studies have shown that recombination occurs between the 3′ core site of two 59-be. In similar experiments, recombination, at a lower frequency, was also demonstrated between the 3' core site of a 59-be and an *attI* site (Collis and Hall, 1992a; Collis *et al.*, 1993).

3.1.1.2 Recombination between two attI sites

Initially it was thought that integrase-mediated recombination was dependant on the participation of 59-be in the reaction. Recently, Hansson *et al.* (1997) and Recchia (1997; cited in Stokes *et al.*, 1997) have shown that recombination occurs between two *attI* sites, albeit at a frequency 100 fold lower than that observed between *attI* and a 59-be. These results indicate that integrase-mediated cointegrate formation is not dependent on the presence of a 59-be.

3.1.1.3 Recombination involving secondary sites

To test whether gene cassettes can be recombined outside of an integron, namely at a secondary site, Francia *et al.* (1993) assayed for the conduction of a non-transferable plasmid, lacking 59-be and *attI* sites, by plasmid R388, which contains an integron and therefore a number of recombination sites (Martinez and de la Cruz, 1988, 1990; Recchia *et al.*, 1994). In these experiments, cointegrate formation involved the *orfA* 59-be on R388 and random sites on the plasmid. Analysis of the random sites revealed a consensus sequence Ga/tTa/ca/t, which was subsequently designated a secondary site (Francia *et al.*, 1993; Recchia *et al.*, 1994). Further analysis of sequence upstream of these simple sites identified an inverted pentanucleotide, the presence of which resulted in an increased efficiency of recognition of the secondary site by integrase (Francia *et al.*, 1997). The inverted pentanucleotide, located 3 - 7 bps upstream of the secondary site, together with the secondary site forms a double site that is recognized more efficiently than the single pentanucleotide comprising a secondary site. Furthermore, a stem-loop structure, involving the double site and a 100-bp sequence located upstream of the active secondary site, increases IntI1 activity at the double site located at the 3′ end of the stem-loop structure. Studies on recombination events involving secondary sites have shown that IntI1-mediated recombination can occur between these sites and the primary *attI* site (Hansson *et al.*, 1997).

3.1.2 Cassette excision by integrase

In addition to cointegrate formation, integrase catalyzes the mobility of gene cassettes via direct excision or insertion of cassettes. Free, circular cassettes (Collis and Hall, 1992a,b), can be inserted at either primary or secondary sites located on a plasmid or in the chromosome. Although deletion of a cassette may occur via formation and resolution of a cointegrate, IntI1-mediated direct excision is most often associated with deletion of gene cassettes (Collis and Hall, 1992a; Hall and Collis, 1995).

Unless otherwise indicated, the conduction and gene cassette excision experiments, to determine the potential mobility of the *aadB* gene cassette, were carried out by myself in Cape Town or in the Department of Molecular Biology, University Cantabria, Santander, Spain, under the supervision of Prof. J.M. García Lobo.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Plasmids and bacterial strains

The plasmids and strains used are listed in Table 3.1. *E. coli* DH5α was used as a host for plasmid construction, and in gene cassette excision experiments. *E. coli* strains UB5201 (F^- *pro met recA56 gyrA* Nal^R) and UB1637 (*his lys trp recA56 rpsL*) were used as recipient and donor strains, respectively, in mating experiments. Bacteria were selected on media containing the following antibiotics: Gm (5 μg/ml); Km (25 μg/ml); chloramphenicol (Cm) (25 μg/ml); ampicillin (Amp) (100 μg/ml); nalidixic acid (Nal) (20 μg/ml); trimethoprim (Tm) (20 μg/ml).

Table 3.1 Plasmids and bacterial strains used.

	Relevant features	Source/ Reference
Plasmids		
pSU2056	Amp^R; *intI1*	García Lobo/ Martinez and de la Cruz, 1990
pSU18	Cm^R	García Lobo/ Bartolomé *et al.*, 1991
pSU18R2	Cm^R; Tn*21*-59-be	García Lobo
pSU1817	Cm^R; Km^R	this study
R388	Tm^R; In3	García Lobo/ Avila and de la Cruz, 1988
Strains		
E. coli UB1637	*his lys trp recA56 rpsL*	García Lobo/ de la Cruz and Grinsted, 1982
E. coli UB5201	F^- *pro met recA56 gyrA* Nal^R	García Lobo/ de la Cruz and Grinsted, 1982

3.2.2 Cassette excision analysis

The 1.7 kb fragment containing the *aadB* gene cassette from pRAY was ligated to pSU18 (Cm^R) to generate pSU1817 (test plasmid) (Appendix C). IntI1-mediated excision of the *aadB* gene cassette from pSU1817 (Cm^R, Gm^R, Km^R) was analyzed by propagating the plasmid in the presence of integrase encoded by pSU2056 (Amp^R) (Martinez and de la Cruz, 1990). Plasmid pSU2056 carries the integrase gene downstream of a strong promoter, which results in the overexpression of integrase. Plasmid DNA was isolated from DH5α cells containing pSU2056 and pSU1817, and re-introduced into DH5α by transformation. Transformants were selected on media containing Cm. To screen for loss of the *aadB* gene cassette from pSU1817, transformants were subsequently replica plated onto LB media containing either Cm or Km. Km^S transformants were indicative of loss of the *aadB* gene cassette.

3.2.3 Conduction assays

To test whether the putative recombination sites associated with the *aadB* gene on pRAY could be recognized by integrase, conduction assays (Martinez and de la Cruz, 1988) were carried out. The conjugative plasmid R388 (Tp^R, Su^R) (Fig. 3.1) in UB1637 contains the integron In3 (Martinez and de la Cruz, 1988, 1990; Recchia *et al.*, 1994). Since In3 contains two inserted gene cassettes, *dhfrII* and *orfA*, it contains three potential target sites for site-specific recombination; namely, the *attI* site 5′ of *dhfrII,* its associated 59-be, and the 59-be contiguous with *orfA*. Plasmid pSU1817, carrying the *aadB* gene cassette from pRAY, and pSU2056 were introduced simultaneously into UB1637, containing plasmid R388. Linkage of the non-transferable test plasmid, pSU1817, to the conjugative R388 to form a cointegrate, and its subsequent transfer to the recipient, UB5201, was tested in conjugation experiments as follows.

Cells from an exponentially growing culture of UB1637 (R388, pSU2056, pSU1817) (0.5 ml) were mixed with stationary-phase UB5201 (Nal^R) (0.5 ml). Bacteria were harvested by centrifugation, resuspended in 50 µl of LB broth (Appendix A), and pipetted onto a 0.22 µm Millipore filter placed on LB agar (Appendix A). Conjugations were carried out at 37∘C for 3 - 4 hours; afterwhich, filters were washed with 1 ml physiological saline and 10-fold dilutions of the suspension were plated onto: (i) LB containing Cm and Nal and (ii) M9 minimal media (Appendix A) (Miller, 1972) containing Tm. Transconjugants containing R388 (Tm^R) were selected on M9 minimal media containing Tm. UB5201 (Nal^R) containing cointegrates consisting of pSU1817/R388 (Cm^R, Tm^R) were selected on LB agar containing Cm and Nal. The conduction frequency of the test plasmids in each assay was calculated as the ratio of Cm^R to Tm^R transconjugants.

Conjugation experiments between the donor strains UB1637 (R388, pSU2056, pSU18) and UB1637 (R388, pSU2056, pSU18R2) and the recipient UB5201 were included as controls. The conduction frequencies of pSU18 (Francia *et al.*, 1997), containing a secondary site, and pSU18R2, containing the 59-be from Tn*21* (Francia and García Lobo, unpublished), were used as reference values.

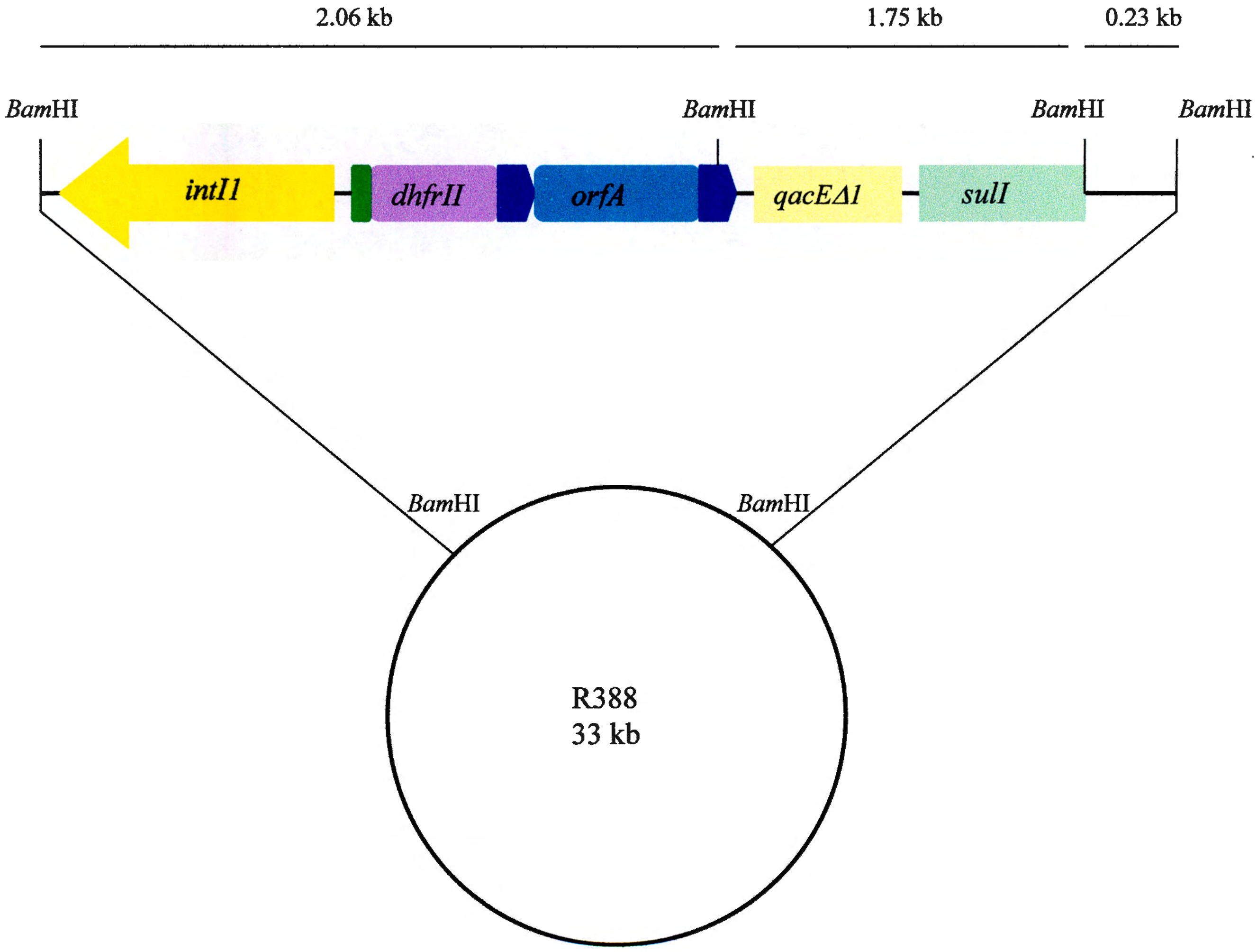


Figure 3.1 Map of plasmid R388 showing ~ 4.0 kb *Bam*HI fragment carrying In3. The integrase gene, *dhfrII* and *orfA* gene cassettes, *qacEΔ1*, and *sulI* genes are shown. The *attI* site (🟩) and the two 59-be (🟦) are potential target sites for IntI1-mediated cointegrate formation. *Bam*HI sites and sizes of *Bam*HI fragments (kb) (Avila and de la Cruz, 1988) are shown.

3.3 RESULTS

3.3.1 Cassette excision studies

Plasmid pSU1817, containing the *aadB* gene cassette from pRAY, was propagated in the presence of excess integrase to assay for excision of the cassette via a single cross-over event. Following the introduction of (pSU2056, pSU1817), transformants were selected on Cm, which selects for the vector marker, and screened for the loss of *aadB* by using Km. Three hundred (300) and, in a separate experiment, 700 Cm^R *E. coli* DH5α (pSU2056, pSU1817) transformants were replica plated onto media containing either Cm or Km. No Km^S transformants were isolated, indicating that either the *aadB* gene cassette could not be excised from pSU1817, or that excision occurred at a low frequency ($< 1.4 \times 10^{-3}$).

3.3.2 Analysis of cointegrate formation

To determine whether the putative recombination sites flanking the *aadB* gene cassette on pRAY could be recognized in an IntI1-mediated cointegrate formation event, conduction assays were carried out. The donor UB1637 strains, containing plasmids R388, pSU2056 and either the test plasmid, pSU1817, or one of the control plasmids (pSU18 or pSU18R2) were mated with the recipient strain UB5201. The frequencies of recombination, calculated as the ratio of Cm^R to Tm^R transconjugants are tabulated below (Table 3.2).

Table 3.2 Conduction frequencies of cointegrates.

Plasmid[a]	Conduction frequency[b] 1	2	3[c]	Average
pSU1817	5.8×10^{-2}	5.4×10^{-1}	[d]7.8×10^{-2}	2.3×10^{-1}
pSU18	1.3×10^{-7}	3.9×10^{-8}	1.3×10^{-6}	4.9×10^{-7}
pSU18R2	3.2×10^{-3}	1.7×10^{-2}	2.0×10^{-2}	1.3×10^{-2}

[a] The donor strain also contained plasmid R388 and pSU2056.
[b] Averages of duplicate platings were scored in each experiment.
[c] Carried out by V. Francia, Santander, Spain
[d] Average of four independent mating experiments

The averages from six individual assays, four of which were carried out by V. Francia (Santander, Spain), were used to determine the conduction frequencies. The frequencies of recombination between R388 and pSU18 (4.9 X 10^{-7}), and R388 and pSU18R2 (1.3 X 10^{-2}), were similar to those which had been observed previously (Francia and García Lobo, unpublished). Recombination between R388 and pSU1817 occurred at a frequency of 2.3 X 10^{-1}, which is comparable to frequencies of recombination between two 59-be (3.7 X 10^{-2}) (Collis and Hall, 1992a; Collis *et al.*, 1993).

To determine the sites involved in cointegrate formation, plasmid DNA was extracted from 24 transconjugants, digested with *Bam*HI, and analyzed by agarose gel electrophoresis. Plasmid R388 contains four *Bam*HI fragments of ~29 kb, 2.06 kb, 1.75 kb, and 0.23 kb (Avila and de la Cruz, 1988) (Fig. 3.1). The potential site-specific recombination sites of In3 on R388 are located in the 2.06 kb (*attI* and 59-be of *dhfrII*) and 1.75 kb (59-be of *orfA*) fragments. Recombination involving any of the sites in the 2.06 kb or 1.75 kb fragments would alter the size of these fragments. *Bam*HI digestion of plasmid DNA from 24 Cm^R transconjugants generated three profiles (Fig. 3.2). Seventy percent (17/24) of the cointegrates had a profile consistent with a recombination event involving *attI* of R388 and the 59-be of *aadB* on pSU1817 (Fig. 3.3a). The second profile (4/24) resulted from a similar recombination event between *attI* of R388 and the 59-be of pSU1817, and excision of the *orfA* gene cassette (Fig. 3.3b). A recombination event between R388 *attI* and the 59-be of pSU1817, combined with excision of *orfA* and the *dhfrII* cassettes, resulted in a third profile (Fig. 3.3c) in 3 out of the 24 cointegrates analyzed. Antibiotic susceptibility testing confirmed that the Tm resistance gene cassette had been excised.

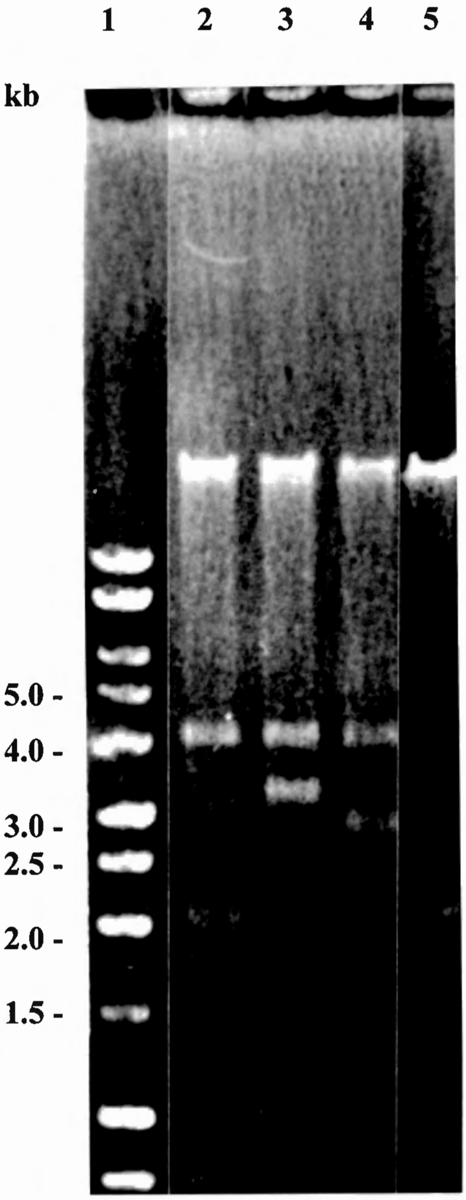


Figure 3.2 Restriction digestion analysis of cointegrates resulting from recombination between pSU1817 and plasmid R388. Plasmid DNA extracted from Cm^RNal^R transconjugants was digested with *Bam*HI and analyzed by electrophoresis through a 1.0 % agarose gel. All three profiles resulted from recombination involving the R388 *attI* site and the 59-be associated with the *aadB* gene on pSU1817. **Lane 1**: 1 kb Ladder (Appendix D); **Lane 2**: Restriction profile of cointegrate resulting from recombination between R388 *attI* and *aadB* 59-be; **Lane 3**: Restriction profile of cointegrate resulting from recombination between R388 *attI* and *aadB* 59-be and excision of *orfA* cassette; **Lane 4**: Restriction profile of cointegrate resulting from recombination between R388 *attI* and *aadB* 59-be and excision of both *orfA* and *dhfrII* cassettes; **Lane 5**: R388. Sizes of relevant fragments in 1 kb Ladder are indicated.

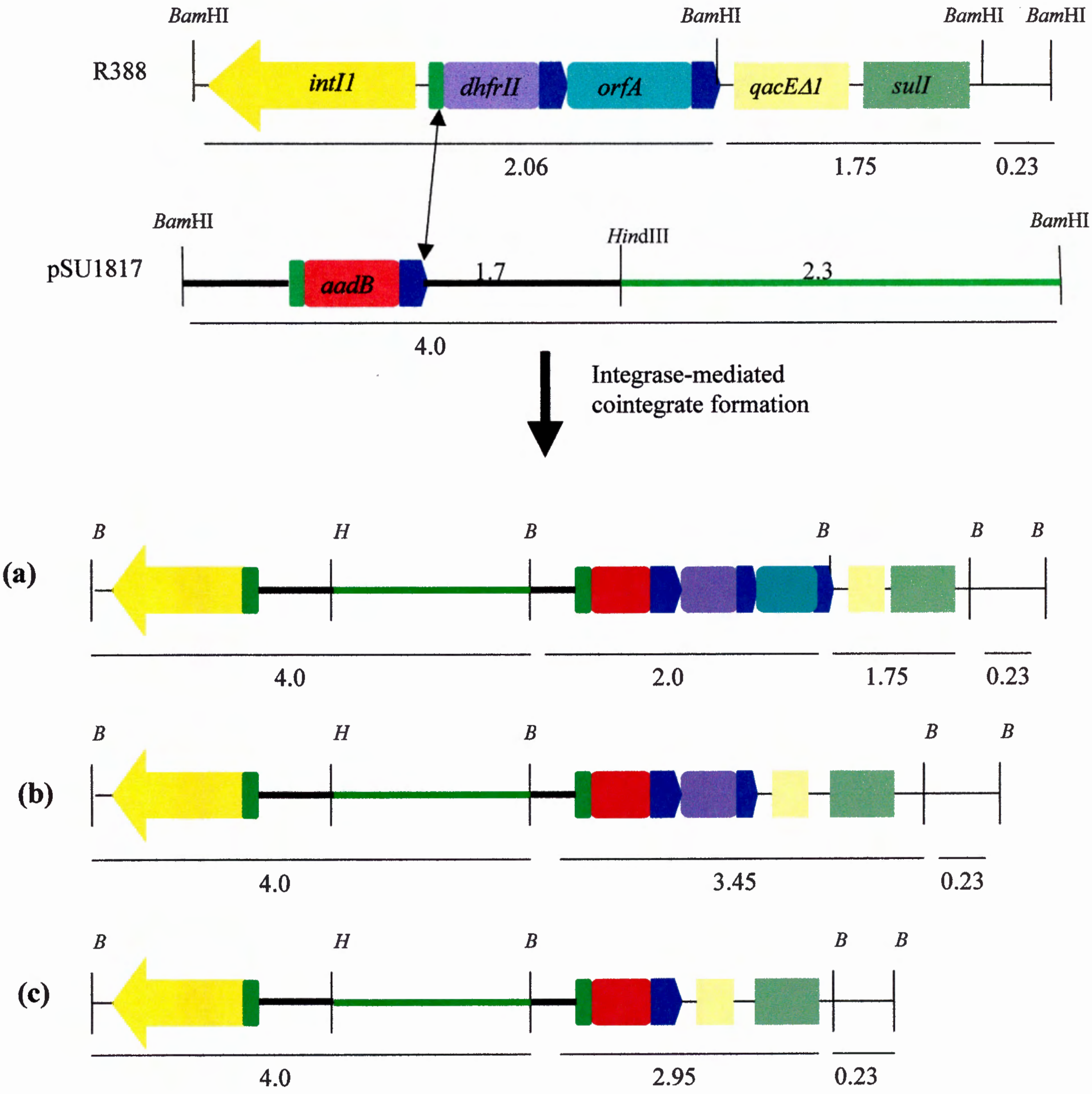


Figure 3.3 Cointegrate formation involving R388 *attI* site 🟩) and *aadB* 59-be 🟦) on pSU1817. Double-headed arrow indicates sites involved in formation of cointegrates. *Bam*HI (B) and *Hin*dIII (H) sites are shown. Sizes (in kb) of *Bam*HI-*Hin*dIII fragments of pSU1817 are shown. Sizes of *Bam*HI fragments are indicated below map of In3 following conduction of pSU1817 by R388. **(a)** recombination between R388 *attI* site and 3' core site of 59-be on pSU1817; **(b)** recombination between R388 *attI* site and 3' core site of 59-be on pSU1817 and excision of *orfA* gene cassette; **(c)** recombination between R388 *attI* site and 3' core site of 59-be on pSU1817 and excision of *orfA* and *dhfrII* gene cassettes.

3.4 DISCUSSION

To determine whether the sites flanking the *aadB* gene cassette on pRAY are recombinationally active, gene cassette excision and cointegrate formation experiments were done. No Km^S transformants were obtained when assaying for gene cassette excision, indicating that either the recombination sites flanking the cassette could not be recognized by integrase, or that recognition occurred at a frequency of $< 1.4 \times 10^{-3}$. Similarly, the *aadB* gene cassette integrated at a secondary site on pIE723 was excised at a low frequency of $< 7 \times 10^{-4}$, and the cassette was considered to be stably integrated (Recchia and Hall, 1995b).

It is noteworthy that different factors, including the position of a cassette in the variable region and the recombination sites involved, influence the frequency of excision of the cassette (Collis and Hall, 1992a; Collis *et al.*, 1993). An *aadA2* cassette in the second or third position in an array of cassettes was excised more frequently (3.7×10^{-2}) than when it was the first or only cassette in the variable region ($< 1 \times 10^{-3}$). This data indicates that excision involving an *attI* site and a 59-be occurs less frequently than excision involving two 59-be and may explain the lack of excision of the *aadB* gene cassette in this study.

The results of the conduction assays suggest that the *aadB* gene cassette is mobile. Cointegrates of R388 and pSU1817, involving the 59-be of the *aadB* cassette and the *attI* site on R388, formed at a frequency of 2.3×10^{-1}, which is similar to the frequency of recombination observed between two primary sites (3.7×10^{-2}) (Collis and Hall, 1992a; Collis *et al.*, 1993).

In similar experiments, the composite 59-be of the *aadB* from pIE723 was conducted at a frequency (2.3×10^{-6}), which was similar to the frequency observed for the vector plasmid pACYC184, suggesting that the composite 59-be is not recombinationally active and that the cassette is not mobile (Recchia and Hall, 1995b).

The possiblity that integrase could recognize the sites flanking the *aadB* gene cassette on pRAY has important implications. Firstly, additional cassettes could be inserted at either of the recombinationally active sites flanking the *aadB* gene, resulting in the acquisition of genes. Secondly, the *aadB* cassette may be transferred to a different location (integron or transferable plasmid), facilitating the dissemination of aminoglycoside resistance.

CHAPTER 4

"I'LL USE THAT PROMOTER, THANKS !"

4.1 INTRODUCTION

Gene cassettes not associated with integrons are dependent on their insertion downstream of suitable promoters to effect transcription of the structural genes. Four putative promoter sequences, CTGACA (N_{16}) TATGTT, TTGGAT (N_{14}) AATAGT, TTGGAT (N_{26}) TATTGG and TTGACA (N_{17}) CAGAAT, were identified upstream of the *aadB* gene cassette integrated at a secondary site on pRAY (Chapter 2). To identify the promoter(s) used in the regulation of the *aadB* gene from *Acinetobacter* strain SUN, the initiation site of the *aadB* transcript was determined by primer extension analysis.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Isolation of RNA

Total RNA was extracted from overnight cultures of *E. coli* (pHS100) and *A. calcoaceticus* (pRAY), using a FastRNA kit (Bio101 Inc., Vista, CA, USA), according to the manufacturer's instructions. Approximately 10^9 bacterial cells were lysed by shaking the sample with a mixture of sizes of silica and ceramic particles in a FastPrep FP120 instrument (Savant Instruments Inc., Farmingdale, NY, USA). Following lysis, the RNA was extracted with phenol, precipitated, and resuspended in diethyl pyrocarbonate (DEPC)-treated water before storing at -70 °C.

4.2.2 Primer extension analysis

The method used in the identification of the *aadB* gene transcription start site was adapted from that of Sambrook *et al.* (1989). One microgram of a 17-mer primer, 5' - CAATATCATCGTGCTTG - 3', which annealed to complimentary base pairs 767 - 783, 119 bases downstream of the ATG initiation codon of the *aadB* gene, was end-labelled with [γ-^{32}P]dATP (100 mCi) using 10 U polynucleotide kinase (Boehringer

Mannheim). Following labelling, the unincorporated nucleotides were removed from the reaction mix using a Promega G25 column, according to the manufacturer's instructions. Approximately 250 ng labelled primer was added to 100 µg total RNA from either *A. calcoaceticus* (pRAY) or *E. coli* (pHS100). RNA and labelled primer was precipitated, resuspended in 30 µl S1 nuclease hybridisation buffer (Appendix A), denatured at 85°C for 5 minutes, and the primer was annealed to the mRNA template overnight. In a pilot experiment the optimal annealing temperatures of the primer to the mRNA from *A. calcoaceticus* and *E. coli* were determined to be 52°C and 48°C, respectively. RNA and annealed primer was precipitated and resuspended in dH_2O containing 40 U RNase inhibitor. The primers were extended on the mRNA templates at 37°C for 60 minutes, using 500 U M-MLV Reverse Transcriptase (Promega, USA) and an equimolar dNTP mixture (1 mM). The cDNA products were precipitated and resuspended in Stop buffer (T7 Sequencing kit, Pharmacia). Following denaturation at 90°C for two minutes, the primer extension products were analyzed by electrophoresis through a 6 % polyacrylamide-urea gel. To map transcriptional start sites, the cDNA products were loaded adjacent to sequencing reactions performed on the corresponding DNA using the same primer as in primer extension reactions.

4.3 RESULTS and DISCUSSION

4.3.1 Identification of transcription start site of *aadB*

Using RNA from *A. calcoaceticus* (pRAY), the primer extension product was mapped to an A (Fig. 4.1) located 277 bps upstream of the *aadB* start codon (Fig. 4.2). This indicates that none of the putative promoters identified in Chapter 2 regulates the *aadB* gene in *Acinetobacter*. The hexamer, TATTCA, 7 bases upstream of the transcription start site, shows good homology to the consensus -10 sequence recognized by $E\sigma^{70}$. However, relative to this -10 hexamer, no -35 hexamer was identified. In this respect, the regulation of *aadB* is similar to that of *aacC2* from *A. baumannii* strain SAK (Elisha and Steyn, 1991c). As structural genes regulated by promoters lacking a -35 sequence may be positively regulated, it was suggested that cAMP/CAP might regulate

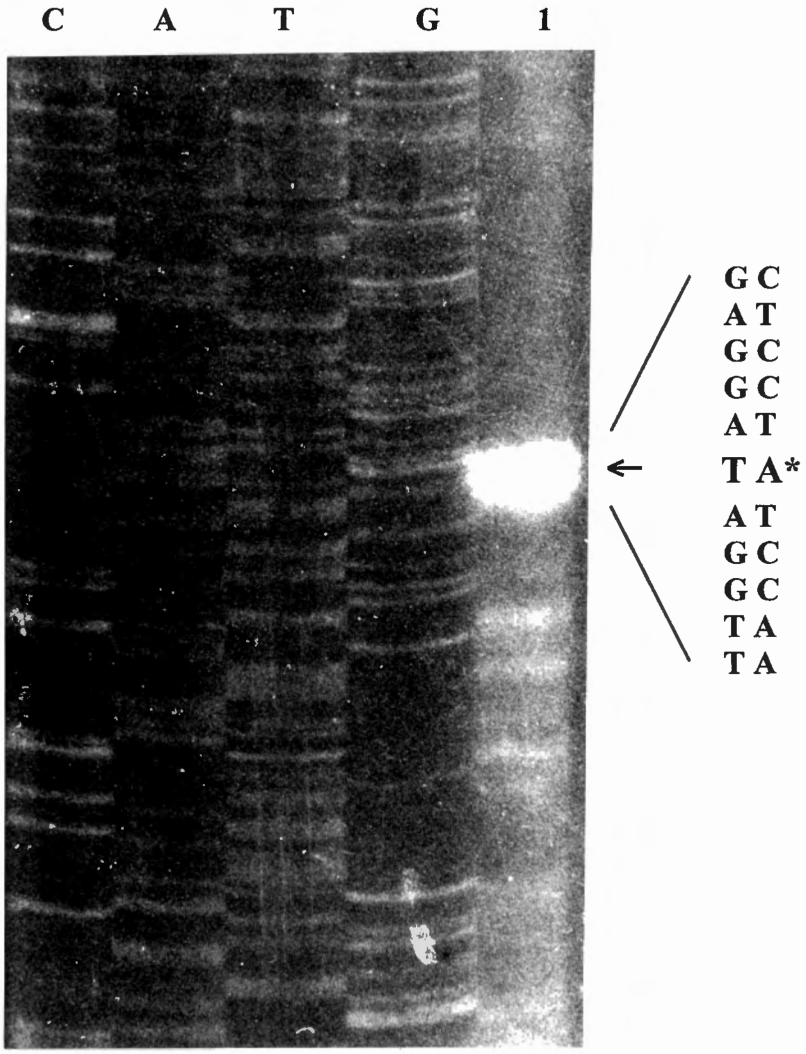
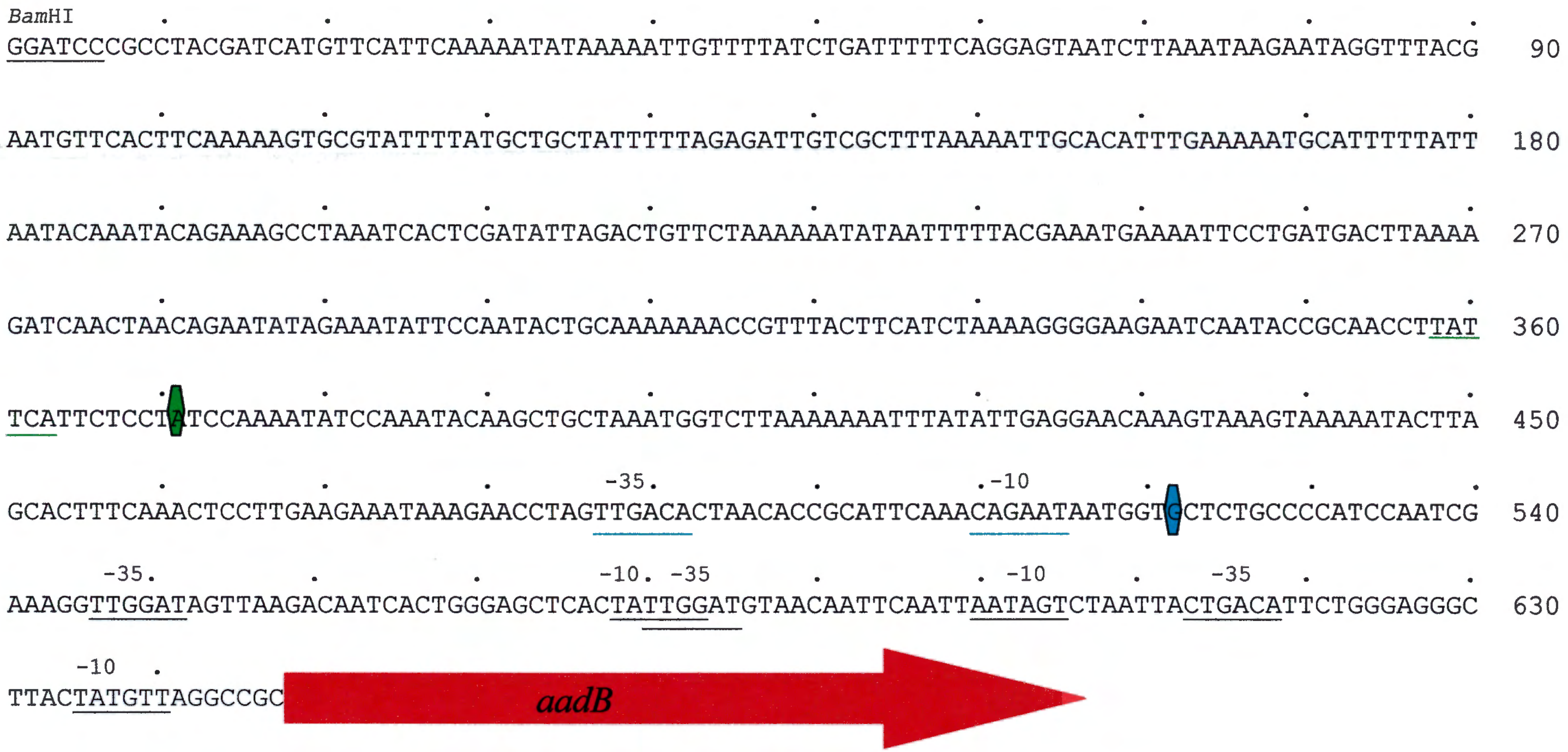


Figure 4.1 Primer extension analysis of the *aadB* transcript in *A. calcoaceticus*. The primer extension product is indicated by an arrow. Lanes CATG contain a dideoxy sequencing ladder obtained with the same primer used in the primer extension studies. Transcription start nucleotide is indicated by an asterisk. Sequence flanking the initiation site is shown.


```
BamHI    .         .         .         .         .         .         .         .         .
GGATCCCGCCTACGATCATGTTCATTCAAAAATATAAAAATTGTTTTATCTGATTTTTCAGGAGTAATCTTAAATAAGAATAGGTTTACG    90

         .         .         .         .         .         .         .         .         .
AATGTTCACTTCAAAAAGTGCGTATTTTATGCTGCTATTTTTAGAGATTGTCGCTTTAAAAATTGCACATTTGAAAAATGCATTTTTATT   180

         .         .         .         .         .         .         .         .         .
AATACAAATACAGAAAGCCTAAATCACTCGATATTAGACTGTTCTAAAAAATATAATTTTTACGAAATGAAAATTCCTGATGACTTAAAA   270

         .         .         .         .         .         .         .         .         .
GATCAACTAACAGAATATAGAAATATTCCAATACTGCAAAAAAACCGTTTACTTCATCTAAAAGGGGAAGAATCAATACCGCAACCTTAT   360

         .         .         .         .         .         .         .         .         .
TCATTCTCCTATCCAAAATATCCAAATACAAGCTGCTAAATGGTCTTAAAAAAATTTATATTGAGGAACAAAGTAAAGTAAAAATACTTA   450

         .         .         .      -35.         .         .-10      .         .         .
GCACTTTCAAACTCCTTGAAGAAATAAAGAACCTAGTTGACACTAACACCGCATTCAAACAGAATAATGGTGCTCTGCCCCATCCAATCG   540

      -35.         .         .      -10. -35     .         . -10     .    -35  .         .
AAAGGTTGGATAGTTAAGACAATCACTGGGAGCTCACTATTGGATGTAACAATTCAATTAATAGTCTAATTACTGACATTCTGGGAGGGC   630

    -10  .
TTACTATGTTAGGCCGC
```

Figure 4.2 Nucleotide sequence of regulatory region upstream of *aadB* gene (indicated by red arrow) from pRAY. The transcription initiation sites in *A. calcoaceticus* and *E. coli* are indicated by a green and blue hexagon, respectively. The -10 hexamer identified upstream of the *Acinetobacter* transcription initiation site is underlined in green and the -35 and -10 hexamers upstream of *E. coli* transcription initiation site are underlined in blue. The remaining three putative promoters identified in Chapter 2 are underlined in black. Dots above the sequence correspond to every 10 nucleotides.

the *aacC2* gene. A common feature of regulatory sequences that bind cAMP/CAP complexes is a conserved TGTGA at variable distances from the -10 hexamer (De Crombrugghe *et al.*, 1984). A sequence with good homology, AGTGA, was noted 12 bases upstream of the -10 region of the *aacC2* promoter from strain SAK (Elisha and Steyn, 1991c), suggesting that this gene might be regulated by cAMP/CAP. However, no sequence with homology to the conserved TGTGA sequence recognized by cAMP/CAP was identified upstream of the *aadB* gene from strain SUN.

Activation of a promoter is not necessarily dependent on auxillary proteins; DNA topology or curvature of a regulatory sequence can play a role in the regulation of a structural gene (Péréz-Martín *et al.*, 1994). In this context, it is interesting to note that the region upstream of the *aadB* gene from strain SUN contains a number of direct and inverted repeat sequences (Fig. 2.3) that may influence secondary and tertiary structure of the DNA sequence. Moreover, the oligo(dA-dT), which is more torsionally deformable than other sequences, is often found in regulatory regions and origins of replication (Cozzarelli and Wang, 1990). It is noteworthy that the region upstream of the -10 hexamer is AT-rich. In addition, tracts of A residues, and certain dinucleotides (AA, AG, CG, GA, or GC), also contribute to the curvature of the DNA. A number of these tracts of A residues and dinucleotides are present upstream of *aadB* (Fig. 4.2). Thus, the topology of the *aadB* regulatory region may influence the transcription of this gene.

In a similar experiment, using RNA from *E. coli* (pHS100), the primer extension product mapped to a G (Fig. 4.3) located 126 bps upstream of the start codon (Fig. 4.2). One of the putative promoters previously identified, TTGACA (N_{17}) CAGAAT, and typical of promoters recognized by $E\sigma^{70}$, was properly aligned with respect to the transcription start site. The -35 (TTGACA) hexamer shows perfect homology to the consensus sequence. The -10 sequence, $\underline{C}A\underline{G}AAT$, differs from the consensus (TATAAT) at two bases (underlined). Instead of a T at positions 1 and 3, the -10 hexamer consists of a C and G, respectively. Eight percent (%) of *E. coli* promoters contain a C at the first nucleotide, while 10 % have a G in the third position (Harley and Reynolds, 1987).

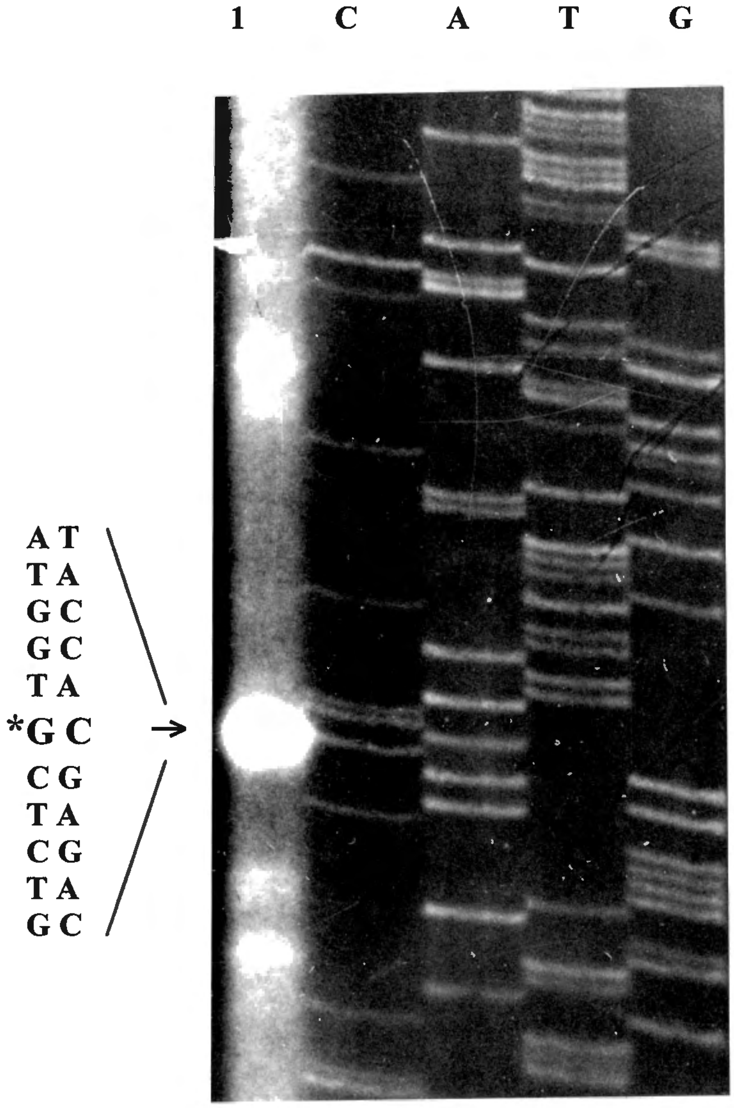


Figure 4.3 Primer extension analysis of the *aadB* transcript in *E. coli*. The primer extension product is indicated by an arrow. Lanes CATG contain a dideoxy sequencing ladder obtained with the same primer used in the primer extension studies. Transcription start nucleotide is indicated by an asterisk. Sequence flanking the initiation site is shown.

The above data suggest that transcription control signals recognized in *Acinetobacter* are different from those recognized in *E. coli.* Support for this suggestion comes from analyses of promoter sequences associated with other genes identified in *Acinetobacter.* Atypically spaced promoter sequences (16 - 23 bp) have been observed in the regulatory regions of esterase genes (Kok *et al.*, 1995) and genes involved in tryptophan biosynthesis in *A. calcoaceticus* (Haspel *et al.,* 1991). Moreover, as discussed in Chapter 1, a number of aminoglycoside resistance genes identified in *Acinetobacter* are regulated by promoters with atypical spacings. The *aac(6')-Ig, aac(6')-Ij*, and *aac(6')-Ik* genes, encoding enzymes with activity against amikacin, were identified in the chromosomes of, and considered intrinsic to, *A. haemolyticus* (Lambert *et al.*, 1993), *Acinetobacter* sp. 13 (Lambert *et al.*, 1994) and *Acinetobacter* sp. 6 (Rudant *et al.*, 1994), respectively. The hexamers of the putative promoters associated with these chromosomally-located genes are separated by 15 - 21 bps. Alternative σ factors, that recognize promoters consisting of atypically spaced hexamers have been described (Thomas and Franklin, 1989), and it may be that the major RNA polymerase in *Acinetobacter* is associated with an alternative σ factor which recognizes such promoters.

It has been suggested that either σ^{70} does not exist in *Acinetobacter,* or that the association between this σ factor and the core enzyme is weak (Kleppe and Kleppe, 1976). The identification of an aminoglycoside resistance gene regulated by a promoter typical of those recognized by σ^{70} (Lambert *et al.*, 1994) suggests that the counterpart of this σ factor is present in *Acinetobacter.* Nevertheless, promoter sequences recognized by σ^{70} may not be the preferred promoters in this organism.

CHAPTER 5

CHARACTERIZATION OF pRAY

5.1 INTRODUCTION

Many studies have highlighted the important role of plasmids in the transfer of genetic material between bacteria. These plasmids often carry metabolic, virulence, or resistance genes, which give the bacterial host a competitive advantage. Extrachromosomal DNA was first isolated from *Acinetobacter* in 1973 (Christiansen *et al.*), yet, despite the high carriage of plasmids by *Acinetobacter* (Gerner-Smidt; 1989), few plasmids from this genus have been characterized.

Most of the early studies on the maintenance of plasmids in *Acinetobacter*, involved well characterized enterobacterial R plasmids in *E. coli* K12 (Towner and Vivian, 1976; 1977). In one study, Towner and Vivian (1976) showed that the broad host range plasmid, RP4, from the incompatability (Inc) group P1, was capable of transfer from *E. coli* K12 to *A. calcoaceticus* EBF65/65. Moreover, RP4 was found to be relatively stable in the *Acinetobacter* host. This study was extended to include plasmids from other Inc groups, including C, F, I, W, and P2 (Towner and Vivian, 1977). However, only Inc P plasmids were capable of transfer to *Acinetobacter*, which led the authors to suggest that *Acinetobacter* was not included in the host range of the plasmids in the other Inc groups. The work of Chopade *et al.* (1985) offered an explanation of these findings. They showed that the recipient, EBF65/65, contained a cryptic plasmid, pAV2, that affected the fertility of plasmids tested in the previous studies. Experiments using a pAV2$^-$ recipient showed that plasmids from a range of Inc groups were capable of transfer from *E. coli* K12 to *A. calcoaceticus*. All of the plasmids transferred were partially unstable in *Acinetobacter*. Although some plasmids could be transferred to further strains of *A. calcoaceticus*, and even to *E. coli*, other plasmids required a mobilizing plasmid for re-transfer to occur.

There is a paucity of data on 'indigenous' or naturally occuring plasmids in *Acinetobacter*. In part, this has been due to the difficulties encountered in transferring plasmids from *Acinetobacter*. In studies conducted by Murray and Moellering (1979; 1980), transfer of Km^R from an *A. baumannii* isolate to *E. coli* and to *Acinetobacter* could not be demonstrated. Curing of a plasmid from an *Acinetobacter* isolate resulted in the loss of resistance markers, suggesting that the markers were plasmid-encoded.

However, as transfer could not be demonstrated, it could not be shown conclusively that the resistance markers were contained on plasmids.

One of the first reports of transfer of an R plasmid between *Acinetobacter* strains was that by Hinchliffe and Vivian (1980) who demonstrated transfer of plasmid pAV1, encoding sulphonamide resistance, from a clinical isolate of *A. calcoaceticus*, strain JC17, to a recipient *A. calcoaceticus*. Plasmid pAV1 could not be transferred to *E. coli*, *P. aeruginosa*, *Klebsiella*, or *Proteus mirabilis*. Strain JC17 contains an additional plasmid, pAV5, which was non-self-transmissible to *Acinetobacter* and *E. coli* (Divers *et al.*, 1984; 1985). However, pAV5 could be mobilized by pAV1 and transferred to an *Acinetobacter* recipient. Following mobilization, pAV5 segregated into two plasmids, pAV51 (Km^R, Neo^R) and pAV52 (Tet^R), which led the authors to speculate that pAV5 is a cointegrate plasmid formed by recombination between two independent replicons.

Similarly, plasmid pIP1858, from a clinical isolate of *A. baumannii*, could not be maintained in *E. coli* (Lambert *et al.*, 1994). However, the nonconjugative pIP1858, carrying an *aac(6')-Ih* gene, could be transferred, by transformation, to an *A. calcoaceticus* recipient, but not to *E. coli*.

An Inc 6-C plasmid, pIP1031, encoding resistance to penicillins, aminoglycoside-aminocyclitols, chloramphenicol, sulphonamides, and trimethoprim, was capable of transfer from a clinical isolate of *A. baumannii* to *E. coli* recipients (Goldstein *et al.*, 1983). Interestingly, pIP1031 was transferable, by conjugation, from only the original *A. baumannii* isolate to *E. coli*. An explanation for this finding was that pIP1031 was unstable in *A. baumannii* because it had been recently acquired by this organism. This plasmid was stable in *E. coli*.

Krcmery *et al.* (1985) have also reported a plasmid encoding Gm^R that was transferred from *Acinetobacter* to *E. coli*. Interestingly, this plasmid was also stable in *P. aeruginosa*. In the same study, amikacin resistance was transferred from a different strain of *Acinetobacter* to *P. aeruginosa*, but not to *E. coli*.

Towner (1991) suggests that as most *Acinetobacter* plasmids are relatively small, they probably lack genes encoding conjugative functions and are therefore not self-transferable. He also proposes that the origin of replication of a large population of *Acinetobacter* plasmids may not be recognized in *E. coli*, and though transferred, are possibly not maintained in the *E. coli* hosts.

That many of the plasmids from enterobacters were unstable in *Acinetobacter* (Chopade *et al.*, 1985) and that some of the naturally occuring plasmids in *Acinetobacter* could not be maintained in *E. coli*, supports Towner's (1996) suggestion that certain plasmid-mediated genetic information is confined to enterobacters, and a set of similar genetic information is confined to acinetobacters. There is, however, a separate group of plasmids capable of transfer from *Acinetobacter* to *Enterobacteriaceae*, and vice versa (Towner, 1996). The similarity of genes encoding enzymic resistance in acinetobacters to those identified in *Enterobacteriaceae* supports this notion.

Few studies have been devoted to characterizing naturally occuring plasmids in *Acinetobacter*. Therefore, pRAY was characterized by sequencing.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Cloning and sequencing of pRAY

The 1.9 kb *Hind*III and 1.2 kb *Bam*HI-*Hind*III fragments (Fig. 5.1) were ligated to appropriately digested pUC19 at 16°C for 30 minutes [2.2.5] to generate pHS400 and pHS300 (Appendix C), respectively. Recombinant plasmids were introduced into *E.coli* DH5α cells [2.2.6] and transformants were screened for the presence of pHS300 and pHS400. Due to difficulties encountered in cloning the remaining fragments, pRAY was linearized with *Bam*HI and ligated to *Bam*HI-digested pUC19, resulting in pHS500 (Appendix C). The inserts of pHS300 and pHS400 were sequenced [2.2.8.1]. Remaining sequencing data was obtained from pHS500. All sequencing data was confirmed on both strands.

5.3 RESULTS

5.3.1 Sequence analysis of pRAY

The nucleotide sequence (6076 bp) of pRAY is shown in Fig. 5.2. Several repeat sequences, ranging in size from 6 - 9 bp were identified (Fig. 2.3). An AT-rich sequence (72 %) starts at nucleotide 5890 and continues through to nucleotide 477 (Fig. 5.3). This sequence includes three DnaA boxes, corresponding to the consensus sequence TTATc/aCAc/aA (Fuller *et al.*, 1984), at nucleotides 357 - 365, 369 - 377, and 378 - 386 (Fig. 5.3). This sequence also contains 8 repeats defined by at least 7 nucleotides out of the 9-bp consensus sequence AAAAAATAT (Fig. 5.3) previously associated with origins of replication of *Acinetobacter* plasmids (Hunger *et al.*, 1990; Minas and Gutnick, 1993). An inverse of this repeat sequence (TTATTTTTT) was identified at nucleotides 5890 - 5898 (Fig. 5.3). Sequences with homology to an anchor sequence (CCGCAAGCG) described by Maas *et al.* (1997), are present at nucleotides 349 - 357, 2067 - 2075, 5543 - 5551, 5872 - 5880 (Fig. 5.3). Sequence with 45 % homology to the basis of -mobility (*bom*) region of pCN3 (Roessler *et al.*, 1985) (Fig. 5.4), a mobilizable ColE1-type plasmid, was identified at nucleotides 5013 - 5129 on pRAY (Fig. 5.2).

An AT-rich sequence (68.4 %) is also present between nucleotides 1341 - 2735 (Fig. 5.2). It contains 7 copies of DnaA boxes with at least 7 of the 9 consensus nucleotides, and 10 copies of repeat sequences with 7 out of 9 bases homologous to the AT-rich repeat sequence, AAAAAATAT (Fig. 5.2), associated with origins of replication in *Acinetobacter*.

There are 10 major ORFs ranging in size from 240 - 1095 bps contained within this sequence (Fig. 5.1 and 5.2). ORF 1 (648 - 1181) contains the *aadB* structural gene (Chapter 2). ORF 2 (5948 - 399) is contained within the putative ori. The translational product of this ORF shows 23 % similarity to the McbG protein (Fig. 5.5) involved in host immunity to a peptide antibiotic, microcin B17, encoded by *E. coli* plasmids (del Carmen Garrido *et al.*, 1988). ORF 2 is preceded by a ribosomal binding site (RBS)-like sequence, GGA, which is complimentary to the 3'-OH terminal sequence of *E. coli*

16S rRNA (5'-GAUCACCUCCUUA-3') (underlined) (Shine and Dalgarno, 1974). ORF 2 is also preceded by -35 and -10 hexamers (Table 5.1) with good homology to the consensus promoter sequences recognized by $E\sigma^{70}$ (Harley and Reynolds, 1987). Interestingly, the hexamers are separated by 26 bps.

Table 5.1 Promoter and RBS-like sequences preceding ORFs identified on pRAY.

ORF	Nucleotide	Size	Preceded by		Similarity to
		(bps)	-35 (N_x) –10[a]	RBS	(%)
1	648 - 1181	534	[b]TATTCA	GGAGG	AAD(2") (99.4)
2	5948 - 399	528	TTGATT (N_{26}) TATTCT	GGA	McbG (23)
3	4549 - 3932	618	TTGACA (N_{16}) TTTGTT	GGAG	MbeA (44)/CppC (47)
4	3966 - 3658	309	TTAAGG (N_{13}) TTACAT	GAG	
5	3830 - 2736	1095	TCGACA (N_{14}) TATCCA	GGAG	
6	3490 - 3224	267			CppB (45)
7	3220 - 2804	417			
8	2625 - 2083	543	TTGTAC (N_{22}) TTGAAT	AGG	
9	1888 - 1649	240			
10	1697 - 1398	300	TTGGCA (N_{12}) TTTATT	GGAGG	

[a] differences to the consensus sequences recognized by $E\sigma^{70}$ are underlined

[b] identified in Chapter 4

The remaining ORFs, ORFs 3 - 10, are transcribed in the opposite orientation (Fig. 5.1). ORF 4 (3966 - 3658), ORF 5 (3830 - 2736), ORF 7 (3220 - 2804), ORF 8 (2625 - 2083), ORF 9 (1888 - 1649), and ORF 10 (1697 - 1398) shared no nucleotide homology to DNA sequences in GenBank. Similarly, the translational products of these ORFs showed no similarity to any protein sequences in GenBank. Four out of these six ORFs (4, 5, 8, 10) are preceded by putative RBS-like sequences which show good homology to the RBS of *E. coli* (Shine and Dalgarno, 1974). In addition, each of these ORFs is preceded by hexamers with good homology to $E\sigma^{70}$ consensus hexamers. Interestingly, these hexamers are also seperated by 12 - 26 bps. No sequences with homology to RBS-like sequences and consensus promoter hexamers were identified in the region prior to ORF 7 and ORF 9.

The translational product of ORF 3 (4549-3932) has a 44 % similarity to the MbeA protein encoded by the ColE1 plasmid identified in *E. coli* (Boyd *et al.*, 1989). Alignment of ORF 3 and *mbeA* gene products (Fig. 5.6) showed that the product of ORF 3 shares a greater homology with the amino terminal of MbeA. The translational product of ORF 3 also has a 47 % similarity to the CppC protein (Fig. 5.7) encoded by pJD1 from *Neisseria gonorrhoeae* (Korch *et al.*, 1985). ORF 3 is preceded by a RBS-like sequence and promoter sequences typical of those recognized by $E\sigma^{70}$. The translational product of ORF 6 (3490-3224) has a 45 % similarity to a portion of the the CppB protein (Fig. 5.8) from pJD1 (Korch *et al.*, 1985). The CppB protein consists of 214 amino acids. Amino acids 26 - 73 of the ORF 6 putative protein (88 amino acids) has similarity to amino acids 94 - 141 of CppB. No transcription and translation initiation signals were identified upstream of this ORF.

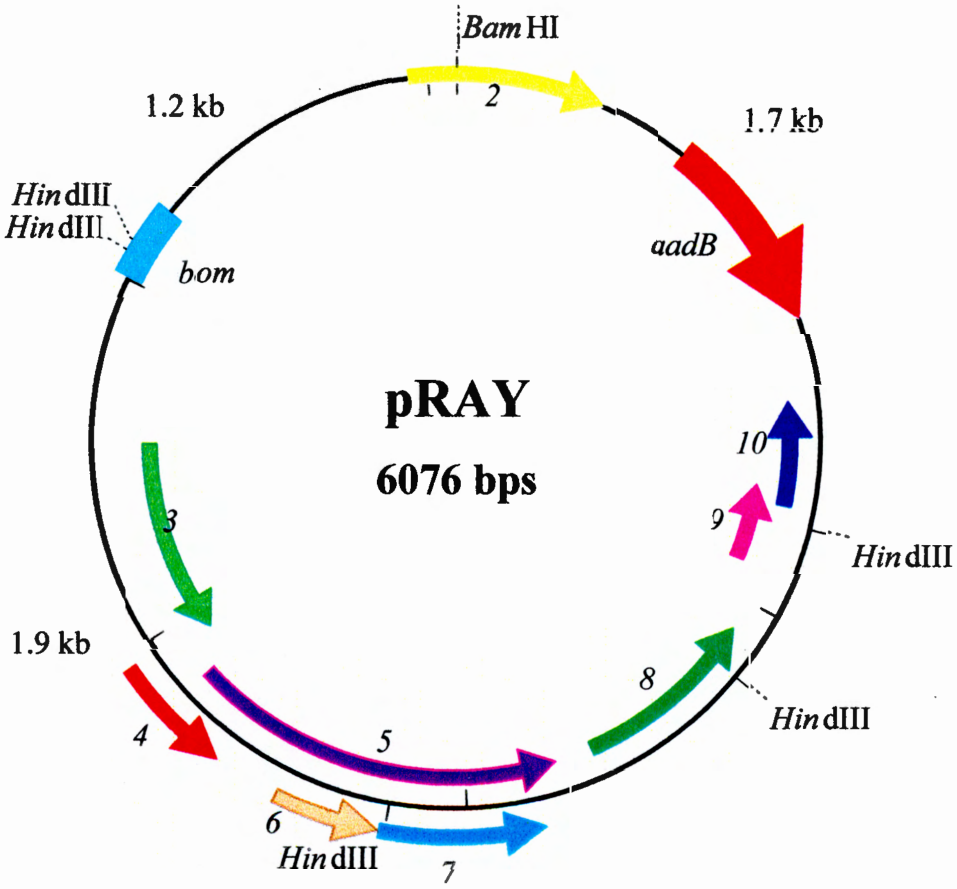


Figure 5.1 Circular map of pRAY. The ORFs identified on pRAYand their orientations are indicated by arrows. The 1.2 and 1.7 kb *Bam*HI-*Hin*dIII fragments, and the 1.9 kb *Hin*dIII fragment, are indicated.

```
GGATCCCGCCTACGATCATGTTCATTCAAAAATATAAAAATTGTTTTATCTGATTTTTCAGGAGTAATCTTAAATAAGAATAGGTTTACG     90

AATGTTCACTTCAAAAAGTGCGTATTTTATGCTGCTATTTTTAGAGATTGTCGCTTTAAAAATTGCACATTTGAAAAATGCATTTTTATT    180

AATACAAATACAGAAAGCCTAAATCACTCGATATTAGACTGTTCTAAAAAATATAATTTTTACGAAATGAAAATTCCTGATGACTTAAAA    270

GATCAACTAACAGAATATAGAAATATTCCAATACTGCAAAAAAACCGTTTACTTCATCTAAAAGGGGAAGAATCAATACCGCAACCTTAT    360

TCATTCTCCTATCCAAAATATCCAAATACAAGCTGCTAAATGGTCTTAAAAAAATTTATATTGAGGAACAAAGTAAAGTAAAAATACTTA    450

GCACTTTCAAACTCCTTGAAGAAATAAAGAACCTAGTTGACACTAACACCGCATTCAAACAGAATAATGGTGCTCTGCCCCATCCAATCG    540

AAAGGTTGGATAGTTAAGACAATCACTGGGAGCTCACTATTGGATGTAACAATTCAATTAATAGTCTAATTACTGACATTCTGGGAGGGC    630

TTACTATGTTAGGCCGCATGGACACAACGCAGGTCGCATTGATACACCAAATTCTAGCTGCGGCAGATGAGCGAAATCTGCCGCTCTGGA    720
     M  L  G  R  M  D  T  T  Q  V  A  L  I  H  Q  I  L  A  A  A  D  E  R  N  L  P  L  W  I

TCGGTGGGGGCTGGGCGATCGATGCACGGCTAGGGCGTGTAACACGCAAGCACGATGATATTGATCTGACTTTTCCCGGCGAGAGGCGCG    810
  G  G  G  W  A  I  D  A  R  L  G  R  V  T  R  K  H  D  D  I  D  L  T  F  P  G  E  R  R  G

GCGAGCTCGAGGCAATAGTTGAAATGCTCGGCGGGCGCGTCACGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCGGGGATGAGTTAC    900
 E  L  E  A  I  V  E  M  L  G  G  R  V  T  E  E  L  D  Y  G  F  L  A  E  I  G  D  E  L  L
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TTGACTGCGAACCTGCTTGGTGGGCAGACGAAGCGTATGAAATCGCGGAGGCTCCGCAGGGCTCGTGCCCAGAGGCGGCTGAGGGTGTCA   990
  D  C  E  P  A  W  W  A  D  E  A  Y  E  I  A  E  A  P  Q  G  S  C  P  E  A  A  E  G  V  I

TCGCCGGGCGGCCAGTCCGTTGTAACAGCTGGGAGGCGATCATCTGGGATTACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGC  1080
  A  G  R  P  V  R  C  N  S  W  E  A  I  I  W  D  Y  F  Y  Y  A  D  E  V  P  P  V  D  W  P

CTACAAAGCACATAGAGTCCTACAGGTTCGCATGCACCTCACTCGGGGCGGAAAAGGTTGAGGTCTTGCGTGCCGCTTTCAGGTCGCGAT  1170
  T  K  H  I  E  S  Y  R  F  A  C  T  S  L  G  A  E  K  V  E  V  L  R  A  A  F  R  S  R  Y

ATGCGGCCTAACAATTCGTCCAAGCCGACGCCGCTTTGCGGCGCGGCTTAACTCAGGTGTTAGGAAACACTGCTACCGTTATCGGATTTA  1260
  A  A  *

CTGCTTTACTACTAACCTTGTTAATTCAACATTTGCAAAATAGTCTGACCTTTCGATTGTTTGGGGTGAAATTCTTATCTTAAAAATCTA  1350

AATATTCTTTTAGATCTAATGCAGATTGCTCGATAAAATCGTATGTATTAATACAACGACAGTAAATAAATTTTGTTAAAAACCTCTAAA  1440

TCATTTTCTAGATTACGGTTAAAAATAATCGGTTCATGATGAGCAATCCTGTTTCTTAATTCACGTAATTGATCTGTCCAATCGTAAAAA  1530

ATTTGCCTGTTTTCAACTGTTGCATTAGGAAAAATAGAATTAAAACATCCCTGCCAAATACGTTCTTCATAATTAGCCATAAGCATCTTT  1620
                                                                         ORF 10 ←
TGCCAGAATACAAATTTAAGCTCAGGGATCATTTTACTAGGCGAATCTGGGAACTTCTCTTTAGCACTATCAAACATACTTTTAGGGCTA  1710

TACCTCCCTCTACGGCTAAGGCTTCGAATAAAGCTTCATTTTCATGCCAACTATTTGTTTCATTACACCTAAAAGATATTTCTATTGCCT  1800
```

ORF 9

GAACAACAGCATTTCTCAGAACAACTTCAAAATAATTTAATAAGTAAAAAAACTTATTGGAAATTATTAAATTACGTTCATAAAGCATCA 1890

ATACATCAGATTTTTCATATGGTTCACCTTTATCTTTATAGAAATTTTCATACTTAGATAAGCGTGGTATAGATAAAGCCTGCTCTATCT 1980

TAAGCACTGCATCATTTGACAATTCCACCGTATATACCCTAATATATAGTTGTAAGTTTGAGAGACTGCGGATCTAAGATCTTCCCTCCC 2070

AAGCGCTAAGATTCAAAAAAGCCTTTCATTAACGTGAAAGGCTTTTTTCTTTTTTTGGTCAATTAAAAGCTTACATATTGATGTAATTGA 2160

AATATTTTCTTGAGGGACATCAAAATAATTAGCTAATAGTCTCTTAGCTTCAGGAATAGATATTTTATCCAGTGTTTTAGAAGAATTATC 2250

TTCATTTAATTTGGTAAATTTCAGCTCATTCAAATCGATATTCATCTCTTCTAATGAACTATAAGAAACTGGATTCCGATTACCATCCCA 2340

CATATTGGGAATAGATATATCAGCATATTCGGAGATATTTATTATCCATCTACCATCTGCATCTTCTTCTTGAGATTGGCTGATACTAGA 2430

TATTTTGCCAACTAAAAATGCAGTGTGATGATCAGCCTCTGGTTCTCCCCAAGATTGTTTCCTATTTTGCACACAAACTAGATAATCGCA 2520

CTTTCCTGCATTATGAGCATTTAGTCTCCAAGACTGACTACCACCTTCTTTTAAAATTCTAGTCTTTCCACGAGCAGTTAGTACTACTAC 2610

ORF 8

ACAGTTATTATTCATAACACCACCAAAATATATAAACAAGCCTATACTACATTCAATTATAGCTAAATTTAACATTAGTACAAGCTAAT 2700

ACTAAGAAATAAACACCAACATTACTAGAAAATAATTACATCCCAAAATCCATACCTCGATCTTGCTTAGGTCGAGGTTTTTCTATCTCT 2790

TGTTTACGCTCATTCATCTGGACGAGATTTTTTTCAATATCTCGGATGTTTCGAGCAAATTCTGCTGCGCTTTGGCTACTTCTGACGAGA 2880

CAGAATGCTGTGTGGTTGAGATTCTCTCCAGAATTTCTTCGTTGTTGGCTGTGATAAGGCTCATCTCTCGGACTTTGCCATTGATCTGCT 2970

CGGTATAGCTTGCTGTACTTCGTTCTAGCTCGTGAAATTGCTTCTGCATTGTCGCTAATGATTTCTGTGCTTGCTGTAAGCTTTGCTGAT 3060

CGCTCTTGATTGTCTTTGATTGTGCTGACAAGGCTTGGATAATTTTGGTTTGCATTGTGTCTAAAGCTTGCTGTTGCTTCTGCAATTGCA 3150

ORF 7 ←

CTTGTTGTTGCATTAATTGTTGCTGTTGTTCTTCGATGATTCTCAATAATTGTGTTTCTAATTCGGTCATCGTTTAATACCCCATTGGAA 3240

TCATGTAGCCGTTCTGCCATTCGCCTGCGTTCTTGTTGATCTGAATACATAGGCGCTGTGCTTTGCTGTCTGGTCGCTTGCATGTGCTGA 3330

TCGTCAAACTGCCTCCCGATCGGTTCAAATGGTCGACCCTCATTTGCTTCTCGTTGATCACGGTATTTAGCGTCTGCATCTCGCTGTACT 3420

ORF 6 ←

TGTTTTTGGTGACGAGACCAAGCGAGAAAATTGCTATAAGCAAAATAACTGCTACTGAAATCAGTCCCATATTCAAAGTGGTAAGTTTTT 3510

TCTGTACCGCTTGATTCTGGGCTTGTTGTTCTTGGCTCTGTATTTGCTCGTTTAATTGCGTTAAATGGCTCAATTTCGATTGCAGCAAGG 3600

TTTGATACAGGCTTAACTGCTTCTCGACCGTCTGGGCGTTGTTTTGAATGCTCTGGGCTATTTTCTCGTCGACTGTTGGATAGCGGTCGC 3690

AGTGATATTGGCTCTAGCAAGCTGTCTCTGATAGTCTGTGACGTTTGTTCGATACCGCTCAGAAGTTGTTCCTCTGTATTCTTCAATTCG 3780

ORF 5

TTGTTGTACTTCTTGTCTATATTCGCCAGTAGCTCTAAAGCCTTGCTCATAAATCTCGCCCTCTAAACGGATGGGACGTTTTGCGCCCTC 3870

ATATGGGTTTTCAATGGATATGGAGTTTTTTACTTGTCGAGTGACATTGATTTGATTCGATTCAAGCCATTGCACCAATTCTGCGATCGG 3960

ORF 4

CAACATTCACCACACCATTGGCAACCGCTCGGTAAACACGTTGATGTAACTGCGCTTTAAAATCCTTAATATCTTTGGGTAGGTTCTTTT 4050

TATTTAAAAATAGCTGTCGATGCTCAGGATCATCGGGGTCATACAGTGAATGTTCGGTATTAGTGATCTGCTTAAATAGATCGACTCGAT 4140

CTAAATCGACTGGCGCATAGAATGGCTGTAGGCGTTTGCCTGTTGAAAGCTCAACATTAGGAATCACAAAATTAAGCTCAAGCCTTGTCT 4230

CACCTGTGTCCTGATTAATTTTGTCTTGGTGCTGTACCCATAAAATTTGATATTGGTCTTTATCTAGTCCCGGGAATAAACATTCCTCAA 4320

AATTTTGCATGATTTTCTGTTTATCCTGGTCAGATAAATCATGCTCATAAAAGGACAAACAACCACTGGTATATTTTTTGGCAAAAGGAC 4410

TGCTATCAATGAGTTGTGCTGTCAGTTCTACATCACCATTGAGCACTTGAGCATGTTCACGCTCTCGATCTTCACCTAACAAATAATCGA 4500

ORF 3 ←

GACAGCCTTTTGATAAACCTGAACCATGTCTAAAGAAATCAACGATCATCTGGACGTAACTCCCGAAGCTGCTGATCTATGGAAATCAAC 4590

AAATGAAGTAACTTCACTTTGTCAAAAGTACCTACCCCTGCAATATCGGTATTCACTGCTTTAGCAATCTGATTCACATTGTTGCCAATC 4680

CGACCAAGCTCTAAAATTAATGCTCGCTCTGTCTTGCTATACGAAGATTCAACAACCTTTGTTGCTTTAATTTTTTGGTCATCTTCTGCT 4770

TTGGAATTATGAATTTTAGTCGCTTTCTCTACTGCTGCTTGTCTAAGCAACTTTGCAATCGAACCATAAGGATTATTGGAATCGAGCAAT 4860

TGATATTCTGATTCTGAAAGTCTGACTTTAAAGGTTCGTTCTCGAACTGCTTTGCTCGTTTGTTTTCTTGTTCCTGTCCTGACATTTCAA 4950

TCTACCCTGCTTTGGCTTTTGTAAGCTTTAAATTGGAAAATCTTTGATTTTTCTGTTTAAAGCTTTTCGGGGGTATCGGGGGTGAAGCCT 5040

GATCAAGGTCACAGACTATCACTGTTTAACGAAGTTAAATAGGGGTAGCTGTGTATCCTTGCTTATTTAAAAATGGACGAGTGAAATTCA 5130

ATAAAAACTAATCAAATGACACGGTATATAAACTCTAGTAGGTATTGGCATGAATCTTGTAAAAACAAATGCGCTCAACTCTTTCTCACC 5220

GTCCCCACATCCCTCGCTTTGCTCGGGCTGATGGTGAACGTTGAGAGAGTTTCGGCAATACTTTCCCTAAGAGGCTTTGATTTAAAAAAA 5310

TCAAATATCCTCTTAAGTATTGCCGATTAACCTGTAATTGGGCTGACGGAGCCACGCATCGGTCGTATCCGCACATGGTTTTGCTGACCA 5400

```
TGCAGACAAGGACTAAAGGTTTTGCACCTTCTCCCACGCGCTCCCTGTCGAAGTATGGTGGTATCTCTGCGAGGATTACTCCAACATGCA   5490

ACTTAACCAACCTATACAACTACTTCCAACGCCTCCGCCTATGTGAGATCCCCTGTAAGCGGTTTAGCCTTCGAGCTATCGCTACGATGC   5580

ACCAGTGACACTTATTCCCTCCACATGCAACGCATATCTTCCTTAAAAAAGGCGCACAGAAGTAGATAAAGCTCGACCGTTAGGTGCTTA   5670

TTTGATGATTTAAACTGCTGAATTAACGAAGGACTTCAAATTGAGGCTTGTCAAACTGTTCTAATTCTTTAAAATAGATAGTGGTACTAG   5760

CCTTTAAGGTGAATATCAGTATGAGTTGCAAGCTCTGCCGTATCTTTCGTTAATTGATGTTGCAAGCATCAAATAACTGTACCCATCGAA   5850

TTTAAAACTAATGGACAGATTTCGCAAGAGACCTGTCCATTATTTTTTTTGATTACACCTAAGAATTAAGCTGAAACTCCTATTCTTTGG   5940
              ORF 2
ACTATTAATGTCAAAACTTCCTAGATTTCATAACAAGCACATCGAAAAACGTAAGTATTCAAATAGCCTCCTAGCCAAATCTATTTCTTC   6030

TAAAGTACGTTTCTCTAATAAAACATTTTTTAATGTTAATTTGAGA                                                6076
```

Figure 5.2 Nucleotide sequence of 6076-bp pRAY. Dots above the sequence correspond to every 10 nucleotides. The AT-rich region, shown in Fig. 5.3, is boxed in red. The direction of transcription of the ORFs identified on pRAY are indicated by an arrow at the start codon of each ORF. Repeat sequences and DnaA boxes present outside of the AT-rich region shown in Fig. 5.3 are highlighted in green and turquoise, respectively. Sequences with homology to the anchor sequence are highlighted in yellow. The region on pRAY with homology to the *bom* region on pCN3 is underlined in purple. The deduced amino acid sequence of the *aadB* gene is shown below the DNA sequence. The -35 and -10 hexamers upstream of the ORFs, excluding *aadB*, are underlined in blue and the RBS-like sequences are underlined in red.

```
TTATTTTTTTTGATTACACCTAAGAATTAAGCTGAAACTCCTATTCTTTGGACTATTAATGTCAAAACTTCCTAGATTTCATAACAAGCA   5979

CATCGAAAAACGTAAGTATTCAAATAGCCTCCTAGCCAAATCTATTTCTTCTAAAGTACGTTTCTCTAATAAAACATTTTTTAATGTTAA   6069

TTTGAGAGGATCCCGCCTACGATCATGTTCATTCAAAAATATAAAAATTGTTTTATCTGATTTTTCAGGAGTAATCTTAAATAAGAATAG     83
        BamHI
GTTTACGAATGTTCACTTCAAAAAGTGCGTATTTTATGCTGCTATTTTTAGAGATTGTCGCTTTAAAAATTGCACATTTGAAAAATGCAT    173

TTTTATTAATACAAATACAGAAAGCCTAAATCACTCGATATTAGACTGTTCTAAAAAATATAATTTTTACGAAATGAAAATTCCTGATGA    263

CTTAAAAGATCAACTAACAGAATATAGAAATATTCCAATACTGCAAAAAAACCGTTTACTTCATCTAAAAGGGGAAGAATCAATACCGCA    353

ACCTTATTCATTCTCCTATCCAAAATATCCAAATACAAGCTGCTAAATGGTCTTAAAAAAATTTATATTGAGGAACAAAGTAAAGTAAAA    443

ATACTTAGCACTTTCAAACTCCTTGAAGAAATAA                                                             477
```

Figure 5.3 AT-rich region (72 %) on pRAY from nucleotides 5890 to 477. This region spans the *Bam*HI site (underlined), designated nucleotide position 1. The AT-rich repeat sequences and the inverse of the repeat sequence are highlighted in green and the DnaA boxes, in turquoise. The anchor sequence is boxed in yellow. ORF 2 is contained within the boxed area (5948 - 399). The -35 and -10 hexamers identified upstream of ORF 2 are underlined in blue, and those identified upstream of the anchor sequence are underlined in purple. Dots above the sequence correspond to every 10 nucleotides.

```
pRAY          CTTTTCGGGGGTATCGGGGGTGAAGCCTGATCAAGGTCAC-AGACTATCACTGTTTAACGAAGTTAAATAGGGGTAGC  5089
               * ** * **** ****** * ****    *   *****    * **  * *  *    *   *   *       **
(pCN3) bom    GTGTTAGCGGGTGTCGGGG-CGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGT--ATACTGGCTTAACCATGC    75

pRAY          TGTGTATCCTTGCTTATTTAAAAATGGACGAGTGAAATTC                                          5129
               *   ***  ***  ***  *  *     * * *  ** *
(pCN3) bom    GGC--ATCAGTGCGGATTGTATGAAAA–GTGCGCCATACCGG                                         114
```

Figure 5.4 Alignment of putative *bom* region (5018-5129) on pRAY and *bom* region of pCN3 (Roessler *et al.*, 1985), a plasmid with a *bom* region homologous to that of ColE1-type plasmids. *, identical nucleotides. Dashes in the sequence indicate gaps introduced to optimize alignments.

```
ORF 2 MSKLPRFHNKHIEKRKYSNSLLAKSISSKVRFSNKTFFNVNLRGSRLRSCSFKNIKIVLSDFSGVILNKNRFTNVHFKKCVFYA   84
               : : ::  : : :     : : . :  :  ..:     : : :   . :      . ::  .     :: :.  .
McbG      MDIIEKRITKRHLSESEL-----SGVNYYNCIFERIQLDNFNFRDCEFEKCRFVNCSIKNLKLNFFKLIDCEFKDCLLQG   75

ORF 2 AIFRDCRFKNCTFE--KC--IFINTNTESLNHSI-LDCSKKYNFYE---MKIPDDLKDQL--TEYRNIPILQKNRLLHLKGEES  158
          :  :  :::    :   :.   .  :. :: : :  .   .:    .  :        ::.:    :         : .
McbG  VNAADIMFP-CTFSLVNCDLRFVDFISLRLQKSIFLSCRFRDCLFEETDLRKSDFTGSEFNNTEFRHSD-LSHCDFSMTEGLDI  157

ORF 2 IPQPYSFSYPKYPNTSC                                                                    175
       :        : :.
McbG  NPEINRILSIKIPQEAGLKILKRMGVVVGG                                                       187
```

Figure 5.5 Alignment of deduced amino acid sequence of ORF 3 and McbG encoded by plasmids identified in *E. coli* (del Carmen Garrido *et al.*, 1988). Colon (:), identical amino acids; dot (.), similar amino acids. Dashes in the sequence indicate gaps introduced to optimize alignments.

```
ORF 3   MIVDFFRHGSGLSKGCLDYLLGEDREREHAQVLNGDVELTAQLIDSSPFAKKYTSGCLSFYEHDLSDQDKQKIMQNFEECLFP    83
        ::: :   : :   : .::::: .: :: : ::.:  :    ::: .::::::::: ::: : .:    . :.:  ::  : :
MbeA    MIVKFHARGKGGGSGPVDYLLGRERNREGATVLQGNPEEVRELIDATPFAKKYTSGVLSFAEKELPPGGREKVMASFERVLMP    83

ORF 3   GLDKDQYQILWVQHQDKINQDTGETRLELNFVIPNVELSTGKRLQPFYAPVDLDRVDLFKQITNTEHSLYDPDDPEHRQLFLN   166
        ::.: :: :::: ::::        :::::::::: :: :::::::.:   :  :.:    . :    : ::  :: :
MbeA    GLEKNQYSILWVEHQDK-------GRLELNFVIPNMELQTGKRLQPYYDRADRPRIDAWQTLVNHHYGLHDPNAPENRRTLTL   159

ORF 3   KKNLPKDIKDFKAQLHQRVYRAVANGVVNVADRRIGAMA                                              205
          :::       . .   .      : .      : :
MbeA    PDNLPETKQALAEGVTRGIDALYHAGEIKGRQDVIQALTEAGLEVVRVTRTSISIADPNGGKNIRLKGAFYEQSFADGRGVRE   242

MbeA    KAERESRIYRENAEQRVQEARRICKRGCDIKRDENQRRYSPVHSLDRGIAGKTPGRGERGDDAAQEGRVKAGREYGHDVTGDS   325

MbeA    LSPVYREWRDALVSWREDTGEPGRNQEAGRDIAETEREDMGRGVCAGREQEIPCPSVREISGGDSLSGERVGTSEGVTQSDRA   408

MbeA    GNTFAERLRAAATGLYAAAERMGERLRGIAEDVFAYATGQRDAERAGHAVESAGAALERADRTLEPVIQRELEIREERLIQER   491

MbeA    EHVLSLERERQPEIQERTLDGPSLGW                                                           517
```

Figure 5.6 Alignment of deduced amino acid sequence of ORF 3 and MbeA protein from ColE1 plasmid identified in *E. coli* (Chan *et al.*, 1985). Colon (:), identical amino acids; dot (.), similar amino acids. Dashes in the sequence indicate gaps introduced to optimize alignments.

```
ORF 3    MIVDFFRHGSGLSKGCLDYLLGEDREREHAQVLNGDVELTAQLIDSSPFAKKYTSGCLSFYEHDLSDQDKQKIMQNFEECLFP    83
         :::  ::  : :   : .::::: ::.:: : .: :: : :: :: :: .::::: ::::: : .     :  .:  ::::.:
CppC     MIVQFFNRGKGGGSGPIDYLLGKDRDREEARLLRGDPEETAALINSSDYAKKYTAGCLSFEESNIPAEQKHALMDSFEECIFA    83

ORF 3    GLDKDQYQILWVQHQDKINQDTGETRLELNFVIPNVELSTGKRLQPFYAPVDLDRVDLFKQITNTEHSLYDPDDPEHRQLFLN   166
         :::::::. ::: : ::        ::::::::::.:: .::::::.:   :  :::  .   :  :   :::::  ::   .
CppC     GLDKDQYNCLWVEHRDK-------GRLELNFVIPNIELLSGKRLQPYYYAADRGRVDAWRTMQNLTHGYSDPDDPAKRQSMTQ   159

ORF 3    KKNLPKDIKD---FKAQLHQRVYRAVANGVVNVADRRIGAMA                                           205
          : ::.   .        : :.             :: :  .
CppC     AKDLPRNTQEAAQSITEHHRRLRSPSPIRQAKKPRRRAGNAGKGRF                                       205
```

Figure 5.7 Alignment of deduced amino acid sequence of ORF 3 and CppC from pJD1 (Korch *et al.*, 1985). Colon (:), identical amino acids; dot (.), similar amino acids. Dashes in the sequence indicate gaps introduced to optimize alignments.

```
ORF 6   26 SEMQTLNTVINEKQMRVDHLNRSGGSLTISTCKRPDSKAQRLCIQINK    73
           .:.  :     : .  .: :      ::.  :   .::: :.:.   :
CppB    94 NELDDLKQQRAEAERTLDLLETKTKGLTLENCPVENSKATRVCVATEK   141
```

Figure 5.8 Alignment of deduced amino acids 26 - 73 of ORF 6 and amino acids 94 - 141 of the 213 amino-acid CppB from pJD1 (Korch *et al.*, 1985). Colon (:), identical amino acids; dot (.), similar amino acids.

```
CppC  MIVQFFNRGKGGGSGPIDYLLGKDRDREEARLLRGDPEETAALINSSDYAKKYTAGCLSFEESNIPAEQKHALMDSFEECIFAG   84
      ::: :  :::::::::.:::::..: :: : .: : :::   ::  . .::::: : ::: :  .:   .  .: :::  .  :
MbeA  MIVKFHARGKGGGSGPVDYLLGRERNREGATVLQGNPEEVRELIDATPFAKKYTSGVLSFAEKELPPGGREKVMASFERVLMPG   84

CppC  LDKDQYNCLWVEHRDKGRLELNFVIPNIELLSGKRLQPYYYAADRGRVDAWRTMQNLTHGYSDPDDPAKRQSMTQAKDLPRNTQ  168
      :.: ::  ::::: ::::::::::::: :: .::::::::  ::: :.::: :  :   :  ::  :  : . :    ::   :
MbeA  LEKNQYSILWVEHQDKGRLELNFVIPNMELQTGKRLQPYYDRADRPRIDAWQTLVNHHYGLHDPNAPENRRTLTLPDNLPETKQ  168

CppC  EAAQSITEHHRRLRSPSPIRQAKKPRRRAGNAGKGRF                                                205
        :  .:     :     :.           ::
MbeA  ALAEGVTRGIDALYHAGEIKGRQDVIQALTEAGLEVVRVTRTSISIADPNGGKNIRLKGAFYEQSFADGRGVREKAERESRIYR  252

MbeA  ENAEQRVQEARRICKRGCDIKRDENQRRYSPVHSLDRGIAGKTPGRGERGDDAAQEGRVKAGREYGHDVTGDSLSPVYREWRDA  336

MbeA  LVSWREDTGEPGRNQEAGRDIAETEREDMGRGVCAGREQEIPCPSVREISGGDSLSGERVGTSEGVTQSDRAGNTFAERLRAAA  420

MbeA  TGLYAAAERMGERLRGIAEDVFAYATGQRDAERAGHAVESAGAALERADRTLEPVIQRELEIREERLIQEREHVLSLERERQPE  504

MbeA  IQERTLDGPSLGW                                                                        517
```

Figure 5.9 Alignment of CppC from pJD1 (Korch *et al.*, 1985) and MbeA from plasmid ColE1 (Chan *et al.*, 1985). Colon (:), identical amino acids; dot (.), similar amino acids.

5.4 DISCUSSION

Essential to the maintenance of a plasmid in its host is an origin (ori) of replication that ensures propagation of the plasmid within the bacterial cell. The ori of replication, *oriV,* is a site at which DNA melting occurs, which permits initiation of replication of the plasmid. Analysis of the region encompassing *oriV* in a number of plasmids has identified several characteristic features associated with initiation of replication. These include an AT-rich region, containing a series of repeat sequences, and may contain a number of DnaA boxes (Bramhill and Kornberg, 1988).

An AT-rich region (72 %) upstream of the *aadB* cassette on pRAY contains features that are reminiscent of origins of replication. Eight copies of an AT-rich repeat sequence, AAAAAATAT, previously identified in regions of *Acinetobacter* plasmids shown to be essential for maintenance of these plasmids in their *Acinetobacter* hosts (Hunger *et al.*, 1990; Minas and Gutnick, 1993), have been identified on pRAY. In addition, sequences with homology to the DnaA boxes, with at least 7 nucleotides out of the 9 consensus nucleotides TTATc/aCAc/aA (Fuller *et al.*, 1984) were identified in tandem array. These DnaA boxes are specific binding sites for the host DnaA protein and binding of DnaA molecules to these sites in the ori causes the DNA to wrap around these protein molecules (Fuller *et al.*, 1984; Kline *et al.*, 1986). The torsional forces result in a conformational change of the heteroduplex, which favours subsequent melting at the repeat sequences of the ori, and allows priming for initiation of replication. Another feature of this region that is suggestive of it being an ori is the presence of an ORF (ORF 2) that may encode a replication initiator protein; however, the translational product of ORF 2 showed no similarity to any replication initiation proteins in GenBank. Nevertheless, initiation of replication of pRAY may be dependent on a plasmid-specific replication protein and host DnaA.

It is interesting to consider the regulation of replication initiator proteins and their role in plasmid replication. Recent studies by Maas *et al.* (1997) have shown that the RepA1 initiator protein binds to an anchor sequence (CCGCAAGCG) within the RepA1 ORF. Binding of RepA1 to the anchor sequence results in steric hindrance, which inhibits initiation of transcription at the RepFIC replicon of the enterotoxin plasmid, EntP307. This steric block is relieved by transcription from an upstream promoter, thereby allowing initiation of replication to proceed.

A further level of regulation of replication initiation is achieved by the regulation of the initiator protein. Binding of the RepE initiator protein of the *E. coli* F plasmid to sequences within the *repE* operator autoregulates RepE expression (Masson and Ray, 1986). Furthermore, sequestering of the initiator protein by its binding to repeat sequences at regions other than in the *oriV*, controls replication via a mechanism referred to as initiator titration (Tsutsui *et al.*, 1983; Chattoraj *et al.*, 1984; Masson and Ray, 1986).

Features of the regulation of replication initiator proteins, as discussed above, are present in pRAY. A sequence (CCGCAACCT) with good homology (differences are underlined) to the anchor sequence (Maas *et al.*, 1997) is present within ORF 2 (349 - 357). Binding of the ORF 2 putative initiatior protein to the anchor sequence would inhibit replication of pRAY. Inhibition could be overcome by transcription from a promoter (TTCCAA (N_{16}) TTTACT) identified upstream (296 - 323) (Fig. 5.3) of the anchor sequence, alleviating steric hindrance. Binding of the ORF 2 gene product to an inverse of the AAAAAATAT repeat (TTATTTTTT) and an anchor sequence identified immediately upstream of the ORF 2 putative promoter (TTGATT (N_{12}) TAAGCT) (296-323) (Fig. 5.3) could effect autoregulation of ORF 2. A further seven copies of this AT-rich repeat sequence in a region 3' of the *aadB* gene cassette (1341 - 1855), may serve to sequester the initiator protein and, in turn, regulate replication of pRAY. Thus, the replication initiator protein may be regulated by one, or a combination of these mechanisms to regulate initiation of replication at the ori of pRAY.

In addition to its regulation by an initiatior protein, transcriptional activity of the ori region is also known to contribute to initiation of replication (Bramhill and Kornberg, 1988). The close proximity of RNA transcripts in the region of the ori assists melting of the AT-rich repeats and the transcriptional activity of this region facilitates the functioning of the initiator protein. Contained within the putative ori of pRAY is the *aadB* gene transcription initiation site (371) and the associated -10 hexamer (358 - 363)

(Chapter 4). The transcriptional activity of the ori of plasmid pWH1277 from *Acinetobacter lwoffii* has been noted previously (Hunger *et al.*, 1990).

Assuming the product of ORF 2 to be an initiator protein, initiation of replication of pRAY could proceed as follows: (i) the gene product of ORF 2 docks at the anchor sequence CCGCAACCT, present within this ORF, thereby inhibiting replication; (ii) inhibition is overcome by transcription from the putative promoter upstream of the anchor sequence, which relieves the steric hindrance, due to the momentum of the replication fork; (iii) the gene product of ORF 2 binds specifically to the 9-mer AT-rich repeats, AAAAAATAT, inducing a conformational change, resulting in formation of an open complex from which replication can proceed. The *Acinetobacter* host's DnaA binds the tandemly arrayed DnaA boxes, facilitating the unwinding of the AT-rich repeat sequences to form the open complex, from which replication of pRAY may proceed. Of course, it may be that ORF 2 does not encode a replication initiator protein, in which case replication of pRAY could be initiated by DnaA.

The similarity (23 %) of the putative protein encoded by ORF 2 to McbG must be considered. The McbG protein, in conjunction with McbE and McbF, contributes to immunity to microcin B17, a peptide antibiotic, synthesized by the bacterial host (del Carmen Garrido *et al.*, 1988). In a model proposed by del Carmen Garrido *et al.* (1988), McbE and McbF are responsible for transporting the synthesized microcin B17 molecules, across the bacterial membrane, to the external environment. McbG is presumed to inactivate intracellular microcin B17, which has by-passed McbE and McbF, to prevent these peptides accessing their target; DNA gyrase. It is interesting to speculate that pRAY may encode a protein which acts in concert with, as yet, unidentified proteins in synthesis of and immunity to peptide antibiotics.

The translational product of ORF 3 showed a 44 % similarity to the amino-terminal of the MbeA protein encoded by the ColE1 plasmid from *E. coli* (Chan *et al.*, 1985). The MbeA protein is one of a family of mobilization (mob) proteins that act in concert and are sufficient to ensure mobilization of a plasmid (Boyd *et al.*, 1989). The amino terminals of the MobA proteins are highly conserved (Boyd *et al.*, 1989), suggesting that ORF 3 encodes a mob protein. MobA proteins generate the initial nick at *oriT* in the basis of mobility (*bom*) region of the strand to be transferred (Derbyshire *et al.*, 1987; Bhattacharjee and Meyer, 1993). An appropriately located *bom* region (5005 - 5208) upstream of ORF 3 showed a 46 % homology to the *bom* region of pCN3 and related ColE1 plasmids (Roessler *et al.*, 1985). Thus, the putative mob protein encoded by ORF 3 may activate transfer of pRAY by generating the initial nick within this *bom* region.

Most mobilizable plasmids require the presence of MobABC and D to be mobilized by a conjugative plasmid (Boyd *et al.*, 1989). None of the other ORFs identified on pRAY encoded proteins with similarity to MobBC or D. Interestingly, the translational product of ORF 3 also showed 47 % similarity to the CppC protein encoded by pJD1, a cryptic plasmid identified in *Neisseria gonorrhoeae* (Korch *et al.*, 1985). Similarly, the product of ORF 6 showed 45 % similarity to CppB, which is downstream of the structural *cppC* gene on pJD1. The *cppC* gene, together with *cppB*, may encode proteins required for mobilization of pJD1, particularly as CppC shows a 49 % similarity to MbeA of ColE1 (Fig. 5.9). Two *mob* genes in tandem array have been identified on pCloDF13 and were shown to be sufficient for mobilization of pCloDF13 (Van Putten et al., 1987). Moreover, the *oriT* of pCloDF13 could not be mobilized by a conjugative plasmid when the ColE1, K, or A mob proteins were supplied *in trans*, and vice versa, suggesting that pCloDF13 belongs to a distinct lineage of mobilizable plasmids (Boyd *et al.*, 1989). Similarly, pRAY may be of a distinct lineage of mobilizable *Acinetobacter* plasmids.

Although the putative origin of replication of pRAY has features similar to those of plasmids known to replicate in *E. coli*, *E. coli* transformants containing pRAY were not isolated. This suggests that pRAY could not be stably maintained in *E. coli*, and that this plasmid belongs to the group of plasmids suggested to be specific to *Acinetobacter* (Towner, 1996).

It is interesting to consider the G + C content of pRAY. The *aadB* gene cassette has a G + C content of 58 %; the remainder of pRAY has a G + C content of 37 %, which is similar to that predicted for both plasmids (36.5–39.5 %) (Goldstein *et al.*, 1983) and the chromosome (38 - 45 %) (Towner *et al.*, 1991) of *Acinetobacter*.

This is the first report of the characterization of an R plasmid from a clinical isolate of *Acinetobacter*.

CHAPTER 6

SUMMARY AND GENERAL CONCLUSIONS

Clinical isolates of *Acinetobacter* were screened for the presence of an *aadB* gene using a gene-specific probe. A positive hybridization signal was obtained with the DNA from two isolates, designated strain SUN and strain CHA. Transformation and hybridization studies showed that the *aadB* genes are plasmid-located. The plasmid from strain SUN was designated pRAY, and from strain CHA, pCAS.

The *aadB* gene from strains SUN and CHA were cloned on 1.7 kb fragments into pUC19 and expressed in *E. coli*. The 1.7 kb insert from strain SUN and a portion of that from strain CHA were sequenced. Analysis of the sequencing data showed that the *aadB* structural genes are flanked by features of gene cassettes: *attI* sites and 59-be. Interestingly, the sequencing data indicated that the *aadB* gene cassettes are not associated with integrons; instead, the cassettes had recombined at secondary sites on pRAY and pCAS, respectively.

Integration of gene cassettes outside of integrons has been described (Francia *et al.*, 1993; Recchia *et al.*, 1994; Recchia and Hall, 1995b; Francia and García Lobo, 1996; Francia *et al.*, 1997; Hansson *et al.*, 1997). A previous study showed that, following integration at a secondary site, the *aadB* gene cassette was stable on pIE723 (Recchia and Hall, 1995b). This was due to the disruption of the 59-be 3' core site, which could no longer be recognized by integrase, the enzyme responsible for catalyzing the insertion and excision of gene cassettes at secondary sites, and on integrons. To determine whether the *aadB* gene cassette on pRAY is mobile, conduction assays and gene cassette excision experiments were carried out. Because the insertion site on pRAY is similar to the preferred insertion site on integrons, the regenerated 3' core site of the *aadB* 59-be from pRAY was recognized in integrase-mediated cointegrate formation events. Therefore, unlike the *aadB* gene cassette on pIE723, the *aadB* gene cassette on pRAY is potentially mobile.

Transcription of a gene cassette that has inserted at a secondary site is dependent on insertion of the cassette downstream of correctly aligned promoter sequences. Four putative promoters, consisting of -35 and -10 hexamers with good homology to the consensus hexamers recognized by Eσ^{70} (Harley and Reynolds, 1987) were identified upstream of the *aadB* gene cassette on pRAY. However, primer extension analysis showed that none of these promoters were recognized in transcription of the *aadB* gene in *Acinetobacter*. Rather, an alternative -10 hexamer (TATTCA) was identified upstream of the transcription initiation site in *Acinetobacter*. No -35 hexamer was present relative to this -10 hexamer. Interestingly, in similar experiments, a promoter typical of the Eσ^{70} promoters was recognized for transcription of the *aadB* gene in *E. coli*, suggesting that transcription initiation signals recognized in *Acinetobacter* are different from those recognized in *E. coli*.

The study of R plasmids in *Acinetobacter* has been hampered by the lack of ready transfer of these genetic elements from *Acinetobacter* to *Enterobacteriaceae*, and vice versa. As naturally occuring plasmids in *Acinetobacter* have not been fully characterized, pRAY was characterized by sequencing.

Analysis of the sequencing data of pRAY identified features consistent with an origin of replication. These include an AT-rich region containing three copies of DnaA boxes in tandem array, as well as eight copies of the repeat sequence, AAAAAATAT, associated with origins of replication of plasmids identified in *Acinetobacter* (Hunger *et al.*, 1990; Minas and Gutnick, 1993). Furthermore, an ORF identified in the region of the putative ori may encode a plasmid-specific replication initiator protein, suggesting that replication of pRAY may be initiated by a plasmid-encoded replication protein and by DnaA. Features suggestive of pRAY being a mobilizable plasmid were also identified. These include the identification of an ORF that encodes a protein with similarity to the mob protein, MbeA, from the mobilizable ColE1 plasmid (Chan *et al.*, 1985), and a region with homology to the *bom* region, an essential determinant for mobilization, of ColE1-type plasmids (Roessler *et al.*, 1985).

The G + C content (58 %) of the *aadB* gene cassette on pRAY suggests an exogenous origin for the cassette as the remainder of pRAY has a G + C content of 37 %, which is within that which is predicted for *Acinetobacter* plasmids (Goldstein *et al.*, 1983). The region flanking the *aadB* gene cassette is AT-rich (72 %) and DNA melting in this region probably facilitated the insertion of the cassette.

Interestingly, no *E. coli* transformants harbouring pRAY were obtained. The atypical spacing of the promoter sequences identified on pRAY may provide a possible explanation. The -35 and -10 hexamers upstream of an ORF in the ori identified on pRAY are separated by 26 bps, which is more than the typical ±17-bp spacing required for recognition of promoter sequences by $E\sigma^{70}$ (Hawley and McClure, 1983). As this ORF is in the ori region, it may encode a replication initiator protein. If this is the case, pRAY may not be replicated in *E. coli* because the promoter sequences of the plasmid-specific replication protein are not recognized in *E. coli*.

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APPENDIX A

Media and Buffers

Media

2 X Yeast-Tryptone (YT) broth

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	1000 ml
Autoclave	

2 X YT agar

2x YT	100 ml
Agarose	1.5 g
Autoclave	

M9 minimal media

$Na_2HPO_4.7H_2O$	32 g
KH_2PO_4	7.5 g
NaCl	1.25 g
NH_4Cl	2.5 g
Distilled water	to 250 ml
Sterilize and add autoclaved	
Agar	3.75g
Distilled water	195 ml
Trimethoprim	4 mg
Add	
CAAS (5 %, filter sterilized)	25 ml
Glucose (1 M, sterilized)	5 ml
$MgSO_4$ (1 M)	0.25 ml
$CaCl_2$ (1 M)	25 µl

LB broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	1000 ml

Autoclave

LB agar

LB broth	100 ml
Agarose	12 g

“sloppy” LB agar

Tryptone	1 g
Yeast extract	0.5 g
NaCl	1 g
Agarose	0.7 g
Distilled water	100 ml

Autoclave

Buffers

Tris-EDTA buffer

Tris-Cl (1M, pH8.0)	1ml
EDTA (0.5M, pH8.0)	0.2ml
Distilled water	98.8ml

Autoclave

S1 nuclease hybridization buffer

Formamide (80 %)	800 µl
PIPES (1 M, pH 6.4)	40 µl
NaCl (5 M)	80 µl
EDTA (0.5 M, pH 8.0)	2 µl
Distilled water	78 µl

Store at –70 °C

APPENDIX B

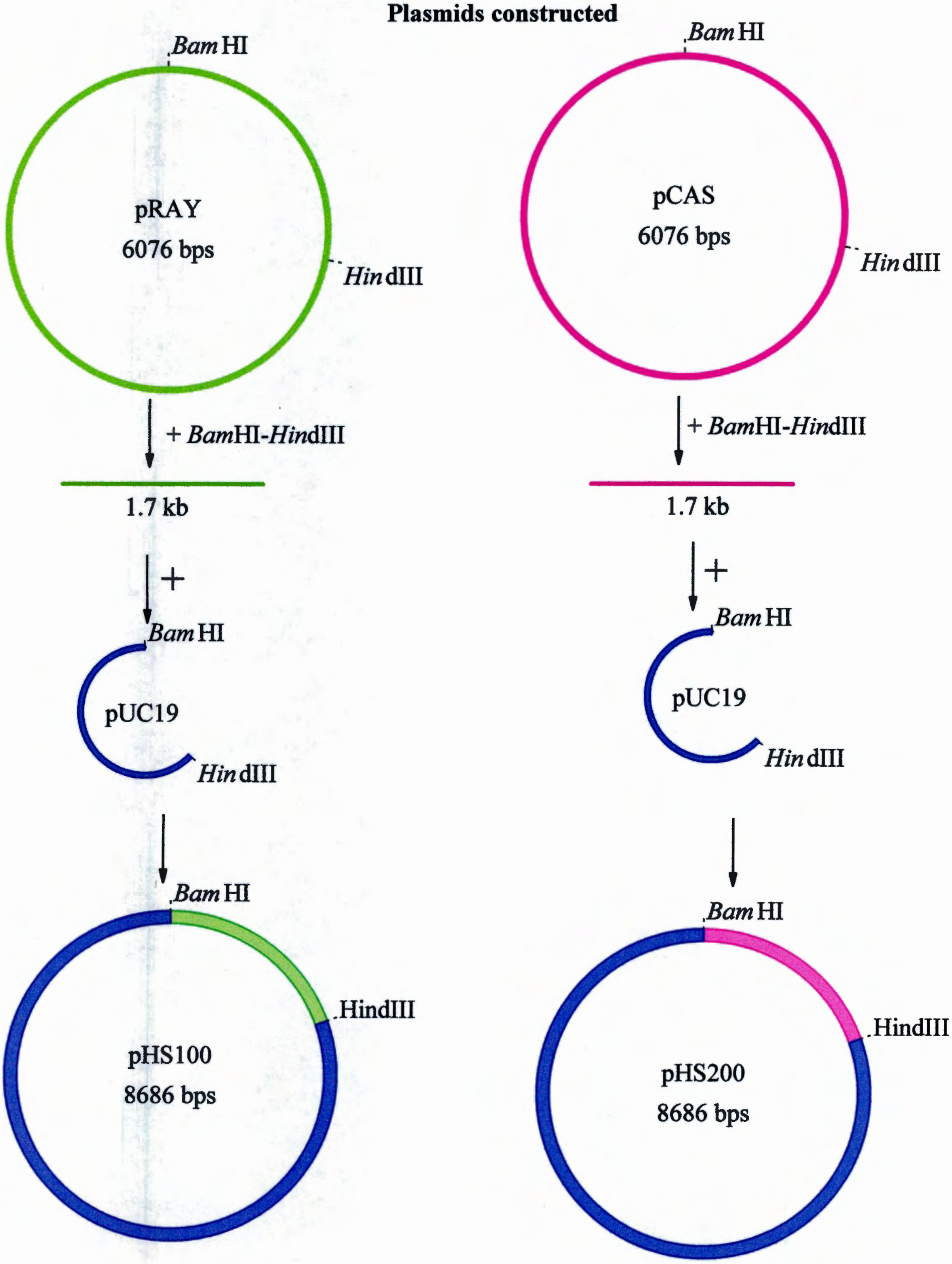
Bacterial strains, plasmids, and bacteriophage DNA used in this study

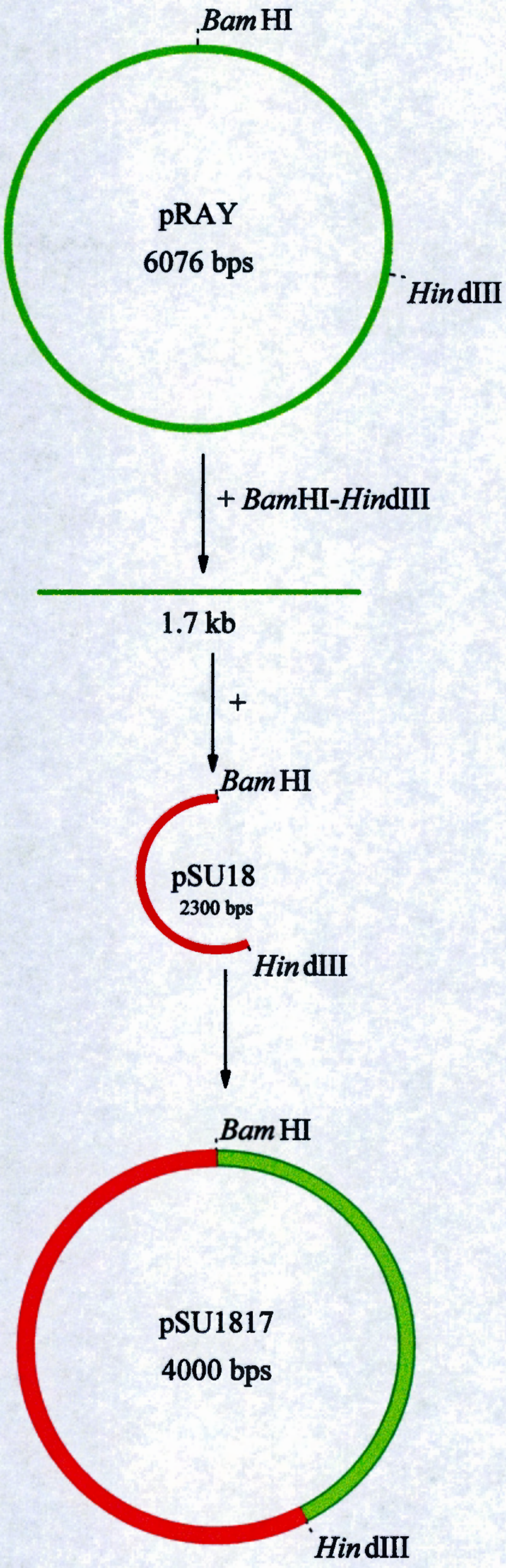
	Relevant characteristics	Source/ Reference
Plasmids		
pUC19	Amp^R	Norrander *et al.*, 1983
pGSH108	*aadB* from strain SAK	Elisha, 1991
pSU2056	Amp^R; *int*	García Lobo/ Martinez and de la Cruz, 1990
pSU18	Cm^R	García Lobo/ Bartolomé *et al.*, 1991
pSU18R2	Cm^R; Tn*21*-59-be	García Lobo
pSU1817	Cm^R; Km^R	this study
R388	Tm^R; In3	García Lobo/ Avila and de la Cruz, 1988
pRAY	Gm^R	this study
pCAS	Gm^R	this study

	Relevant characteristics	**Source/ Reference**
Bacteriophage DNA		
M13mp18 and 19		Yanisch-Perron *et al.*, 1985
Strains		
Acinetobacter		
strain CHA	Gm^{R}, Tob^{R}, Km^{R}	this study
strain SUN	Gm^{R}, Tob^{R}, Km^{R}	this study
strain BD413 C91	Str^{R}, Rif^{R}	Dr A. Vivian, Bristol polytechnic, Bristol, U.K.
E. coli		
strain LKIII	derivative of K514, *recA*, *lac2Δ* M15	Zabeau and Stanley, 1982
strain DH5α	*recA*$^{-}$	Hanahan, 1983
strain UB1637	*his lys trp recA56 rpsL*	García Lobo/ de la Cruz and Grinsted, 1982
strain UB5201	F^{-} *pro met recA56 gyrA* Nal^{R}	García Lobo/ de la Cruz and Grinsted, 1982

APPENDIX C

Plasmids constructed

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+ BamHI-HindIII
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+
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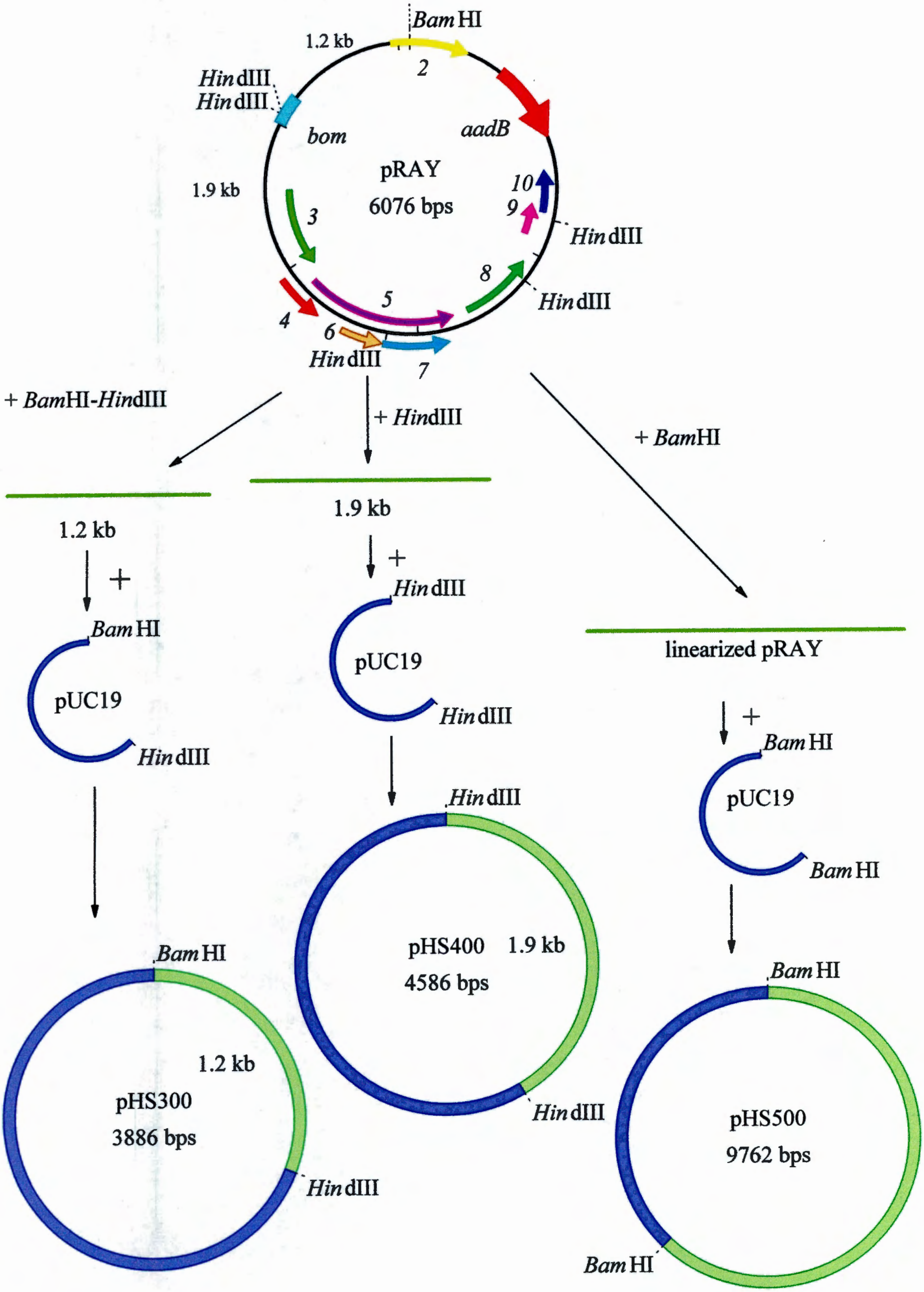
HindIII
BamHI
pSU1817
4000 bps
HindIII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3
9
HindIII
8
5
HindIII
4
6
HindIII
7
+ BamHI-HindIII
+ HindIII
+ BamHI
1.2 kb
1.9 kb
+
+
HindIII
BamHI
pUC19
HindIII
pUC19
HindIII
linearized pRAY
+
BamHI
pUC19
BamHI
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pHS400
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HindIII
BamHI
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HindIII
BamHI
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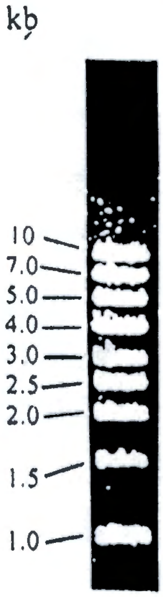
9762 bps
BamHI

APPENDIX D

Molecular weight markers used

10 kb BioMarker



1 kb Ladder