THE LOCATION, EXPRESSION AND REGULATION OF THREE ANTIBIOTIC RESISTANCE GENES IN *ACINETOBACTER*

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PREFACE

Clinical isolates of *Acinetobacter* often exhibit resistance to many antibiotics, including the aminoglycosides and chloramphenicol. Yet very little is known about this resistance at the molecular level. A strain of *Acinetobacter baumannii* (strain SAK) was isolated from a tracheal aspirate; it is resistant to gentamicin, tobramycin, netilmicin, streptomycin, chloramphenicol and suphamethoxazole.

This study examines the nature of the resistance to gentamicin, tobramycin and chloramphenicol at three levels: the biochemical mechanism of resistance, the location of the resistance genes, and the expression and regulation of these genes.

Two aminoglycoside resistance genes, and a chloramphenicol resistance gene have been identified in strain SAK. The aminoglycoside resistance genes, *aadB* and *aacC*, encode AAD(2") and AAC(3), respectively. Both of the enzymes have activity against gentamicin and tobramycin.

DNA:DNA hybridization with specific DNA probes indicates that the *aadB* gene is in the chromosome, whereas the *aacC* and *cat* genes have a plasmid and a chromosomal locus.

Each of these genes was cloned and expressed in *E.coli*. Interestingly, although the *aadB* gene is functional in *E.coli*, RNA studies indicate that it is not expressed in strain SAK. Only *aacC* gene transcripts were detected in this strain. Thus, strain SAK contains a seemingly redundant *aadB* gene.

DNA sequencing data show that the *aadB* gene is part of a Tn*21*-like transposon; similarly, the *cat* gene is also part of a transposon which may be identical to Tn*2670*. In a limited study, it was not possible to activate the *aadB* gene in strain SAK. There is DNA sequencing evidence to suggest that the *aacC* gene may be linked to an IS, and that it may have a catabolite sensitive promoter.

Papers based on the work described in this dissertation have been accepted for publication in: Plasmid (Elisha,B.G. & Steyn,L.M., 1991); Current Microbiology (Elisha,B.G. & Steyn,L.M., 1991); FEMS Symposium Series: The Biology of *Acinetobacter*. K.J.Towner & C.A.Fewson (eds.). Plenum Press. New York. (Elisha,B.G. & Steyn,L.M., 1991).

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ABBREVIATIONS

AAC	aminoglycoside acetyltransferase
AAC(3)	aminoglycoside 3-*N*-acetyltransferase
aacC	gene encoding 3-*N*-acetyltransferase
AAD	aminoglycoside adenylyltransferase also called aminoglycoside nucleotidyltransferase (ANT)
AAD(2")	aminoglycoside 2"-*O*-adenylyltransferase
aadB	gene encoding 2"-*O*-adenylyltransferase
AME	aminoglycoside modifying enzyme
APH	aminoglycoside phosphotransferase
ATP	adenosine-5'-triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
CAP	catabolite activator protein
CAT	chloramphenicol acetyltransferase
cat	gene encoding CAT activity
CoA	acetyl coenzyme A
cpm	counts/minute
DEPC	diethylpyrocarbonate
DNAaseI	deoxyribonuclease I
EDTA	ethylenediaminetetra-acetic acid
IPTG	isopropyl-ß-D-galactopyranoside
IR	inverted repeat
IS	insertion sequence
kb	kilobase
kDal	kilodalton
K_m	Michaelis-Menten constant
mg	milligram
MIC	minimum inhibitory concentration
ml	millilitre
MOPS	morpholino-propane sulphonic acid
mRNA	messenger RNA

ORF	open reading frame
PEG	polyethelene glycol
SDS	sodium dodecyl sulphate
TLC	thin layer chromatography
Tn	transposon
μ	(prefix) micro (10^{-6})
X-gal	5-bromo-4-chloro-3indolyl ß-galactoside

CONTENTS

FIGURES

TABLES

CHAPTER 1

GENERAL INTRODUCTION

"I saw living a great number of minute animalcules; some thousands, indeed, in a drop of water. I then poured some of this water into a porcelain teacup; adding a quantity of coarsely pounded pepper; and I stirred the pepper in the water to discover whether the said animalcules would keep alive in the peppery water, or whether they should die."

Antoni Van Leeuwenhoek.

A. AIM OF THIS WORK

Although we have replaced the pepper of Van Leeuwenhoek with a myriad of antibacterial agents, the question remains the same: will the bacteria succumb, or will they survive? As indicated by Greenwood (1989a), it was predicted that antibiotics would bring about the disappearance of bacterial infections but regrettably this has not proved to be the case. Resistance mechanisms exist to all classes of antimicrobial agents currently in clinical use. In many cases the incidence of resistance is related to antibiotic usage (Levy, 1982; McGowan, 1983; Saunders, 1984). Furthermore, the use of antibiotics in the hospital environment has contributed to the emergence of opportunistic pathogens such as *Acinetobacter* (Finland, 1970; McGowan, 1983; Rowe & Threlfall, 1984; Mayer & Zinner, 1985). The multiple antibiotic resistance of *Acinetobacter* has often presented a formidable problem to the physician prescribing antibiotic therapy (Murray & Moellering, 1979; Bergogne-Berezin, 1985; Joly-Guillou *et al*, 1987; Peacock *et al*, 1988). Although the problem of multi-resistant *Acinetobacter* is recognised, little is known about this resistance at the molecular level. In part, this has been due to the difficulties encountered in locating these genes in this organism (Vivian *et al*, 1981; Chopade *et al*, 1985). The work described in this dissertation focuses on the identification, genetic location, expression and regulation of the genes responsible for resistance to the aminoglycoside antibiotics and chloramphenicol in a clinical isolate of *Acinetobacter*.

B. THE GENUS *ACINETOBACTER*

B.1. Bacteriology

Acinetobacter species are aerobic, Gram-negative organisms which are rod shaped during exponential growth, but become almost spherical in the stationary phase (Baumann *et al*, 1968). Thus, they are often described as cocco-bacilli (Juni, 1978). They characteristically occur in pairs, although some chain formation has been observed (Baumann *et al*, 1968). *Acinetobacter* species are non-spore forming and non-flagellated, but are said to show "twitching" movement due to the presence of fimbriae (Baumann *et al*, 1968). Most strains grow well at 37^{o}C (Bouvet & Grimont, 1986). All strains are catalase positive, and indole and oxidase negative. Some strains are able to form acid from glucose oxidatively, while others are not (Baumann *et al*, 1968; Bouvet & Grimont, 1986).

B.2. Taxonomy

The classification of *Acinetobacter* has proved problematic for the taxonomists. Although members of the genus have been studied for many years, it was not clear until relatively recently that strains called *Bacterium anitratum*, *Herellea vaginicola*, *Mima polymorpha*, *Moraxella glucolytica*, *Moraxella lwoffii*, *Micrococcus cerificans*, *Diplococcus mucosus* and *Achromobacter* were all the same organism (Juni, 1978). Another name was added to the list when the generic name, *Acinetobacter*, was first introduced by Brisou & Prevot to differentiate the non-motile members of the genus *Achromobacter* from the motile members (Juni, 1984).

Largely due to the studies of Baumann *et al* (1968), many of the taxonomic problems were clarified. Organisms previously placed in a variety of genera were shown to be related and could, therefore, be classified in a single genus which they proposed should be called *Acinetobacter*. *Acinetobacter calcoaceticus* was proposed as the type species, and the epithet *anitratum* was suggested as a synonym or a variety. Baumann *et al* (1968) also proposed that two other species, *A.lwoffii* and *A.haemolysans*, and one sub-species, *A.haemolysans haemolyticus*, be recognised. Only one species,

however, is described in Bergy's Manual of Systematic Bacteriology, namely, *Acintobacter calcoaceticus* (Juni, 1984). Medical microbiologists have not always supported the single species notion; two variants of the type species are frequently described in the literature: var *anitratus* (forms acid from glucose oxidatively) and var *lwoffii* which does not oxidize glucose [see 1.B.4].

The heterogeneity of the genus *Acinetobacter* is no longer in question. Bouvet and Grimont (1986) have studied the DNA relatedness, in parallel with the biochemical reactions of many *Acinetobacter* strains. As a result of their study, the genus *Acinetobacter* has been delineated into a number of species: *A.calcoaceticus*, *A.baumannii*, *A.lwoffii*, *A.haemolyticus*, *A.junnii* and *A.johnsonnii*. Clinical isolates have been identified to any of the species, although the predominant strain in hospital outbreaks has been *A.baumannii*.

B.3. Natural habitat and human carriage

Acinetobacter species are ubiquitous in nature. They have been isolated from soil and water (Baumann, 1968), and in addition, they are part of the normal flora of the skin, conjunctiva, nose and pharynx of healthy adults (Taplin *et al*, 1963; Pederson *et al*, 1970; Al-Khoja & Darrell, 1979).

B.4. *Acinetobacter* infections

Infections with *Acinetobacter* commonly occur in hospitals, with the immunocompromised patient, and those patients receiving intensive care, being most at risk of infection with this organism (Altman & Sacks, 1979; Bergogne-Berezin *et al*, 1987; Peacock *et al*, 1988). There have been reports of *Acinetobacter* causing meningitis (Ghoneim & Halaka, 1980; Allen & Green, 1982; Buisson *et al*, 1990); respiratory tract infections (Buxton *et al*, 1979; Sherertz & Sullivan, 1985; Buisson *et al*, 1990); pneumonia (Allen & Green, 1987); septicaemia (Ramphal & Kluge, 1979; Allen & Green, 1982; Sherertz & Sullivan, 1985; Peacock *et al*, 1988, Buisson *et al*, 1990); urinary tract infections (French *et al*, 1980; Sherertz & Sullivan, 1985;

Buisson *et al*, 1990) and skin and wound infections (Sherertz & Sullivan, 1985; Allen & Green, 1987; Buisson *et al*, 1990).

The epidemiology of this organism has not been clearly established, although outbreaks of nosocomial infections have been associated with room humidifiers (Smith & Massanari, 1977); respirometers (Cunha *et al*, 1980), angiography catheters (Shawker *et al*, 1974) and a contaminated water bath used to warm bottles of peritoneal dialysis fluid (Abrutyn *et al*, 1978). In one instance, foam mattresses were implicated in an outbreak in a burns unit (Sherertz & Sullivan, 1985). Cross infection via the hands of medical and nursing staff has been suggested as a possible route of transmission (Buxton *et al*, 1978; French *et al*, 1980). In this respect, Ayliffe *et al* (1988) have shown that var *anitratus* survived hand disinfection better than most Gram-negative bacilli. In another study carried out by Musa *et al* (1990), var *anitratus* survived better on the finger tips than did var *lwoffii*. This is a possible explanation as to why var *anitratus* is more frequently implicated in hospital outbreaks (Musa *et al*, 1990). Although *Acinetobacter* is usually found in moist areas, there is one report in which spread of the infection was shown to be air borne (Allen & Green, 1987).

A seasonal pattern of infections caused by *Acinetobacter* has been described. While reviewing nosocomial infections reported to The Centres for Disease Control, Atlanta, Georgia, USA, Retailliau *et al* (1979) observed that the rate of *Acinetobacter* infection was twice as high in late summer as in the late winter. A seasonal pattern at the Middlesex Hospital, London, U.K. has been reported by Holton (1982). In this case there were more isolates in autumn and winter.

Although *Acinetobacter* infections are usually associated with hospitalized patients there are reports of community acquired pneumonia (Rudin *et al*, 1979) and urinary tract infections (Hoffmann *et al*, 1982).

C. THE AMINOGLYCOSIDE ANTIBIOTICS

C.1. Structure and site of action

The aminoglycoside aminocyclitol antibiotics comprise a large family of antimicrobial agents which are used primarily for serious, often life-threatening, Gram-negative infections. They can be divided into two main groups on the basis of whether they contain streptidine or 2-deoxystreptamine (Davies & Smith, 1978; Foster, 1983). Streptomycin is the only member of the streptidine containing group that is of any clinical importance. Within the 2-deoxystreptamine group, there are two sub-classes. In one of these sub-classes, which includes neomycin, the 2-deoxystreptomine ring is substituted at positions 4 and 5 (Davies & Smith, 1978; Foster, 1983). The other group, which is clinically more important, has substitutions at positions 4 and 6 (Davies & Smith, 1978; Foster, 1983). This group includes gentamicin, tobramycin, netilmicin, kanamycin and amikacin. This study focuses on resistance to gentamicin and tobramycin. All of the aminoglycosides, irrespective of their structural classification, inhibit protein synthesis by binding to the ribosome (Tai & Davies, 1985).

C.2. Resistance to the aminoglycosides

Resistance to the aminoglycosides can be divided into three categories: 1) alteration of the antibiotic target site; 2) diminished uptake of the aminoglycoside by the bacterial cell; and 3) inactivation of the aminoglycoside by aminoglycoside modifying enzymes (AMEs) (Davies & Smith, 1978; Phillips & Shannon, 1984). Most clinically significant resistance to the aminoglycosides is due to AME activity (Davies & Smith, 1978; Phillips & Shannon, 1984).

C.3. Aminoglycoside modifying enzymes

C.3.a. Classification of aminoglycoside modifying enzymes

Three classes of AME have been described (Davies and Smith, 1978; Foster, 1983; Phillips & Shannon, 1984). They are classified according to their mechanism of substrate modification.

1. Acetyltransferases (AAC), which use CoA as a co-substrate to catalyze the acetylation of the susceptible amino groups on the aminoglycoside. The acetyltransferases identified thus far only modify the 2-deoxystreptamine containing antibiotics. They comprise AAC(3) which acts at the 3 position on the 2-deoxystreptamine ring, AAC(2') and AAC(6') which act at the 2' and 6' position on the amino sugar, respectively (Davies & Smith, 1978).

   Isoenzymes that possess different substrate profiles may exist for a given enzyme activity (Davis & Smith, 1978; Foster, 1983; Phillips & Shannon, 1984).

2. Adenylyltransferases (AAD) use ATP as a co-substrate to catalyse the adenylylation of susceptible hydroxyl groups. These, too, are classified according to the site they modify on the aminoglycoside molecule. Two members of this group have activity against the deoxystreptamine containing antibiotics: AAD(2") and AAD(4'). AAD(2") is the only member of the group that has activity against gentamicin. AAD(4') has been identified recently in Gram-negative organisms and has activity against tobramycin and amikacin but not gentamicin (Kettner *et al*, 1989; Jacoby *et al*, 1990).

3. The phosphorylases (APH) use ATP as a co-substrate to modify hydroxyl groups on the aminoglycoside molecule. A number of phosphorylases have been described, but only APH(3') modifies 2-deoxystreptamine containing antibiotics, and then only those compounds that contain a 3'-OH group, namely, neomycin, kanamycin and amikacin (Bongaerts & Vliegenthart, 1988).

Some of the enzymes and their substrate profiles are shown in Table I. It is clear from Table I that an antibiotic can be inactivated by more than one enzyme and that each enzyme can modify more than one antibiotic.

TABLE I

EXAMPLES OF AMINOGLYCOSIDE MODIFYING ENZYMES THAT HAVE BEEN IDENTIFIED IN GRAM-NEGATIVE BACTERIA[a]

Enzyme	Usual Substrate[b]
Acetyltransferases	
2' - acetyltransferase [AAC(2')]	Gentamicin
3 - acetyltransferase [AAC(3)]	Gentamicin, tobramycin, netilmicin, kanamycin, neomycin
6' - acetyltransferase [AAC(6')]	Tobramycin, amikacin, kanamycin, neomycin
Adenylyltransferases	
2" - adenylyltransferase [AAD(2")]	Gentamicin, tobramycin, kanamycin
4' - adenylyltransferase [AAD(4')]	Tobramycin, amikacin, kanamycin, neomycin
Phosphorylases	
3' - phosphorylase [APH(3')]	Kanamycin, neomycin, amikacin

(a) Enzymes that have activity against the streptidine group of antibiotics have been excluded from this list.

(b) These vary with the isozymic form of the enzyme.

C.3.b.i. Assays for aminoglycoside modifying enzymes

Several assays for AMEs have been described (Davies, 1980), but a general outline of only the radio-enzyme assay will be described here. This assay, also known as the phosphocellulose binding assay (Ozanne *et al*, 1969) takes advantage of the fact that aminoglycosides are positively charged and have a high affinity for ion exchange paper such as phosphocellulose (Davies & Smith, 1978). It can be used for all AMEs by measuring the transfer of radio-label from the co-substrate to the antibiotic, and measuring the amount of radio-activity bound to the aminoglycoside which in turn is bound to the phosphocellulose paper (Davis & Smith, 1978). An enzyme is identified by its co-substrate requirement and aminoglycoside substrate specificity.

C.3.b.ii. Limitations of the enzyme assay

A major limitation of this assay is that it is dependent on an enzyme being produced in detectable amounts. Extremely low yields can hinder the detection of enzymes (Williams & Northrop, 1976). Tenover *et al* (1984) were unable to detect AAD(2") activity in *Providencia stuartii* and *Proteus mirabilis*, however, the gene encoding this enzyme was detected with a DNA probe specific for the AAD(2") structural gene, *aadB*. A similar finding has been reported by Gootz *et al* (1985) in *Pseudomonas aeruginosa*.

A further disadvantage is that the definitive identification of an enzyme can be complicated when a resistant strain contains two or more enzymes which use the same co-substrate (Davies & Smith, 1978), or different mechanisms of substrate modification, but share similar substrate profiles (Coombe & George, 1981; Shimizu *et al*, 1985). Nevertheless, enzyme assays have identified a number of enzymes with activity against the aminoglycoside antibiotics in *Acinetobacter* and these are listed in Table II.

TABLE II

AMINOGLYCOSIDE MODIFYING ENZYMES THAT HAVE BEEN IDENTIFIED IN *ACINETOBACTER* spp.

Enzyme	Reference
AAC(2')	Dowding (1979)
AAC(3)I	Murray & Moellering (1980); Bergogne-Berezin *et al* (1980). Gomez-Lus *et al* (1980); Devaud *et al* (1982); Van de Klundert (1984); Phillips *et al* (1986); Lovering *et al* (1988.
AAC(6')	Shannon *et al* (1978); Murray & Moellering (1979); Krcemery *et al* (1985); Phillips *et al* (1986); Lovering *et al* (1988).
AAD(2")	Murray & Moellering (1980). Phillips *et al* (1986);
AAD (3")(9)	Shannon *et al* (1978); Gomez-Lus *et al* (1980); Murray & Moellering (1980); Shimizu *et al* (1981); Devaud *et al* (1982); Goldstein *et al* (1983).
APH(3')I	Gomez-Lus *et al* (1980); Bergogne-Berezin *et al* (1980); Devaud *et al* (1982); Goldstein *et al* (1983). Divers *et al* (1985).
APH (3')II	Murray & Moellering (1979);
APH(3')III	Murray & Moellering (1980).
APH(3')VI	Lambert *et al* (1988).
APH (3") or (6)	Shannon *et al* (1978).
APH (3")	Elisha & Steyn (1989).

C.3.c. The role of aminoglycoside modifying enzymes in bacterial resistance

To understand how AMEs effect bacterial resistance it will be necessary to give a simple description of the uptake of aminoglycosides. Aminoglycosides enter the bacterial cell in three phases: an initial binding of the antibiotic to the cells, which is followed by two energy dependent phases, called energy dependent phase I (EDPI), and energy dependent phase II (EDPII) (Bryan & Van Den Elzen, 1977; Dickie *et al*, 1978; Taber *et al*, 1987). Once across the cytoplasmic membrane (EDPI), the aminoglycoside interacts with the ribosomes. This results in a dramatic increase in the rate of antibiotic uptake (EDPII) which leads to the death of the cell. AMEs effect resistance by covalently modifying the aminoglycoside, thereby reducing its affinity for the ribosomes (Benveniste & Davis, 1973). Since the accelerated rate of aminoglycoside uptake (EDPII) needed for killing is dependent on an interaction between the antibiotic and the ribosome, and since the modified aminoglycoside is unable to bind to the ribosome, aminoglycoside uptake remains depressed in bacteria that contain AME activity, and the cells survive (Kagan & Davies, 1980).

It is important to note that although these enzymes modify the antibiotic, they do not protect the bacterial cell by destroying the aminoglycoside in the surrounding medium; only the drug that enters the cell is modified (Davies, 1986). This is in contrast to the ß-lactamases and chloramphenicol acetyltransferase where modified antibiotic can be found in the culture medium (Davies, 1986).

C.3.d. Location of the aminoglycoside modifying enzymes in the bacterial cell

All the evidence indicates that the AMEs are located in the cytoplasm, and are probably associated with the cytoplasmic membrane of the cell, where they would be accessible to ATP and CoA (Dickie *et al*, 1978).

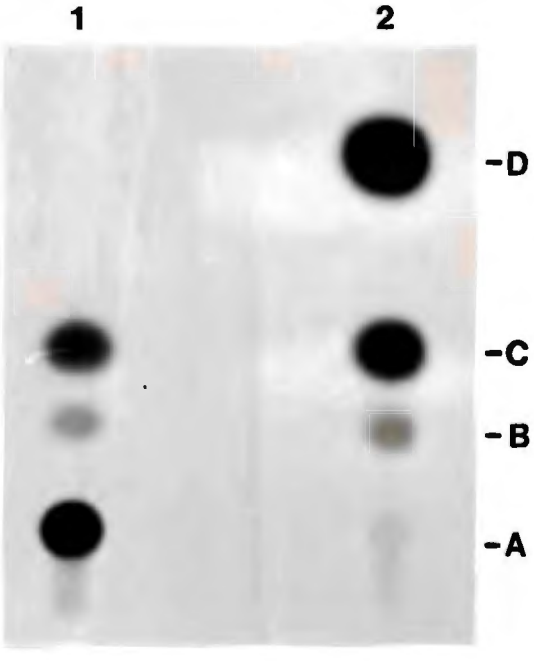

Fig.1. Assay of CAT activity

A cell-free lysate was prepared from strain SAK and was assayed for CAT activity with [^{14}C]chloramphenicol as the substrate. Chloramphenicol and its acetylated forms were separated by TLC and detected by autoradiography. (A) chloramphenicol; (B) 1-acetate chloramphenicol; (C) 3-acetate chloramphenicol; (D) 1,3-diacetate chloramphenicol. Lane 1, acetylated forms of chloramphenicol after incubation with the lysate prepared from strain SAK; lane 2, after incubation with CAT which was present in lysates prepared from *E.coli*(JR66). JR66 encodes resistance to chloramphenicol in addition to resistance to the aminoglycosides (see Appendix 2).

D. GENE TRANSFER STUDIES TO LOCATE THE AMINOGLYCOSIDE AND CHLORAMPHENICOL RESISTANCE GENES IN STRAIN SAK

In a bacterial cell, the genes specifying resistance to antibiotics may be located in the chromosome, or on a plasmid, or both. Plasmid mediated antibiotic resistance has caused most problems in the hospital environment. In the main, this is due to the ability of plasmids to move efficiently from one bacterium to another, and so disseminate the antibiotic resistance genes. This ability of plasmids to move between different bacterial cells by conjugation, has been used to demonstrate plasmid associated antibiotic resistance genes. However, not all plasmids are self-transmissible; in which case a different method of gene transfer - transformation - may be used to demonstrate the presence of plasmid linked antibiotic resistance genes. Each of these methods of gene transfer was used to ascertain whether the gentamicin, tobramycin and chloramphenicol resistance genes are plasmid encoded in strain SAK.

D.1. Methods used in the gene transfer studies

D.1.a. Isolation of DNA

Plasmid DNA for use in the transformation studies [see D.1.c.] was extracted from strain SAK as follows. Cleared lysates were prepared from 150ml of an overnight culture of strain SAK by treating the cells with lyzozyme (which was added as a dry powder, 5-10mg), 1% Triton-X100 (V/V) and 0.4% (W/V) sodium deoxycholate in 0.01M Tris-Cl, pH8.0, 0.001M EDTA as described by Clewell and Helinski (1969). Plasmid DNA was separated from chromosomal DNA by two ultra-centrifugal spins in caesium chloride/ethidium bromide density gradients. Although not used in this experiment, the less dense chromosomal DNA band from the first gradient was also isolated. Since the yields of plasmid DNA were low, plasmid and RNA was harvested from the first gradient and separated from any residual chromosomal DNA in a second density gradient.

D.1.b. Conjugation experiments

Conjugation experiments were done to determine whether strain SAK carries resistance to gentamicin, tobramycin and chloramphenicol on a self-transmissible plasmid. In these studies strain SAK was crossed with two recipients, *E.coli* J53/Nal^r and *Acinetobacter* C91 BD413, which were obtained from Prof.J.Coetzee and Dr.A.Vivian, respectively. An *Acinetobacter* recipient was included in these experiments because the transfer of resistance genes from *Acinetobacter* to *E.coli* has not always been successful [see 1.E.1.]. Nalidixic acid resistant mutants of C91 were obtained by growing C91 in the presence of 100μg/ml of the antibiotic; these were resistant to 150μg/ml nalidixic acid. The conjugation experiments were carried out as follows. Broth cultures of strain SAK and the recipient strains were grown at 37^{o}C to the exponential phase. Equal volumes of donor and recipient cells were mixed on Iso-sensitest medium (IST, Oxoid) and left at room temperature for 2hrs, followed by overnight incubation at 37^{o}C. After incubation the cells were washed off the medium with 10ml of saline, harvested by centrifugation and suspended in 1ml of saline. An aliquot of 0.5ml was plated onto Luria agar containing chloramphenicol and nalidixic acid at 25μg/ml and 150μg/ml, respectively. The remaining 0.5ml was spread onto Luria agar containing gentamicin (5.0μg/ml) and nalidixic acid (150μg/ml).

In a parallel control experiment *E.coli* J53(RP4) (which was also obtained from Prof.J.Coetzee) was used as the donor in crosses with *E.coli* J53/Nal^r or C91 BD413/Nal^r. In this instance, selection was for ampicillin resistant transconjugants.

D.1.c. Transformation experiment

Only one recipient was used in the transformation experiments: *Acinetobacter* C91 BD413. Since this strain is naturally competent (Juni, 1972), no special treatment was required to make it competent. An overnight culture of C91 in 5ml of Luria broth was used to inoculate 50ml fresh Luria broth supplemented with glucose (0.5ml of 50% (W/V)). After 2hrs incubation at 37^{o}C an aliquot of 1ml was added to approximately 200ng of plasmid DNA; the mixture was incubated for a further 3hrs. An aliquot of

the transformation mix (0.5ml) was plated onto Luria agar containing gentamicin (5.0μg/ml), and onto Luria agar containing chloramphenicol (25μg/ml).

D.2. Results and discussion

There was no detectable transfer from strain SAK, to *E.coli* J53/Nal or to *Acinetobacter* C91 BD413/Nal, of a plasmid encoding resistance to gentamicin or chloramphenicol. Ampicillin resistant transconjugants were, however, isolated from the *E.coli*(RP4) X *E.coli* J53/Nal and *E.coli*(RP4) X *Acinetobacter* C91 BD413/Nal matings.

These data suggest that the gentamicin and chloramphenicol resistance genes are not carried on a self-transmissible plasmid in strain SAK. Although there are reports of conjugation experiments having established the genetic basis of antibiotic resistance in *Acinetobacter* [see 1.E.1.], there are also a number of reports in which conjugation experiments were unsuccessful in locating antibotic resistance genes in this organism (Murray & Moellering, 1979; French *et al*, 1980; Allen & Green, 1987; Buisson *et al*, 1990).

The plasmid DNA isolated from strain SAK did not transform *Acinetobacter* C91 to either gentamicin, or chloramphenicol resistance. This suggests that either the resistance genes are not carried on a plasmid, or that the plasmid is not capable of transforming C91.

E. THE CONSTRUCTION AND USE OF ANTIBIOTIC RESISTANCE GENE PROBES TO IDENTIFY THE CORRESPONDING GENE IN STRAIN SAK

The enzyme assays did not elucidate the mechanism of gentamicin and tobramycin resistance in strain SAK; however, it was possible to identify CAT activity as the mechanism of resistance to chloramphenicol. Furthermore, gene transfer experiments failed to determine the genetic basis of the resistance.

Since DNA probes have proved useful in the identification of genes encoding AMEs, they were used to elucidate the mechanism and genetic basis of aminoglycoside resistance in strain SAK. Indeed, the *aphA6* gene, which has activity against amikacin, and was cloned from *Acinetobacter*, has been used to probe clinical isolates of *Acinetobacter* (Buisson *et al*, 1990; Lambert *et al*, 1990). The choice of probe was based on the observation that the only enzyme with activity against gentamicin and tobramycin that has been demonstrated in local isolates of *Acinetobacter* is AAD(2"). Thus, a probe specific for this modifying enzyme was constructed, and was used in hybridization experiments, to detect the corresponding gene in DNA prepared from strain SAK. Similarly, a DNA probe for CAT_I was constructed to determine the genetic basis of this resistance in strain SAK. A CAT_I gene was chosen because this gene is frequently present in Gram-negative bacilli (Shaw, 1983).

E.1. Methods used in the construction and application of the DNA probes

E.1.a. Bacterial transformation

E.coli recipient cells were made competent by the method of Mandel and Higa (1970). The ligation mixes were added to 100μl of competent cells. The mixture was incubated on ice for 30-90mins and heat shocked at 42^{o}C for 3mins. Luria broth (1ml) was added to the cells which were incubated at 37^{o}C for 3hrs (for gene expression). The transformation mix was plated onto selective media containing gentamicin (5μg/ml).

E.1.b. Isolation of DNA

Plasmid and chromosomal DNA was separated in a caesium chloride/ethidium bromide density gradient [see 2.D.2.a.]. For rapid, small scale preparations of DNA from recombinant plasmids the method of Birnboim and Doly (1979) was used.

E.1.c. Restriction endonuclease digestion

DNA (500ng-1.0μg) was digested with the required enzyme and incubated for 1-2hrs at the temperature and buffer reaction conditions specified by the manufacturers.

E.1.d. Agarose gel electrophoresis (AGE)

DNA fragments were separated in horizontal agarose gels (0.8% or 1.0%) in 0.4M Tris-acetate, 0.001M EDTA buffer. After electrophoresis, the gels were stained with ethidium bromide and the DNA was visualized by U.V.light transillumination (302nm).

E.1.e. Extraction of DNA from agarose

A slice of agarose containing DNA was cut out and the DNA was extracted with phenol (Seth,1984).

E.1.f. DNA ligations

Approximately 500ng of insert DNA was ligated to 200ng of vector in 1xligase buffer (supplied by the manufacturer of the ligase) in the presence of 1 unit of T4 ligase. The ligations were incubated at 15^{o}C for 16-18hrs.

E.1.g. Controlled progressive deletions with exonuclease III

Recombinant pUC18 plasmids were digested with restriction enzymes that generated an exonuclease III susceptible end adjacent to the insert DNA, and a 3' overhang to protect the vector DNA (Henikoff, 1984). After initiation of the exonuclease III digestion, aliquots of the reaction mix were removed every 30secs for 12mins. The single stranded DNA in each aliquot was digested with S1 nuclease, and the DNA ends were repaired with DNA polymerase. The plasmid was then religated and introduced into an *E.coli* recipient.

E.1.h. Preparation of DNA blots for hybridization

Plasmid and chromosomal DNA which had been denatured at 90^{o}C for 5mins was filtered onto nylon membranes using a dot blot apparatus (Biorad). After blotting, the membranes were rinsed in 1.5M NaCl, 0.5M NaOH for 1min to ensure denaturation of the DNA. Neutralization was for 1min in 1.5M NaCl, 0.001M EDTA, Tris-HCl, pH7.2. The membrane was air dried and the DNA was fixed to the membrane by irradiation with U.V.light (302nm) for 5mins.

When necessary Southern blots were prepared as follows. DNA was separated by AGE, after which the DNA was depurinated in 0.2M HCl, denatured in 1.5M NaCl, 0.5M NaOH and transferred to a nylon membrane (Southern, 1975) by the alkaline transfer method described by the manufacturers of Hybond-N. After transfer, the DNA was fixed to the membrane as described above.

E.1.i. Radio-labelling of DNA for use as DNA probes

DNA was radio-labelled with ^{32}P-dCTP by nick translation. Unincorporated nucleotides were removed by centrifugation of the reaction mixes at 600g for 5mins through a Sephadex G-50 column in an MSE bench top centrifuge. The specific activity of the radio-labelled probes was 0.5-1x10^8 dpm μg^{-1} of DNA.

E.1.j. DNA hybridizations

The method of Johnson *et al* (1984) was used. Briefly, the membranes containing the DNA were pre-hybridized for 2-5hrs at 42^{o}C in 6xSSC, 0.25% non-fat milk powder. The membranes were hybridized overnight in sealed plastic bags heated in a shaking water bath at 42^{o}C. The hybridization buffer contained 3xSSC, 0.25% non-fat milk powder, 50% formamide and the radio-labelled probe. Following hybridization the membranes were washed twice with shaking in 2xSSC, 0.1%SDS, 0.25% non-fat milk powder for 30mins at room temperature. This was followed by two washes of 30min at 55^{o}C in 0.1xSSC, 0.1% SDS. The membranes were exposed to Agfa Curix X-ray film for 1-3days at -70^{o}C.

E.2. Construction of a DNA probe for the detection of the *aadB* gene

The gentamicin/tobramycin resistance gene (*aadB*) encoding AAD(2") activity, was isolated from the multi-resistance plasmid, JR66, which encodes resistance to these antibiotics in addition to kanamycin and neomycin (Benveniste & Davies, 1971). JR66 DNA was cut with *Eco*RI and the restriction fragments were cloned into the *Eco*RI site of pBR322. Following transformation of *E.coli* HB101, gentamicin resistant colonies were selected; all were resistant to tobramycin as well, and harboured an identical

recombinant plasmid, designated pGSH101, which contained a 5.3kb DNA insert (Fig.2). A *Sac*I fragment of 1.8kb was deleted from pGSH101 by a partial *Sac*I digestion and ligation without altering the gentamicin/tobramycin resistance phenotype (pGSH102). The 3.5kb *Eco*RI insert from pGSH102 was subcloned in pUC18 (pGSH103). This subcloning was a prerequisite for the controlled, progressive deletion with exonuclease III that was used to reduce the size of the insert further and still retain a functional *aadB* gene. For the exonuclease III digestion, pGSH103 was cut with *Pst*I to generate a 3'-cohesive end adjacent to the vector DNA, while *Sma*I was used to generate an exonuclease III susceptible end adjacent to the insert. No gentamicin/tobramycin resistant transformants were obtained when more than 1.2kb of the insert DNA was deleted. One of the smallest gentamicin resistant plasmids (pGSH104) contained an insert of 2.3kb and a *Bam*HI/*Hin*dIII fragment (1.8kb) was subcloned in pUC18 (pGSH105). A second digestion with exonuclease III was performed to delete insert DNA at the opposite end of the *aadB* gene. *Bam*HI was used to generate a susceptible site while *Kpn*I was used to protect the vector; 0.7kb of the insert DNA was deleted before loss of AAD(2") activity occurred. The size of the final reduced DNA insert containing the functional *aadB* gene was 1.1kb (pGSH106). The insert DNA was cut out of pGSH106 with *Eco*RI and *Hin*dIII and purified by AGE. The purified DNA was radio-labelled and used as probe.

E.3. Construction of a DNA probe for the detection of the *cat* gene

The plasmid, $pA_{10}cat_2$ (Rosenthal *et al*, 1983), contains the gene encoding CAT type I. A 520-bp fragment, which contains part of the 5' end of the *cat* structural gene, was cleaved from this plasmid by digestion with *Hin*dIII/*Nco*I and radio-labelled for use as a probe.

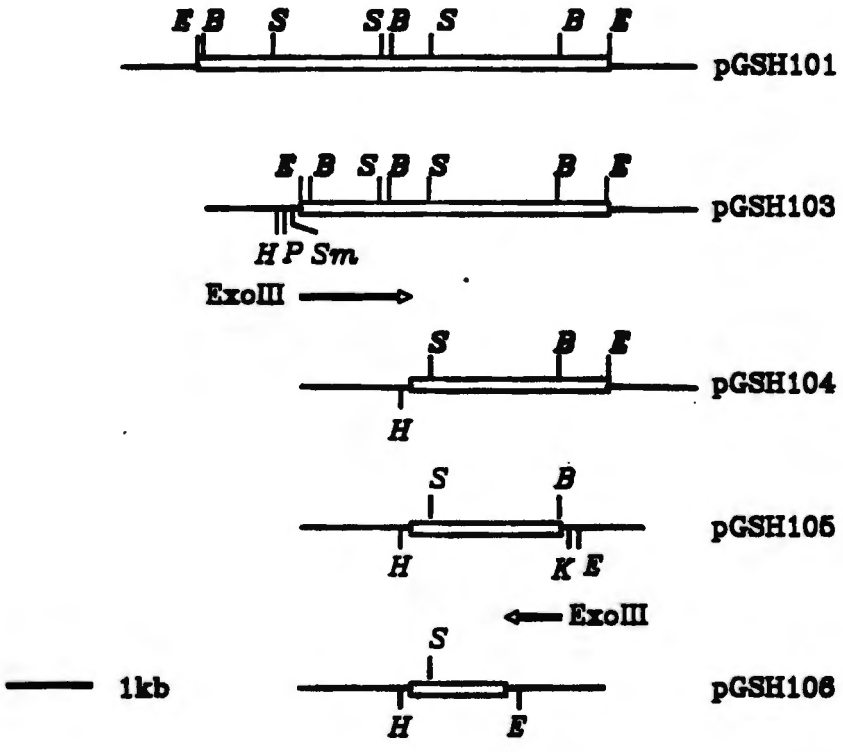

Fig.2. Construction of the AAD(2") gene (*aadB*) probe

▭, insert DNA; —, vector DNA. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; ExoIII, exonuclease III. The vector restriction sites are shown below the bar. The *Bam*HI sites were mapped on pGSH103 and are therefore inferred in pGSH101.

E.4. Results

The *aadB* and *cat* gene probes were used to detect the corresponding gene, and to determine whether they are located in the chromosome or on a plasmid, or both, in strain SAK. In these experiments DNA extracted from a sensitive *Acinetobacter* (strain YON) was included as a negative control. Strain YON is a spontaneous, susceptible derivative of a previously resistant clinical isolate.

E.4.a. Hybridization with the aadB probe

The *aadB* probe hybridized to JR66, the source of the *aadB* gene. There was no hybridization to DNA from strain YON, however, a signal was generated by the chromosomal DNA preparations (Fig.3), but not from the plasmid preparations of strain SAK.

E.4.b. Hybridization with the cat probe

The *cat* probe gave no hybridization signal with the sensitive strain YON, while a signal was generated by $pA_{10}cat_2$, the source of the probe. The probe hybridized to chromosomal and plasmid DNA prepared from strain SAK. Since the plasmid DNA preparation also contained RNA, the signal obtained was weaker than could be expected (Fig.4).

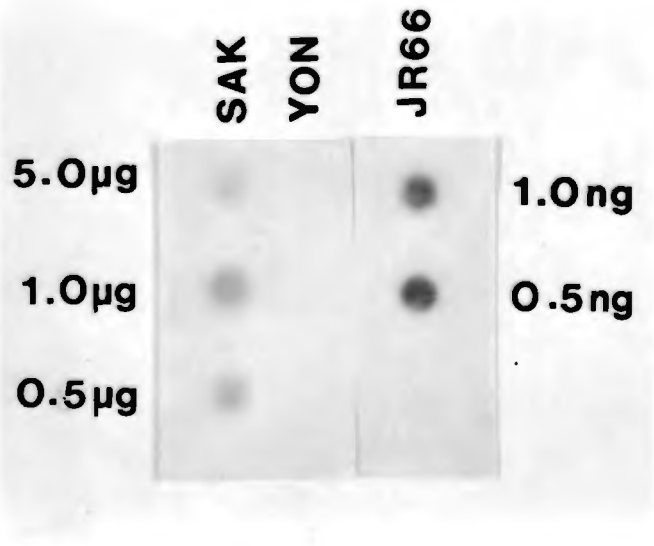

Fig.3. Chromosomal DNA dot blots hybridized with the *aadB* probe
A dot blot was prepared from chromosomal DNA isolated from strain SAK and from the gentamicin, tobramycin sensitive isolate, strain YON. DNA from plasmid JR66, the source of the *aadB* gene was also blotted onto the membrane. The dot blot was probed with the *aadB* probe (1.1kb *Eco*RI/*Hin*dIII fragment from pGSH106). Autoradiography was for 18hrs.

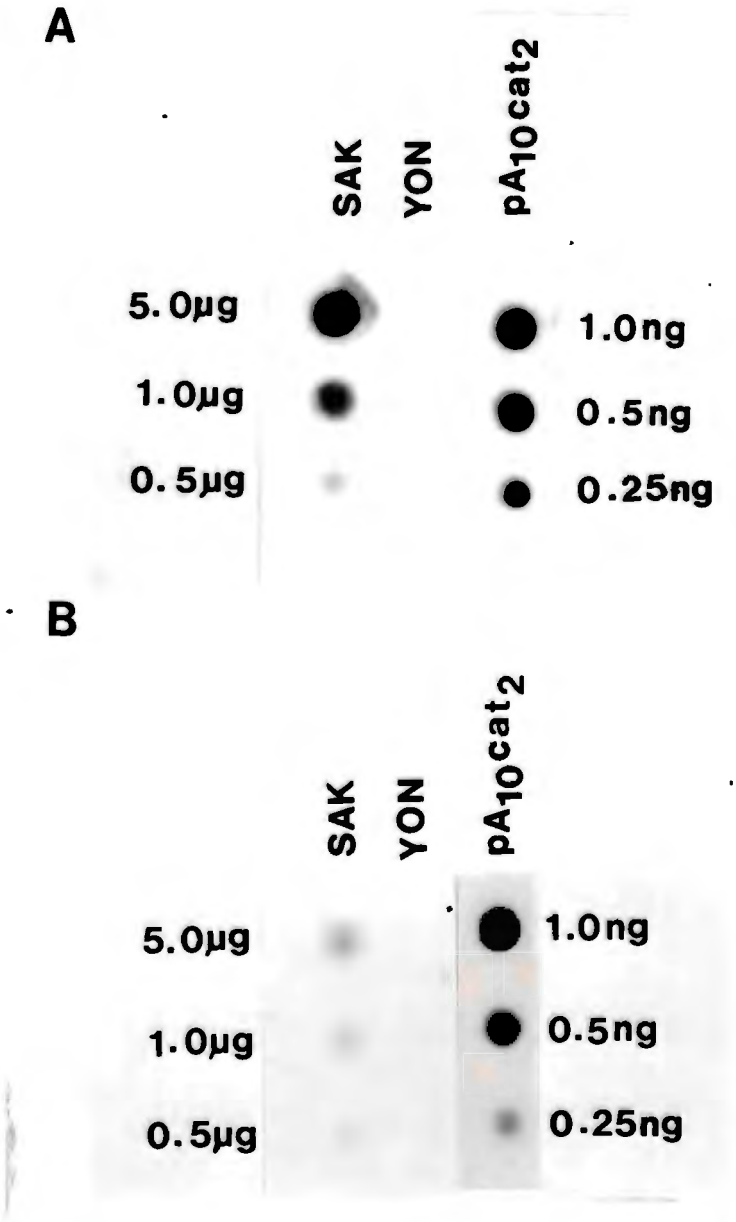

Fig.4. Chromosomal and plasmid DNA dot blots hybridized with the *cat* probe

Dot blots were prepared from chromosomal and plasmid DNA isolated from strain SAK and from the chloramphenicol sensitive isolate, strain YON. The plasmid preparation from strain SAK contained some residual RNA, therefore, the quantity of nucleic acids blotted onto this membrane includes residual RNA as well as plasmid DNA. DNA from plasmid $pA_{10}cat_2$, the source of the *cat* gene, was also blotted onto the membranes. The autoradiographs show the chromosomal DNA dot blot (A) and the plasmid DNA dot blot (B) hybridized with the *cat* probe (520-bp *Hin*dIII/*Nco*I fragment from $pA_{10}cat_2$). Autoradiography was for 72hrs. Since the plasmid DNA preparation from strain SAK also contained RNA, the signal was weaker than could be expected.

F. CONCLUSIONS

Three, different approaches were used to investigate the biochemical mechanism and the genetic basis of gentamicin, tobramycin and chloramphenicol resistance in *Acinetobacter* strain SAK: 1) Assays for antibiotic modifying enzymes; 2) gene transfer experiments and 3) DNA:DNA hybridization with specific antibiotic resistance gene probes. CAT activity was shown to be the mechanism of chloramphenicol resistance, but AMEs with activity against gentamicin and tobramycin were not detected in lysates prepared from strain SAK. The genetic basis of the resistance could not be determined by conjugation and transformation experiments. However, DNA:DNA hybridization experiments detected chromosomal sequences that hybridized to the *aadB* probe, whereas the *cat* probe hybridized to plasmid and chromosomal DNA sequences.

CHAPTER 3

THE MOLECULAR CLONING AND CHARACTERIZATION OF THE AMINOGLYCOSIDE RESISTANCE GENES FROM STRAIN SAK

On the basis of the results described in the previous chapter it could be presumed that strain SAK contains a *cat* gene in the chromosome as well as on a plasmid, whereas the *aadB* gene is only present in the chromosome. Further support for this interpretation was sought from molecular cloning experiments. Moreover, it was necessary to clone the resistance genes to characterize them further. In this chapter, the characterization of the aminoglycoside resistance genes will be described, while the characterization of the chloramphenicol resistance genes will be considered in Chapter 5.

A. CLONING OF THE GENTAMICIN/TOBRAMYCIN RESISTANCE GENES

To clone the *aadB* gene, chromosomal DNA from strain SAK was digested with either, *Pst*I, *Eco*RI, *Hin*dIII or *Bam*HI and ligated in the respective sites in pUC18. The recombinant plasmids were introduced into *E.coli* DK1 by transformation. Gentamicin/tobramycin resistant transformants were obtained from all of the digests. None of the transformants investigated was resistant to chloramphenicol. DNA was extracted from three of the transformants (2 containing a *Hin*dIII insert, and 1 containing a *Bam*HI insert), a Southern blot was prepared [see 2.E.1.b.] which was hybridized to the *aadB* probe. The *Bam*HI insert, and one of the *Hin*dIII inserts, gave strong hybridization signals, whereas no signal was generated from the other *Hin*dIII insert (Fig.5).

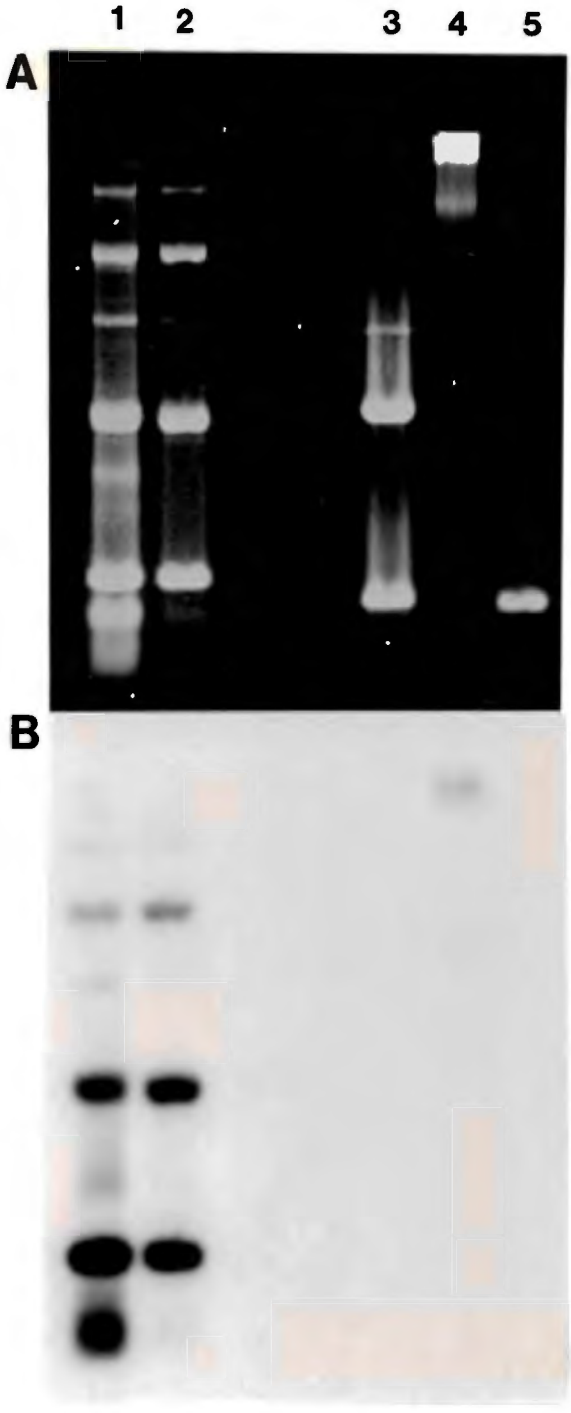

Fig.5. Hybridization of the gentamicin, tobramycin resistant recombinant plasmids constructed from chromosomal DNA prepared from strain SAK to the *aadB* probe

(A) Plasmid DNA was isolated from three gentamicin, tobramycin resistant transformants. Lane 1, recombinant plasmid containing a *Bam*HI insert; lane 2, recombinant plasmid containing a *Hin*dIII insert; lane 3, *Hin*dIII digest of a recombinant plasmid containing a *Hin*dIII insert; lane 4, plasmid JR66, the souce of the *aadB* gene; lane 5, linear pUC18. The DNA was separated by electrophoresis in a 0.8% agarose gel at 20V (1V/cm) for 16hr.

(B) The autoradiograph of the Southern blot of the DNA shown in (A) hybridized with the *aadB* probe (1.1kb *Eco*RI/*Hin*dIII fragment from pGSH106). Autoradiography was for 3.5hrs.

Thus, strain SAK contains at least two gentamicin/tobramycin resistance genes. Although the presence of more than one AME in bacterial strains is not unusual (Groot Obbink *et al*, 1983; Mayer, 1986; Lovering *et al*, 1988), this finding was not anticipated, since AAD(2") activity is the only AME that has been previously demonstrated in local isolates of *Acinetobacter*. It raises the question as to whether the products of both genes contribute to the resistant phenotype, or whether the product of one of the genes is predominantly responsible for the resistance to gentamicin/tobramycin.

B. LOCALIZATION OF THE GENTAMICIN/TOBRAMYCIN RESISTANCE GENE ON THE CLONED INSERTS

To characterize the resistance genes further, it was necessary to locate the functional resistance genes on the cloned inserts.

The clone containing the *Bam*HI insert of 2.3kb was selected for further manipulations. A 1.9kb *Bam*HI/*Hin*dIII fragment was cut out of this insert and subcloned in pUC18. This recombinant plasmid was designated pGSH108, and conferred gentamicin,tobramycin and kanamycin resistance, but not resistance to neomycin and netilmicin, on *E.coli* DK1.

One of the recombinant plasmids contained a *Hin*dIII insert that did not hybridize to the probe. The size of this insert is 5.8kb, which is larger than the estimated 2.3kb for the *Hin*dIII insert that hybridized to the probe. To locate the gentamicin resistance gene in the larger clone, a similar approach to that adopted in the preparation of the *aadB* probe was followed, namely, to reduce the size of the insert by exonuclease III digestion until it contains the functional resistance gene alone. As in the previous exonuclease III digestion experiments *E.coli* HB101 was used as the host. In the first exonuclease digestion, *Kpn*I was used to generate an exonuclease III refractory end adjacent to the vector DNA, while *Bam*HI was used to generate the susceptible site

adjacent to the insert DNA. The insert was reduced to 2.1kb without altering the gentamicin/tobramycin resistance phenotype. To digest the opposite end it was necessary to subclone the 2.1kb *Eco*RI/*Hin*dIII fragment into pMTL23 (Chambers *et al*, 1988). It was then possible to do a second digestion with exonuclease III. This time, *Hin*dIII was used to generate a susceptible site, while *Pst*I was used to protect the vector DNA. A further 1.3kb was deleted from the insert before the gentamicin/tobramycin resistance phenotype was lost. This recombinant plasmid was designated pGSH110, and conferrred resistance to gentamicin,tobramycin and netilmicin, but not resistance to kanamycin and neomycin on *E.coli* HB101.

C. ASSAY OF AMINOGLYCOSIDE MODIFYING ENZYME ACTIVITY IN LYSATES PREPARED FROM *E.COLI* DK1(pGSH108) AND *E.COLI* HB101(pGSH110)

To identify the AME encoded by pGSH110, and to confirm that pGSH108 carried AAD(2") activity, cell free lysates were prepared from *E.coli* containing the respective plasmids and assayed for AME activity [see 2.C.2.]. AAD(2") activity was detected in lysates prepared from *E.coli* (pGSH108), since there was an 83-fold increase in the ^{14}C-ATP counts bound to the membrane in the presence of sisomicin (Table IV). In contrast, the enzyme encoded by pGSH110 is an AAC, as there was a 107-fold increase in the counts bound to the membrane when ^{14}C-CoA was used as the co-substrate (Table IV). Because acetyltransferases comprise AAC(3), AAC(2') and AAC(6'), it was necessary to determine which of the positions on the aminoglycoside molecule was modified. Since the spectrum of aminoglycosides required to determine the site of enzymatic modification is not readily available, the assistance of Dr.S.Raja of Bristol Laboratories was enlisted. The enzyme encoded by pGSH110 was identified as AAC(3) at Bristol Laboratories. This group of acetyltransferases can be subclassified into seven isoenzymes. The subclassification of the AAC(3) encoded by pGSH110 will be considered in the following chapter.

TABLE IV

ASSAY OF AMINOGLYCOSIDE MODIFYING ENZYMES IN *E. COLI* DK-1 (pGSH108) & *E. COLI* HB101 (pGSH110)

(i) Assay of acetyltransferase activity

	Substrate	
	None	Sisomicin
E. coli DK1(pGSH108)	1823	1866
E. coli HB101(pGSH110)	1024	109643

(ii) Assay of adenylyltransferase activity

	Substrate	
	None	Sisomicin
E. coli DK1(pGSH108)	133	11096
E. coli HB101(pGSH110)	101	108

Results are expressed as cpm bound to phosphocellulose paper.
Aminoglycoside modification was assumed to have occurred if the cpm obtained in the presence of the substrate were at least twice the cpm obtained in its absence.

D. IDENTIFICATION AND CHARACTERIZATION OF A PLASMID LINKED *aacC* GENE

The serendipitous identification of the *aacC* gene in the chromosome of strain SAK raised the possibility that this gene could also be plasmid linked. To investigate this possibility, plasmid DNA isolated from strain SAK was used to prepare dot blots [see 2.E.1.]. Chromosomal DNA from strain SAK was included as a positive control, while DNA prepared from the aminoglycoside sensitive strain YON was used as a negative control. A 0.8kb *SphI/SalI* fragment, which contains a portion of the structural *aacC* gene (Figs.8 & 9), was isolated from pGSH110, radio-labelled and hybridized to the DNA dot blots [see 2.E.1.].

The *aacC* gene probe did not hybridize to plasmid and chromosomal DNA from strain YON; it did, however, hybridize to plasmid DNA as well as chromosomal DNA from strain SAK (Fig.6). Thus, strain SAK has an *aacC* gene that has both a plasmid and a chromosomal locus, in addition to an *aadB* gene that has a chromosomal locus only.

The *aacC* gene was cloned from the plasmid DNA. A similar approach to that adopted for cloning the chromosomal aminoglycoside resistance genes was used. Plasmid DNA from strain SAK was digested with *Hin*dIII, *Sph*I or *Sal*I and cloned in pUC18. Gentamicin/tobramycin resistant *E.coli* DK1 transformants were isolated only from the *Hin*dIII and *Sal*I gene banks. None of the recombinants investigated carried resistance to chloramphenicol.

A representative of each of the recombinants hybridized to the *aacC* probe. Furthermore, a 120kb plasmid was shown to be the source of the *Hin*dIII fragment (Fig.7). Thus, the *aacC* gene is carried on a 120kb plasmid as well as in the chromosome of strain SAK.

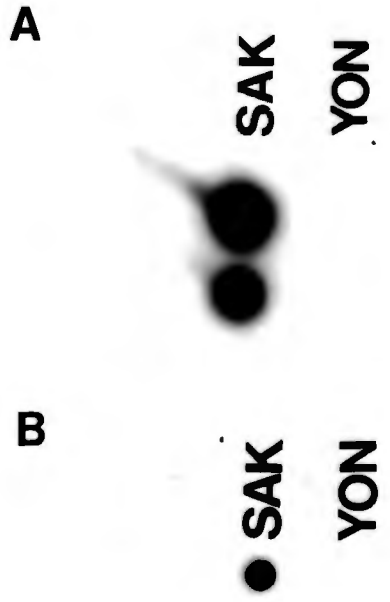

Fig.6. Chromosomal and plasmid DNA dot blots hybridized with the *aacC* gene isolated from strain SAK

Chromosomal and plasmid DNA was prepared from strain SAK and from the gentamicin, tobramycin sensitive strain YON. Two dilutions of DNA (1 and 0.5μg) were blotted onto a nylon membrane. The autoradiographs show the chromosomal DNA dot blot (**A**) and the plasmid DNA dot blot (**B**) hybridized to a portion of the *aacC* structural gene (801bp *SphI*/*SalI* fragment from pGSH110) isolated from strain SAK. Autoradiography was for 3hrs and 18hrs, respectively.

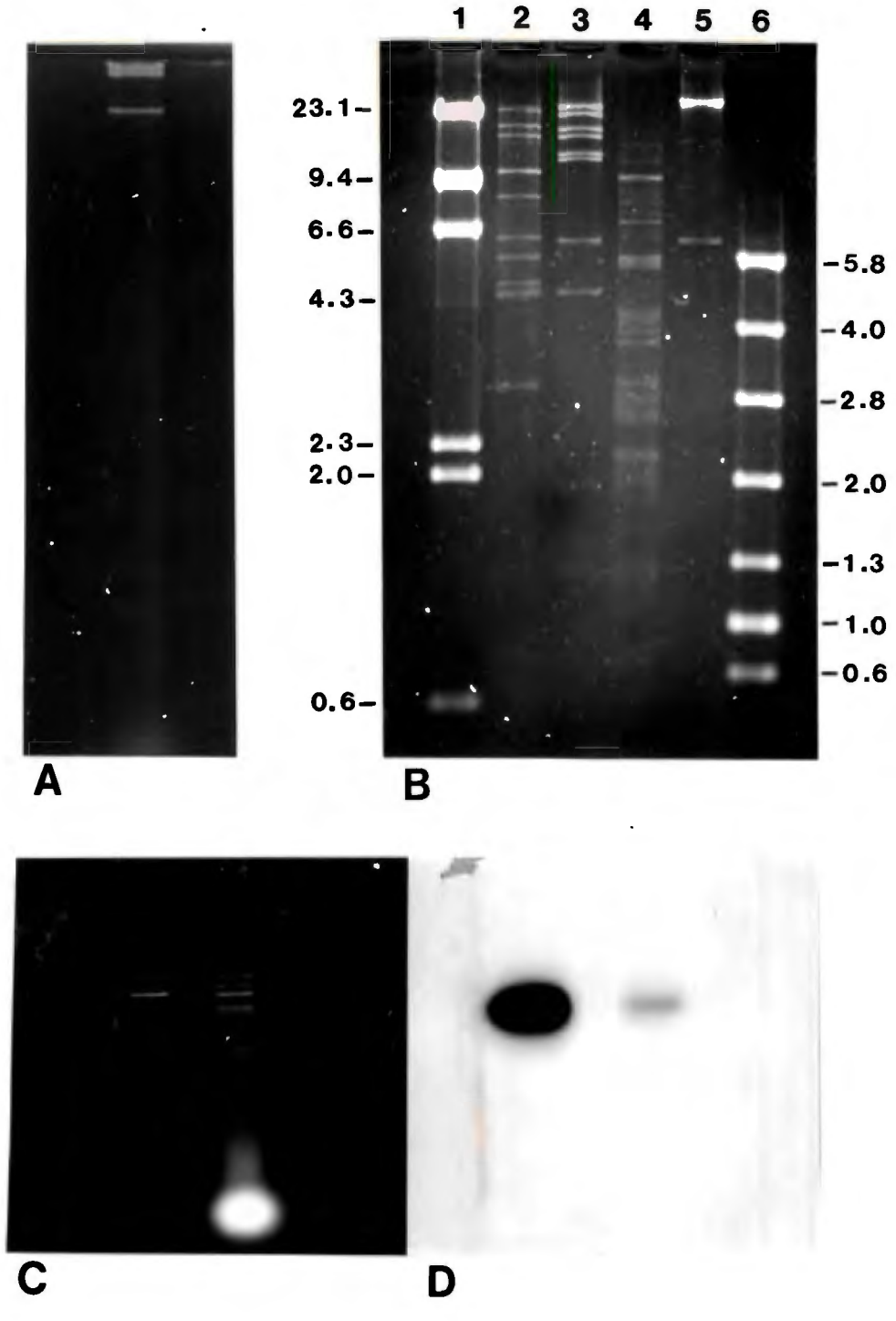

Fig.7. Identification of the plasmid carrying the *aacC* gene

(A) Plasmid profile of strain SAK. (B) Restriction endonuclease digests of the plasmid DNA shown in (A). Lane 1, *Hin*dIII digest of lambda; lanes 2-5, plasmid DNA digested with *Sph*, *Sal*I, *Hin*dIII and *Pst*I, respectively; lane 6, *Eco*RI digest of the molecular size marker (see Appendix 2). The sizes (kilobases) of the marker fragments are indicated. Electrophoresis was for 16hrs at 1V/cm in a 0.8% agarose gel. (C) The *aacC* gene was cloned from plasmid DNA on *Hin*dIII fragment. Lane 1, gel purified *Hin*dIII fragment; lane 2, *Hin*dIII digest of the plasmid DNA from strain SAK. The DNA was separated in a 0.8% agarose gel at 7V/cm for 2hrs, using a mini-submarine gel apparatus. (D) The autoradiograph of the Southern blot of the DNA shown in (C) hybridized with the 0.8kb *SphI/SalI aacC* gene probe. Autoradiography was for 18hrs.

E. DISCUSSION

The problems associated with the detection of AME activity have already been discussed [see 1.C.3.b.ii.]. The demonstration of AAD(2") and AAC(3) activity in the lysates prepared from *E.coli* DK1(pGSH108) and *E.coli* HB101(pGSH110), respectively, was probably due to the high copy number of the plasmids and concomitant high *aadB* and *aacC* gene dose. A similar observation was made by Prince and Jacoby (1982) when they cloned a resistance gene from a 28kb plasmid, harboured by an isolate of *Pseudomonas aeruginosa*, into a high copy number plasmid in *E.coli*. This approach facilitated the detection of AAC activity (4.5-fold above background) that was barely detectable (1.98-fold above background) in the lysates prepared from the *Pseudomonas aeruginosa*.

The presence of two aminoglycoside resistance genes in an organism is not an infrequent occurrence (Groot Obbink *et al*, 1983; Mayer, 1986; Lovering *et al*, 1988). There is a suggestion that *Acinetobacter* may contain more than one type of AME activity (Joly-Guillou *et al*, 1987). The identification of an *aadB* gene, and an *aacC* gene in strain SAK raises the question as to whether both AAD(2") and AAC(3) activity contribute to the gentamicin/tobramycin resistance phenotype. It is interesting and instructive to note that strain SAK is sensitive to kanamycin; normally the *aadB* genotype is associated with kanamycin resistance (Van de Klundert *et al*, 1984; Bongaerts & Vliegenthart, 1988). Indeed, pGSH108, which carries the *aadB* gene cloned from strain SAK confers effective resistance to kanamycin on *E.coli* DK1. There are examples in the literature of bacterial strains that are susceptible to certain aminoglycosides, in spite of the fact that the strain possesses an enzyme for modifying the drug (Haas *et al*, 1976; Allen *et al*, 1982). This point will be returned to in Chapter 6.

CHAPTER 4

THE SUBCLASSIFICATION AND REGULATION OF THE *aacC* GENES ISOLATED FROM STRAIN SAK

A. INTRODUCTION

Two questions arising from the data presented in the previous chapter will now be considered.

1. To which of the isoenzymes does the AAC(3) isolated from strain SAK belong?

2. How is the *aacC* gene regulated, and allied to this; are the plasmid and the chromosomally located genes similarly regulated?

The AAC(3) group of enzymes consists of seven isoenzymes (I, Ia, II, III, IV, V and VII) (Vliegenthart *et al*, 1989). Isoenzymes Ia and III are confined to *Pseudomonas* spp. (Miller *et al*, 1980; Bryan, 1984) while VII has been identified in a strain of *Streptomyces* (Lopez-Cabrera *et al*, 1989). The sequences of *aacC1* [AAC(3)I] (Tenover *et al*, 1989; Wohlleben *et al*, 1989), *aacC2* [AAC(3)II] (Vliegenthart *et al*, 1989), *aacC3* [AAC(3)III] (Allmansberger *et al*, 1985), *aacC4* [AAC(3)IV] (Brau *et al*, 1984) and *aacC7* [AAC(3)VII] (Lopez-Cabrera *et al*, 1989) have been published. It is important to note that the published sequences for the *aacC2* and the *aacC3* genes are identical. AAC(3)I activity has been previously described in *Acinetobacter*, but the same is not true for the other subclasses. Isoenzymes I,II,III,IV and V can be differentiated according to their substrate affinity profiles (Table V). Because some of the substrates required for this type of differentiation are not readily available, the *aacC* gene isolated from strain SAK was sequenced, and the data was compared with published sequences for the AAC(3) group of enzymes.

TABLE V

SUBSTRATE PROFILES OF THE AAC(3) GROUP OF ENZYMES

Enzyme	Substrate						
	Sis	Gm	Tob	Km	Neo	Dib	Apra
AAC(3) I[(a)]	++	++	-	-	-	±	-
II	++	++	++	±	-	++	-
III	++	++	++	++	++	++	-
IV	++	++	++	±	++	++	++
V	++	++	++	-	-	++	-

(a) Subclassification of the AAC(3) isoenzymes (I to III) is according to Biddlecombe *et al* (1976); AAC(3)IV is according to Davies & O'Connor (1978); AAC(3)V is according to Gomez-Lus *et al* (1980).

Abbreviations: Sis, sisomicin; Gm, gentamicin; Tob, tobramycin; Km, kanamycin; Neo, neomycin; Dib, dibekacin; Apra, apramycin.

Symbols: -, no acetylation; ±, poor acetylation; +, good acetylation; ++, best acetylation.

Although much is known about the transcriptional control signals in *E.coli*, there is a paucity of data on the corresponding signals in *Acinetobacter*. Most of the genes in *E.coli* are transcribed by the major RNA polymerase, E sigma^{70}, which recognizes promoters that contain two conserved 6-bp sequences located approximately 35-bp (TTGACA) and 10-bp (TATAAT) upstream from the transcriptional start site (Hawley & McClure, 1983). Another conserved promoter feature is the 17-bp spacer between the -10 and the -35 sequences (Hawley & McClure, 1983). It is possible that the equivalent of *E.coli* E sigma^{70} does not exist in all species of Gram-negative bacilli (Thomas & Franklin, 1989). In this respect, studies done on the properties of RNA polymerases isolated from *Acinetobacter* suggest that there are differences between those of *Acinetobacter* and the RNA polymerases of *E.coli* (Kleppe & Kleppe, 1976). Firstly, although the core RNA polymerases of both organisms resemble one another, the corresponding *E.coli* sigma^{70} subunit was not demonstrated in *Acinetobacter*. This would suggest that either sigma^{70} does not exist in *Acinetobacter*, or that the association between sigma^{70} and the core RNA polymerase is weaker than that in *E.coli* (Kleppe & Kleppe, 1976). Secondly, salt stimulates the RNA polymerase of *E.coli*, whereas it inhibits the *Acinetobacter* counterpart.

B. NUCLEOTIDE SEQUENCING OF THE *aacC* GENES

B.1. Methods used in the preparation of the sequencing gels and the DNA template for the sequencing reactions

B.1.a. Sequencing gels

Buffer gradient gels (8%); non-gradient gels (6%) or polyacrylamide (6%) and formamide (40%) gels were used where appropriate.

B.1.b. Preparation and sequencing of single stranded DNA template

Gel purified DNA fragments were ligated to the replicative forms of M13mp18 and 19 [see 2.E.1.]. Ligation mixes were incubated at 15^{o}C overnight and introduced into competent *E.coli* LK111 cells by transformation [see 2.E.1.]. The LK111 cells were incubated on ice for 1hr, after which they were heat shocked at 42^{o}C for 3mins. IPTG (10μl, 20mM), X-gal (50μl, 2% in dimethylformamide), 0.2ml of an exponential culture of *E.coli* LK111 cells and 3ml of "sloppy" Luria agar were added to the transformed cells, mixed and poured onto an agar base to obtain a uniform lawn and incubated at 37^{o}C overnight.

An agar plug containing a single, white plaque was inoculated into 1.5ml of Luria broth which had been inoculated with 10μl of an overnight culture of *E.coli* LK111 cells. These cultures were incubated at 37^{o}C with vigorous shaking for 4.5hrs, after which the LK111 cells were removed by centrifugation. The resultant supernatant fluid containing the phage was used to infect LK111 cells. Single stranded template was prepared as follows. Luria broth (100ml) was inoculated with 1ml of an overnight culture of LK111 cells and incubated with vigorous shaking at 37^{o}C until an OD_{600} of 0.1-0.2 was reached. At this point, phage (100μl) was added to the growing cells and incubation was continued for a total of 4.5hrs. The LK111 cells were removed by centrifugation. The virus which had extruded from the bacterial cells was concentrated from 40ml of the supernatant fluid by precipitation with PEG (5.18ml, 40%PEG) in the presence of 4.0M NaCl (6.45ml). The viral precipitate was harvested by centrifugation and suspended in 500μl of 10mM Tris-Cl,pH7.5, 10mm NaCl, 0.5mM EDTA.

The viral DNA template for the sequencing reactions was obtained by phenol extraction followed by ethanol precipitation. The DNA sequence was determined by the dideoxy-chain termination method (Sanger *et al*, 1977) using Sequenase (U.S.Biochemicals) and ^{35}S-ATP. Internal oligonucleotide primers were used when necessary to ensure that both strands of the DNA were sequenced. The primer was annealed to the single stranded template, and the sequencing reactions were carried out according to the manufacturers instructions. The sequencing reactions were loaded onto polyacrylamide/urea gels and the bands were visualized by autoradiography following electrophoresis.

B.1.c. Preparation and DNA sequencing of double stranded template

When double stranded template was used in the sequencing reactions, it was prepared by the mini-preparation method of Birnboim and Doly (1979).

Sequenase was also used for sequencing the double stranded templates. The primer was annealed to the template as follows. Plasmid DNA (5μg) in sterile, distilled water was denatured at 37^{o}C for 30min by the addition of 3μl 1N NaOH. The mixture was neutralized by adding 7μl of 10mM TrisCl,pH8.0, 1mM EDTA and 2μl 1N HCl. The primer [1μl(7ng/μl)] was added and incubated at 37^{o}C for 30min. The DNA template/primer was precipitated with alcohol at -70^{o}C for 10min and pelleted by centrifugation. The DNA pellet was dissolved in 8μl of sterile, distilled water, to which was added 2μl of 5x Sequenase buffer (U.S.B.). The sequencing reactions were carried out as described by the manufacturers of the Sequenase kit and the bands were separated by electrophoresis in a polyacrylamide/urea gel.

B.1.d. Nucleotide sequence data analysis

Nucleotide sequences were analysed with computer programmes distributed by the Molecular Biology Computer Research Resource (MBCRR), Harvard School of Public Health, or with the Sequence Analysis Software Package Version 6.2 of Genetics Computer Group Inc. DNA sequence alignments were optimized using the Lipman and Pearson (1985) algorithm.

B.2. Results

B.2.a. Nucleotide sequence analysis of the aacC gene cloned from the chromosomal DNA.

The DNA sequencing strategy is shown in Fig.8. Overlapping fragments restricted from pGSH110, or from the pUC18 recombinant containing the *Eco*RI/*Hin*dIII insert after the first exonuclease III digestion [see 3.B.], were subcloned in M13mp18 and 19. The sequence was confirmed on both strands, except where indicated. The nucleotide sequence of the *aacC* gene from strain SAK is shown in Fig.9.

Analysis of the sequence revealed an ORF of 858 nucleotides, which would encode a protein of 30.5Kdal, starting with an ATG at 252-254 and terminating with the codon TAG (1110-1112). The ORF is preceded by a ribosome binding site (AGGAG) (Gold *et al*, 1981) at nucleotides 239-243. Upstream of the *aacC* coding sequence there are two regions (TATAGT) and (TAGAGT) at nucleotides 219-224 and 199-204, respectively, which show homology to the -10 consensus sequence (TATAAT) for E.*coli* promoters (Hawley & McClure, 1983). However, no sequence with good homology to the -35 hexamer (TTGACA) could be detected relative to either of these putative -10 regions. A further interesting feature of the regulatory region is the presence of an almost perfect direct repeat which encompasses the -10 regions.

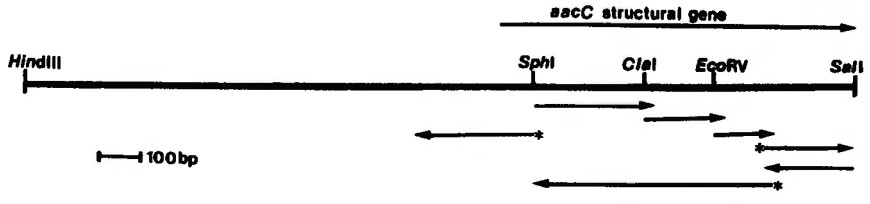

Fig.8. DNA sequencing strategy of the *aacC* gene

The arrows below the bar indicate the direction and extent of the sequenced region. ◄——✱, oligonucleotide primer. The location of the *aacC* gene and the direction of its transcription is indicated by an arrow above the bar. The *Sph*I and *Sal*I sites which define the portion of the *aacC* structural gene used as a probe are indicated.

```
                .         .         :         .         .         .
   1  TTTGTCGTGTACAGAGAAATGGCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCT
                .         .         .         .         .         .
  61  GGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACAC
                .         .         .         .         .         .
 121  AACCGGTGCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGGTCCTCCACCT
                .       . -10     .       . -10     .         .
 181  CGGTCAAGCAGCGAGAGGTAGAGTGATATCTCGATAGGTATAGTGTTTTGCAGTTTAGAG
       SD       .         .         .         .         .         .
 241  GAGATATCGCGATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCC
                  M   H   T   R   K   A   I   T   E   A   I   R   K   L   G   V   Q
                .         .  SphI   .         .         .         .
 301  AAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAG
        T   G   D   L   L   M   V   H   A   S   L   K   A   I   G   P   V   E   G   G
                .         .         .         .         .         .
 361  GAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGCACTGTGATGG
        A   E   T   V   V   A   A   L   R   S   A   V   G   P   T   G   T   V   M   G
                .         .         .         .         .         .
 421  GATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGGTTGGATG
        Y   A   S   W   D   R   S   P   Y   E   E   T   L   N   G   A   R   L   D   D
                .         .         .         .         .         .
 481  ACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGTGGGT
        K   A   R   R   T   W   P   P   F   D   P   A   T   A   G   T   Y   R   G   F
                .         .         .         .         .         .
 541  TCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCG
        G   L   L   N   Q   F   L   V   Q   A   P   G   A   R   R   S   A   H   P   D
                .         .         .         .         .         .
 601  ATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCG
        A   S   M   V   A   V   G   P   L   A   E   T   L   T   E   P   H   E   L   G
                .         .         .         .         .         .
 661  GTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCC
        H   A   L   G   K   G   S   P   V   E   R   F   V   R   L   G   G   K   A   L
                .         .         .         .         .         .
 721  TGCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGG
        L   L   G   A   P   L   N   S   V   T   A   L   H   Y   A   E   A   V   A   D
                .         .         .         .         .         .
 781  ATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAG
        I   P   N   K   R   W   V   T   Y   E   M   P   M   L   G   R   N   G   E   V
                .         .         .         .         .         .
 841  TCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCG
        A   W   K   T   A   S   E   Y   D   S   N   G   I   L   D   C   F   A   I   E
                .         .         .         .         .         .
 901  AAGGAAAGCCGGATGCGGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATC
        G   K   P   D   A   V   E   T   I   A   N   A   Y   V   K   L   G   R   H   R
                .         .         .         .         .         .
 961  GAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGT
        E   G   V   V   G   F   A   Q   C   Y   L   F   D   A   Q   D   I   V   T   F
                .         .         .         .         .         .
1021  TCGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACACGAAG
        G   V   T   Y   L   E   K   H   F   G   A   T   P   I   V   P   A   H   E   A
                .         .         .       SalI
1081  CCGCCCAGCGCTCTTGCGAGCCTTCCGGTTAGAGGCCGTCGAC  1123
        A   Q   R   S   C   E   P   S   G   *
```

FIG.9.

Fig.9. Nucleotide sequence and the derived amino acid sequence of the *aacC* gene from strain SAK

The sequence between nucleotides 252-1110 contains the *aacC* structural gene. Dots above the nucleotides correspond to every 10 nucleotides. There is a 99% sequence similarity between this region and the DNA sequence of the *aacC2* structural gene (Vliegenthart *et al*, 1989), also designated *aacC3* (Allmansberger *et al*, 1985). The sequence from strain SAK contains a G, and not an A, at position 675. The structural gene is preceded by a ribosome binding site (SD) indicated in bold type at 239-243. Two sequences, indicated in bold type, TATAGT at nucleotides 219-224, and TAGAGT at nucleotides 199-204, are potential -10 hexamers. The almost perfect direct repeats which span the -10 hexamers are underlined. The regulatory region contains a putative CAP binding site, AGTGA, at nuleotides 202-206.

B.2.b. Comparison of the aacC sequence from strain SAK with `the published sequences for the aacC group.

The nucleotide sequence of the *aacC* gene of strain SAK was compared with the published AAC(3) gene sequences. It showed 99% similarity with the DNA sequence of the *aacC2* gene (Vliegenthart *et al*, 1989) also designated *aacC3* (Allmansberger *et al*, 1985). The only difference in the structural gene sequence is a transition, at position 675, of a G to an A, which results in a lysine residue in place of glutamic acid. The promoter regions of the *aacC* genes described by Vliegenthart *et al* (1989) and Allmansberger *et al* (1985) are different from each other, and from the *aacC* gene cloned from strain SAK (Fig.10). The *aacC2* gene has two potential -10 boxes, one of which has an optimally spaced -35 box (Vliegenthart *et al*, 1989). One of the *aacC3* designates, pWP14a, has only one -10 region with an optimally spaced -35 hexamer, while another, pWP113a, has a potential -10 box but it does not have a -35 region (Allmansberger *et al*, 1985). In this respect, the regulatory region of pWP113a resembles the corresponding region of the *aacC* of strain SAK, although the nucleotide sequence of this region is different in both cases.

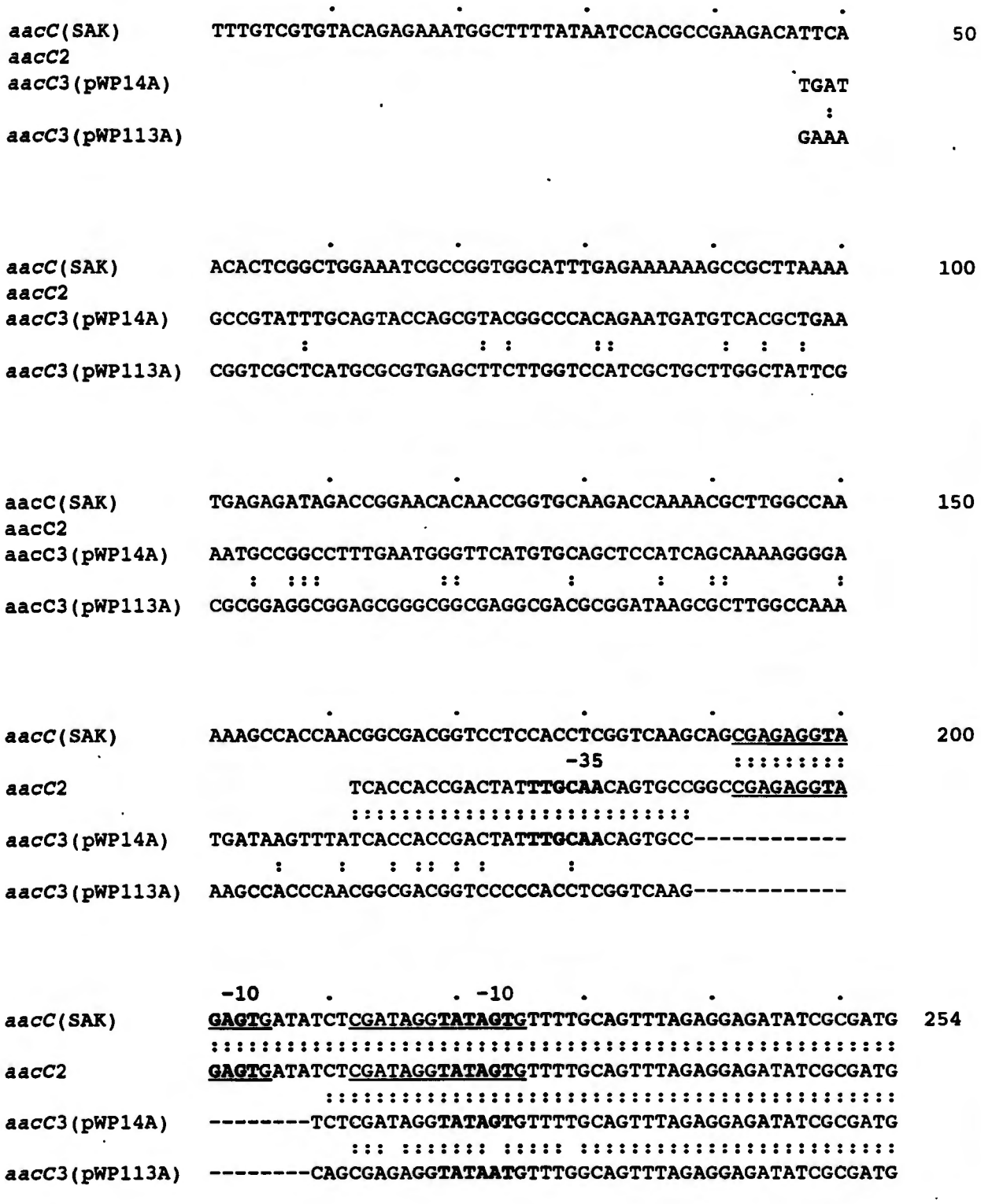
aacC(SAK) TTTGTCGTGTACAGAGAAATGGCTTTTATAATCCACGCCGAAGACATTCA 50
aacC2
aacC3(pWP14A) TGAT
aacC3(pWP113A) GAAA
aacC(SAK) ACACTCGGCTGGAAATCGCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAA 100
aacC2
aacC3(pWP14A) GCCGTATTTGCAGTACCAGCGTACGGCCCACAGAATGATGTCACGCTGAA
aacC3(pWP113A) CGGTCGCTCATGCGCGTGAGCTTCTTGGTCCATCGCTGCTTGGCTATTCG
aacC(SAK) TGAGAGATAGACCGGAACACAACCGGTGCAAGACCAAAACGCTTGGCCAA 150
aacC2
aacC3(pWP14A) AATGCCGGCCTTTGAATGGGTTCATGTGCAGCTCCATCAGCAAAAGGGGA
aacC3(pWP113A) CGCGGAGGCGGAGCGGGCGGCGAGGCGACGCGGATAAGCGCTTGGCCAAA
aacC(SAK) AAAGCCACCAACGGCGACGGTCCTCCACCTCGGTCAAGCAGCGAGAGGTA 200
-35
aacC2 TCACCACCGACTATTTGCAACAGTGCCGGCCGAGAGGTA
aacC3(pWP14A) TGATAAGTTTATCACCACCGACTATTTGCAACAGTGCC----------
aacC3(pWP113A) AAGCCACCCAACGGCGACGGTCCCCCACCTCGGTCAAG----------
-10 -10
aacC(SAK) GAGTGATATCTCGATAGGTATAGTGTTTTGCAGTTTAGAGGAGATATCGCGATG 254
aacC2 GAGTGATATCTCGATAGGTATAGTGTTTTGCAGTTTAGAGGAGATATCGCGATG
aacC3(pWP14A) --------TCTCGATAGGTATAGTGTTTTGCAGTTTAGAGGAGATATCGCGATG
aacC3(pWP113A) --------CAGCGAGAGGTATAATGTTTGGCAGTTTAGAGGAGATATCGCGATG

FIG.10.

Fig.10. Comparison of the regulatory region of the *aacC* gene from strain SAK with the corresponding region described for *aacC2* and *aacC3*

The promoter regions of the *aacC* from strain SAK, the *aacC2* ((Vliegenthart *et al*, 1989) and two *aacC3* genes (Allmansberger *et al*, 1985) are compared. Dots above the nucleotides correspond to every 10 nucleotides. Identical nucleotides are indicated witha colon (:). The -10 hexamer, TATAGT, at nucleotides 219-224 is common to the *aacC* from strain SAK, *aacC2* and the *aacC3* from pWP14A, while the hexamer of the *aacC3* from pWP113A reads TATAAT (indicated in bold type). Relative to this -10, only the *aacC3* from pWP14A has a -35 hexamer, TTGCAA (indicated in bold type), which has good homology to the consensus sequence for this region. The *aacC* from strain SAK and the *aacC2* contain an alternative -10 sequence, TAGAGT (indicated in bold type), at nucleotides 199-204. Relative to this -10 hexamer, only the *aacC2* gene has a -35 hexamer, TTGCAA, at nucleotides 176-181. The *aacC* from strain SAK has no sequence with homology to the -35 consensus sequence (TTGACT) in this region.

B.2.c. Comparison of the region upstream from the aacC structural gene from strain SAK with DNA sequences in the Genbank Database

To investigate whether the 170-bp region upstream from the functional *aacC* gene has sequence similarity with other known sequences, this region was compared with known sequences in the Genbank Database. Interestingly, the sequence between nucleotides 1-155 has a 54.8% identity with the right end of IS*981* (Fig 11). This may imply that the *aacC* gene is carried on a transposon in strain SAK.

B.2.d. Nucleotide sequence analysis of the aacC gene cloned from the plasmid DNA.

To ascertain whether the promoter region of the plasmid located *aacC* gene is similar to that of the chromosomally located gene, it was necessary to sequence the control region of the *aacC* gene cloned from plasmid DNA. The DNA sequence of the regulatory region of the *aacC* gene carried by the 5.8kb *Hin*dIII insert [see 3.D.] was determined. The DNA sequence of the control region was identical to the corresponding region of the chromosomally derived *aacC* gene. This suggests that the regulation of the differently located genes may be identical.

Fig.11. Comparison of the region upstream from the *aacC* gene from strain SAK with the DNA sequence of IS*981*

The complete DNA sequence (1222-bp) of IS*981* is shown. The first 170bp of the *aacC* sequence shown in Fig.9. were compared with this sequence. Nucleotides 1-155 have a 54.8% similarity with one end of IS*981*. Dots above the sequence (and where necessary below the sequence) correspond to every 10 nucleotides. Idential nucleotides are indicated with a colon (:). The sequence alignment was optimized using the algorithm of Lipman and Pearson (1985).

```
   1  GATGTTATCCTTAAATCTTAGAGTCACTATTGTATAATTTAGACAAAGGACAAAAACATG

  61  CAAAAACGCTACTCAAAAGAATTTAAAGAAACCCTTATCGCCTTCTATCATTCTGGTCAA

 121  TCCGTCACCCAGCTGTCTAAAGAATATGACGTGGCCCCTGCAACAATTTATAAATGGATA

 181  GACCTCTACTCTAAATCTAATGAAAGCTCCGTCTCTAAAGCTGATTTTCTAGAATTAAAA

 241  AGACAACTGGCTAAAGTTAAGGAAGAACGAGACATCTTAAAAAAAGTATTGACCATATTC

 301  GCCGAGAAAAAGAAGTGAGTGCTGCGGATATGGCTCAAACCATACAAACTTTAGCACTCA

 361  ATGTCAGACTAAGCTGTCAACTCCTTGATGTTCCTGAATCAAGTTATTATGAACGGATTA

 421  ACCGACACCCATCTAAAACTCAATTAAGGAGACAATACCTGTCACTCAAAATTTCTCAAC

 481  TCTTCAATGCTAACCGAGGAATCTATGGTGCTCCTAAAATTCATCATCTTCTACTTAAAC

 541  AAGGGGAAAAAGTCGGGTTAAAACTGGTACAGAAGCTAATGAAGCAACTTCAACTCAAGT

 601  CTGTAGTCATTAAGAAATTTAAGCCTGGATACTCACTAAGTGATCACATCAATCGAAAAA

 661  ATCTCATACAGACTGAACCTACAAAGAAAAATAAGGTTTGGTCAACCGACATTACTTATA

 721  TTCCTACTCAACAAGGATGGGCTTATCTCTCAACCATTATGGATCGTTATACTAAAAAAG

 781  TCATTGCTTGGGATTTGGGCAAGCGAATGACTGTAGAATTAGTGCAAAGAACTTTAAATA

 841  AGGCCATTAAATCACAAGACTATCCAGAAGCTGTTATTCTTCATTCTGACCAAGGAAGCC

 901  AGTATACGAGTCTAGAGTATGAAGAGTTGCTTAAGTATTATGGGATGACTCACTCTTTCA

 961  GTCGAAGGGGATACCCTTATCATAATGCCAGTCTTGAATCTTGGCATGGACATTTAAAAA

1021  GAGAGTGGGTGTACCAATTTAAATATAAGAACTTTGAAGAAGCCTATCAGAGTATTTTCT
                                                          :: ::
   1                                                     TTTGTCG

1081  GGTACATCG--AAGCCTTTTATAATTCAAAACGAATCCATCAAAGTTTAGGGTATCTTAC
       :::::  :  : : :::::::::: ::   ::::  :::  ::   : :: :      :
   8  TGTACAGAGAAATGGCTTTTATAATCCACGCCGAAGACATTCAACACTCGGCTGGAAATC

1139  ACCTAATCAATTTGAAAAGGTAAGTGCTTAAAAT---AAATAGATTAAAATTCTACGTTT
       ::      :::::: ::   :   :::::::::   : :::::    ::  : ::   :
  68  GCCGGTGGCATTTGAGAAAAAAGCCGCTTAAAATGAGAGATAGACCGGAACACAACCGGT

1196  GTTACTCTAAAA-ACTTGACTTAACGTC               1222
      :  :  : ::::  :::: :  ::   :
 128  GCAAGACCAAAACGCTTGGCCAAAAAGCCACCAACGGCGACGG  170
```

FIG.11.

C. DISCUSSION

The complete DNA sequence similarity between the *aacC2* (Vliegenthart *et al*, 1989) and the *aacC3* (Allmansberger *et al*, 1985) genes is remarkable. Vliegenthart *et al* (1989) offer three lines of evidence which suggest that the sequence described in their study encodes AAC(3)II activity. A comparison of the substrate profile of the product of their cloned *aacC2* gene with those of reference strains containing *aacC2* and *aacC3* genes suggested that their cloned gene encodes AAC(3)II activity. In support of this, hybridization experiments demonstrated that an internal fragment of the cloned structural gene hybridized with the reference strains containing *aacC2* only, and not with those containing *aacC3*. Thirdly, AAC(3)III activity is thought to be confined to *Pseudomonas* spp.; the *aacC3* genes described by Allmansberger *et al* (1985) were isolated from *Klebsiella pneumoniae* and *Serratia marcescens*.

Vliegenthart *et al* (1989) suggest that the original strains studied by Allmansberger *et al* (1985) were not fully tested for the presence of AMEs using a broad range of aminoglycoside substrates. They suggest that the original strains of Allmansberger *et al* (1985) contain undetected APH(3') activity which is responsible for the resistance to neomycin, and possibly kanamycin, in addition to AAC(3)II activity, and that only the *aacC2* gene was cloned and sequenced. This would seem to be a plausible explanation for the discrepant data.

Further confusion surrounds the AAC(3) group of enzymes. This concerns the isoenzyme AAC(3)V. The substrate profile of AAC(3)V is similar to that of AAC(3)II. The isoenzymes are distinguished by their respective activities against kanamycin (Table V). AAC(3)II has activity (albeit, poor activity) against kanamycin, while AAC(3)V does not. Vliegenthart *et al* (1989) have suggested that the classification of AAC(3)V as a separate isoenzyme needs further study. The literature testifies to this. Barg (1988) contends that the AAC(3)III enzyme described by Allmansberger *et al* (1985) is, in fact, an AAC(3)V. Although the gene encoding

this activity has not been sequenced, Barg(1988) has demonstrated that a probe constructed for the *aacC5* gene hybridized to plasmids containing the *aacC3* gene described by Allmansberger *et al* (1985). Similar claims have been made by Martin *et al* (1987). The *aacC5* gene described by this group was shown to be related to the *aacC3* gene (Allmansberger *et al*, 1984).

Thus, it appears likely that the *aacC2*, *aacC3* and *aacC5* genes encode one and the same enzyme. This confusion underlines the importance of establishing standards for the classification of enzymes into new subclasses. It would appear that the substrate range and the concentration dependent MIC profile (Miller *et al*, 1980; Shannon & Phillips, 1982; Bongaerts & Vliegenthart, 1988) are not sufficient to do this.

Although little is known about the transcriptional control signals in *Acinetobacter*, it is, nevertheless, surprising to find that the *aacC* gene of strain SAK lacks a -35 consensus sequence. McClure (1985) and Raibaud and Schwartz (1984) have suggested that promoters with a poor homology to the consensus sequence in this region are frequently controlled by activator proteins. One such protein is the catabolite activator protein (CAP), which binds to cAMP to form an active complex. de Crombrugghe *et al* (1984) have shown that 13 out of 17 regulatory sequences which bind the CAP/cAMP complex contain a conserved sequence, TGTGA, at variable distances from the -10 region; although in most well documented cases, the activator site is located immediately upstream of, or within the -35 region. A similar sequence, AGTGA, is present at nucleotides 202-206 in the *aacC* sequence from strain SAK (Fig.9). It is well known that *cat* is subject to catabolite repression (Harwood and Smith,1971; de Crombrugghe *et al*, 1973; Le Grice & Matzura, 1981), but only two aminoglycoside modifying enzymes, AAD(3") (Harwood & Smith, 1971) and APH(3')II (Davies & Smith, 1978), are known to be subject to catabolite expression. Further studies are required to prove conclusively that the *aacC* gene has a catabolite sensitive promoter.

Why the *aacC* gene in strain SAK, or indeed any antibiotic resistance gene should be subject to a form of control associated with the availability of energy sources is puzzling. Perhaps as Shaw (1983) reasons, special metabolic demands are placed on organisms that inhabit ecological niches where antibiotics are found. *Acinetobacter* is a common soil organism and it may be that this control aspect in strain SAK is related to the origins of the organism.

On the other hand, promoters that do not posses a consensus -35 region can function efficiently without the aid of ancillary proteins (Reznikoff *et al*, 1985). In these cases, the importance of nucleotides, T and G, at positions -15 and -14, respectively, have been demonstrated (Keilty & Rosenberg, 1987). Relative to either of the potential -10 regions, the *aacC* of strain SAK contains a G at -14 but it does not contain the requisite T at -15.

The DNA sequencing data presented here does not provide information on whether the major RNA polymerase of *Acinetobacter* is different from that of *E.coli*. Neverthless, for the sake of clarity, the possibility that this could be the case will be dealt with here.

Only one antibiotic resistance gene from *Acinetobacter* has been sequenced, namely, *aphA6*, which has activity against amikacin (Martin *et al*, 1988). It is instructive to examine the regulatory region of this gene (Fig.12). Presumptive -35 (GTGAAC) and -10 (TTTACT) sequences have been identified for this gene. However, although optimally spaced, neither of the suggested sequences, particularly the -35 hexamer, shows a striking homology to the consensus sequences for similar regions in *E.coli*. Sequences with a better homology to the -35 and -10 boxes are present in the regulatory region of *aphA6*, but they are not separated by 17-bp. The sequences, TTGCTA at nucleotides 18-23, and TAAGAT at 37-42, are only 13-bp apart.

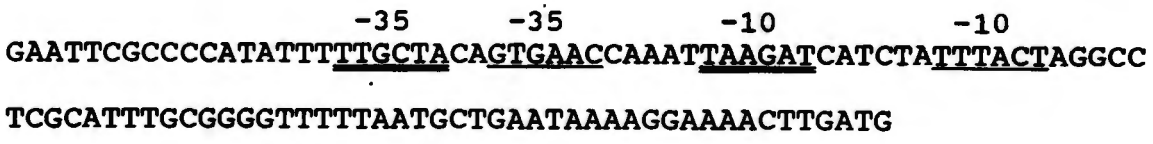

Fig.12. The regulatory region of *aphA6*

The regulatory region of *aphA6* (Martin *et al*, 1988) is shown. The promoter sequences, TTTACT (-10) and GTGAAC (-35) suggested by Martin *et al* (1988) are underlined. Alternative sequences, TAAGAT (-10) and TTGCTA (-35) are indicated by double underlines.

Thus, it is possible that *Acinetobacter* contains an RNA polymerase that recognizes promoter sequences which, although similar to those recognized by E sigma^{70}, are spaced differently. In this respect, the promoters of the genes involved in the regulation of tryptophan synthesis in *Acinetobacter*, while having homology to the E sigma^{70} consensus sequences, do not share an identical spacer requirement. The *trpE* promoter has only 16 nucleotides between the -35 and -10 boxes (Hillen, W. Abstract. FEMS Workshop on the Biology of Acinetobacter (1990) Paris, France), while the *trpFB* promoter has a 19 nucleotide spacer (Kishan & Hillen, 1990). Clearly, to gain a better understanding of the regulation of genes in *Acinetobacter*, it will be necessary to analyse the DNA sequences of many genes. It will also be important to determine the role of alternative sigma factors in the regulation of gene expression in this organism.

The possibility that the *aacC* gene is linked to a sequence which is similar to IS*981* and, therefore, perhaps part of a transposon, could explain the dual location of this gene. IS*981* was isolated from *Lactococcus lactis* and is related to the IS*2* and IS*3* family of IS elements. Further DNA sequencing would have to be done to confirm that the *aacC* gene in strain SAK is, indeed, contained in a transposon. The *aacC3* genes of pWP14a and pWP116a are linked to IS*140* (Allmansberger *et al*,1984). In fact, IS*140* provides the -35 region of the presumptive promoter. The *aacC5* described

by Martin *et al*, is also part of a composite transposon, Tn*2922*. In this case the insertion sequences are related to IS*26*, IS*140* and IS*15*-Δ.

CHAPTER 5

THE MOLECULAR CLONING AND CHARACTERIZATION OF THE CHLORAMPHENICOL RESISTANCE GENES FROM STRAIN SAK

CAT activity was detected in the lysate prepared from strain SAK [see 2.C.3.]. The hybridization experiments showed that the gene encoding this activity has both a plasmid and a chromosomal locus [see 2.E.4.b.]. The *cat* gene was cloned to facilitate the characterization of this gene.

A. CLONING AND CHARACTERIZATION OF THE *cat* GENE FROM PLASMID AND CHROMOSOMAL DNA

The partial library prepared from the chromosomal DNA from strain SAK [see 3.A.] was screened for the presence of chloramphenicol resistance genes. *E.coli* DK1 transformants which were resistant to chloramphenicol (25μg/ml) were obtained only from the *Hin*dIII recombinants. None of the transformants investigated was also resistant to gentamicin/tobramycin. Recombinant plasmids were isolated from six transformants, all of the plasmids contained an insert of the same size. The chromosomal DNA from strain SAK was shown to contain a single *Hin*dIII fragment (>5.8kb) that hybridized to the *cat* probe (Fig.13). One recombinant was selected for further manipulations. It was shown to contain a *Hin*dIII insert of 11.0kb and an internal 5.0kb *Sph*I fragment that hybridized to the *cat* probe was subcloned in pUC18. This recombinant conferred chloramphenicol resistance on the *E.coli* DK1 host. The *cat* gene was further localized to a 1.8kb *Pst*I fragment, which when cloned in pUC18 conferred effective resistance on *E.coli* DK1. This recombinant plasmid was designated pGSH201. The genesis of the recombinants is shown in Fig.14,A,B,C.

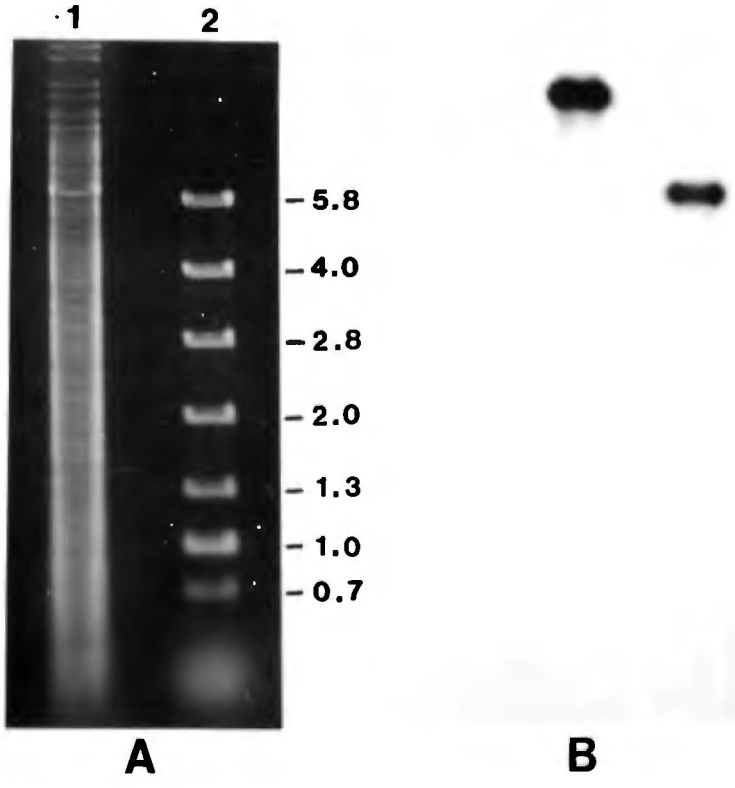

Fig.13. Chromosomal DNA from strain SAK digested with *Hin*dIII and hybridized to the *cat* probe

(A) Chromosomal DNA from strain SAK was digested with *Hin*dIII. Lane 1, *Hin*dIII digest of the chromosomal DNA; lane 2, *Eco*RI digest of the molecular weight marker DNA. The size of the fragments is given in kilobases. Electrophoresis was for 16hrs at 1V/cm in a 0.8% agarose gel. **(B)** The autoradiograph of the Southern blot of the DNA shown in (A) hybridized to the *cat* probe (520bp *Hin*dIII/*Nco*I fragment from $pA_{10}cat_2$) is shown. The *cat* probe hybridized to the 5.8kb fragment in the molecular weight marker. This fragment contains the *cat* gene of pAT15 (see Appendix 2). The probe also hybridized to a single *Hin*dIII of >5.8kb in the digest of the chromosomal DNA from strain SAK.

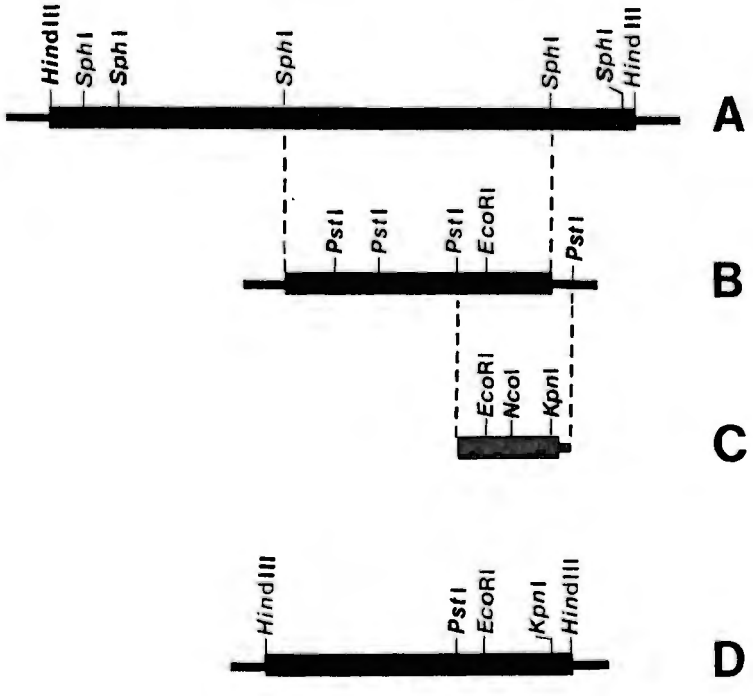

Fig.14. Restriction endonuclease maps of the recombinant plasmids encoding chloramphenicol resistance

, cloned DNA; , pUC18.

(A) 11.0kb *Hin*dIII fragment cloned from chromosomal DNA.

(B) 5.0kb *Sph*I fragment subcloned from the 11.0kb *Hin*dIII fragment.

(C) 1.8kb *Pst*I fragment subcloned from the 5.0kb *Sph*I fragment (pGSH201).

(D) 5.0kb *Hin*dIII fragment cloned from plasmid DNA.

Since the *cat* probe hybridized to plasmid DNA sequences, the gene bank prepared from this DNA [see 3.D.] was screened for chloramphenicol resistant transformants. *E.coli* DK1 transformants which were resistant to chloramphenicol (25μg/ml) were isolated from the *Hin*dIII, *Sph*I and *Sal*I recombinants. One of the recombinants containing a 5.0kb *Hin*dIII insert was characterized further (Fig.14,D). Like the *aacC* gene, the *cat* gene was shown to be contained on the 120kb plasmid harboured by strain SAK. It is important to note that the chromosomal DNA does not contain a 5.0kb *Hin*dIII fragment which hybridizes to the *cat* probe (Fig.13). This confirms that the cloned inserts are derived from separate locations, namely, plasmid and chromosome. Thus, the 120kb plasmid harboured by strain SAK encodes resistance to chloramphenicol, gentamicin and tobramycin. The resistance genes do not appear to be closely linked, since the recombinant plasmids carry either chloramphenicol or gentamicin/tobramycin resistance, but not both.

To verify that the cloned sequences encode CAT activity, crude lysates were prepared from *E.coli* DK1(pGSH201), and from the *E.coli* containing the 5.0kb *Hin*dIII insert cloned from the plasmid DNA, and assayed for activity of this enzyme [see 2.C.]. As expected, the lysates catalyzed the acetylation of chloramphenicol, indicating that the inserts encode CAT activity.

B. NUCLEOTIDE SEQUENCE ANALYSIS OF THE *cat* GENE

The dual location of the *cat* gene suggested that it might be carried on a transposon. Furthermore, Tn*9* which carries resistance to chloramphenicol is flanked by two directly repeated IS*1* elements which contain unique *Pst*I restriction sites encompassing a fragment of 1.87kb (Alton & Vapnek, 1979). To ascertain whether the 1.8kb *Pst*I fragment cloned from the chromosome of strain SAK contains Tn*9*, this DNA was sequenced [see 4.B.1.].

B.1. DNA sequence analysis of the *cat* gene cloned from chromosomal DNA.

The DNA sequencing strategy is shown in Fig.15. Overlapping fragments were restricted from pGSH201 and subcloned in M13 mp18 and 19. The sequence was obtained from both strands. The nucleotide sequence of the 1.8kb *Pst*I insert of pGSH201 is shown in Fig.16. There is 100% sequence homology between the first 181 nucleotides and the left end of IS*1* (Ohtsubo & Ohtsubo, 1978). There is 99.9% sequence homology of the region between nucleotides 182-1284 and the region between the IS*1* elements of Tn*9* (Alton & Vapnek, 1979). The sequence cloned from strain SAK contains a T at position 1104 which is not present in Tn*9* (Alton & Vapnek, 1979). This sequence contains the entire *cat* structural gene beginning with ATG (405-407) and terminating at the ochre stop codon (1062-1064). The remainder of the sequence (1284-1874) is not similar to Tn*9*. Moreover, the sequences diverge at position 1284 which, in Tn*9*, is the first base of IS*1* (right).

The 590-bp region (1284-1874) which is not similar to Tn*9* was compared with known DNA sequences in the Genbank Database GCG Software package. The sequence between nucleotides 1676-1713 has 100% homology with the 38-bp inverted repeat (IR) of Tn*21*. In Tn*21* one of the IRs is linked to, and contains the stop codon (TTA) of the transposase gene, TnpA (Ward & Grinsted, 1987). The other IR is linked to the gene encoding mercury resistance. In the sequence described here, the 160-bp region (1714-1874) which is contiguous with the putative IR has a 100% homology with the *tnpA* gene of Tn*21*. This overall gene organization implies that the *cat* gene in strain

SAK is linked to a Tn*21*-like transposon and to IS*1*. The 392-bp region from 1284-1675 is unique. Analysis of this region shows that it has a G+C content of 48% which is more akin to that of Tn*9* (46%) than to Tn*21* (59%). Based on codon usage and the presence of ORFs, it does not appear to encode any polypeptides.

B.2. DNA sequence analysis of the *cat* gene cloned from plasmid DNA.

The restriction map of the 5.0kb *Hin*dIII fragment cloned from plasmid DNA (Fig.14,D) suggests that the plasmid located *cat* gene is part of a similar genetic structure. To confirm this supposition, it was necessary to demonstrate that the 5.0kb *Hin*dIII insert contains the region which includes the IR and *tnpA* sequences. This was shown to be the case by DNA sequencing. Thus, although located on different replicons, the *cat* genes are contained in similar genetic structures.

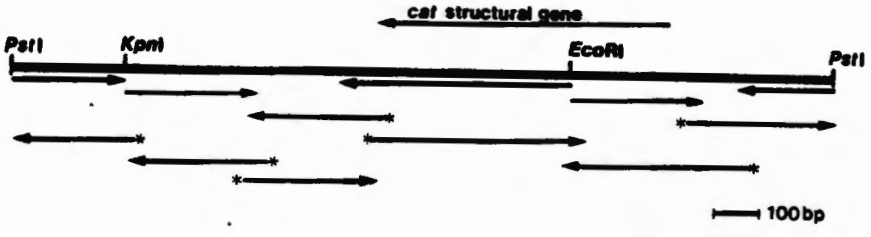

Fig.15. DNA sequencing strategy of the 1.8kb *PstI* fragment carrying the *cat* gene

The arrows below the bar indicate the direction and extent of the sequenced regions. ◄——* , oligonucleotide primer. The location of the *cat* gene and the direction of its transcription is indicated by an arrow above the bar.

```
   1  CTGCAGTTGCCATGTTTTACGGCAGTGAGAGCAGAGATAGCGCTGATGTCCGGCGGTGCT

  61  TTTGCCGTTACGCACCACCCCGTCAGTAGCTGAACAGGAGGGACAGCTGATAGAAACAGA
                                                          T

 121  AGCCACTGGAGCACCTCAAAAACACCATCATACACTAAATCAGTAAGTTGGCAGCATCAC

 181  CCGACGCACTTTGCGCCGAATAAATACCTGTGACGGAAGATCACTTCGCAGAATAAATAA

 241  ATCCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACG

 301  TTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCG

 361  TATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCA
                                                  M  E  K  K  I  T

 421  CTGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTC
        G  Y  T  T  V  D  I  S  Q  W  H  R  K  E  H  F  E  A  F  Q

 481  AGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAA
        S  V  A  Q  C  T  Y  N  Q  T  V  Q  L  D  I  T  A  F  L  K

 541  AGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCC
        T  V  K  K  N  K  H  K  F  Y  P  A  F  I  H  I  L  A  R  L
                    EcoRI
 601  TGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGG
        M  N  A  H  P  E  F  R  M  A  M  K  D  G  E  L  V  I  W  D

 661  ATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCT
        S  V  H  P  C  Y  T  V  F  H  E  Q  T  E  T  F  S  S  L  W

 721  GGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGT
        S  E  Y  H  D  D  F  R  Q  F  L  H  I  Y  S  Q  D  V  A  C

 781  GTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCT
        Y  G  E  N  L  A  Y  F  P  K  G  F  I  E  N  M  F  F  V  S

 841  CAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACT
        A  N  P  W  V  S  F  T  S  F  D  L  N  V  A  N  M  D  N  F

 901  TCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGC
        F  A  P  V  F  T  M  G  K  Y  Y  T  Q  G  D  K  V  L  M  P

 961  CGCTGGCGATTCAGGTTCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTA
        L  A  I  Q  V  H  H  A  V  C  D  G  F  H  V  G  R  M  L  N

1021  ATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTT
        E  L  Q  Q  Y  C  D  E  W  Q  G  G  A  *
                       X
1081  ATTGGTGCCCTTAAACGCCTGGTTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATG

1141  GCAGAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCAGGGCAGGGTCGTTAAATAGC

1201  CGCTTATGTCTATTGCTGGTTTACCGGTTTATTGACTACCGGAAGCAGTGTGACCGTGTG

1261  CTTCTCAAATGCCTGAGGCCAGTTTGCTCAGGCTCTCCCCGTGGAGGTAATAATTGACGA

1321  TATGATCATTTATTCTGCCTCCCAGAGCCTGATAAAAACGGTTAGCGCCGGGGTTGGATT
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                .         .         .         .         .         .
1381  TTTCAGCGTTCCAGCTAAGGCTAAGGCATTCCTGTTCAAGCGCAAGCCGGGCTATAAAGC
                .         .         .         .         .         .
1441  GCATTATCGCTTTACCCGTGCCCTTATTTCGATCGCACTGAGAAACATACAGCTCTTTAA
                .         .         .         .         .         .
1501  TATGCAGCTGACCGCTGTATCGGGGCGAGGGATAAAGAATATTGCAACATGCCAGGCCAG
                .         .         .    KpnI .         .
1561  TAATGTTATTGCCGCAGCGCGCTCTGATCACCAGGGTACCGGAAAGCCGGTTGAATAACT
                .         .         .         .         .         .
1621  TTTTACAGAGATAATCCTTCATCAACGCTTCCTGAATGATACCTTCGCCATAATAGGGGT
      ----------.         .         .         .         .         .
1681  CGTCTCAGAAAACGGAAAATAAAGCACGCTAAGCCGGTTGCAGAGGCCGTAGCGGCCTGA
                .         .       ter       .         .         .
1741  ACTTCCCCGCGCCGATCTTGGCGCTGCTGCGCCATAGGTAATCACCGGTCAGGTTGATGT
                .         .         .         .         .         .
1801  GCTCCCAGCCGAGTGGCGACAGGTACTGCAATAGCGAGTCATCGACGGCATGACCATTGC
                .
1861  CGCGCAACGCATGC
```

Fig.16. Nucleotide sequence analysis of the *cat* gene and its flanking regions

The DNA sequence of the 1.8kb *Pst*I fragment is shown. Only one of the *Pst*I sites, at nucleotides 1-6, is present. The *Pst*I site at the other end of the fragment has been deleted, since this site was shown to originate in pUC18 (see Fig.14). Dots above the sequence correspond to every 10 nucleotides. Identity with IS*1* ends at nucleotide 181 (indicated by a vertical line). The left end of the IS*1* component of Tn*9* contains a T which is absent in IS*1* (Ohtsubo & Ohtsubo, 1979) and in the sequence presented here. This is indicated by a T above the sequence. The region between the two vertical lines is similar to the region between the IS*1* elements of Tn*9*. The only difference is that an additional T which is present in the strain SAK sequence, is absent in Tn*9* (indicated by an X). This region contains the entire *cat* structural gene. The sequence which is identical to the IR of Tn*21* is indicated by a line above the sequence. The region between nucleotides 1714-1874 has homology with the *tnpA* gene. The termination of this gene is within the IR and is indicated by ter below the sequence. Although TAA is indicated, the actual stop codon is TTA, as *tnpA* is transcribed from the opposite strand.

C. DISCUSSION

Analysis of the DNA sequence of a *cat* gene cloned from the chromosomal DNA prepared from strain SAK revealed that the 5'end of the *cat* gene is linked to IS*1*, whereas the 3'end is linked to sequences which show 100% sequence homology with the 38-bp IR and part of the *tnpA* gene of Tn*21*. A similar linkage has been described for the *cat* gene contained within the resistance determinant (r-determinant) of plasmids belonging to incompatibility group FII (IncFII) (Womble & Rownd, 1988). One such plasmid is NR1.

Plasmid NR1 is the originally recognised resistance factor which was first encountered in strains of *Shigella* in 1959 (Nakaya *et al*, 1960). It carries a r-determinant which is 23.3kb in size and encodes resistance to chloramphenicol, streptomycin, the sulphonamides and mercury (Nakaya *et al*, 1960; Watanabe & Fukasawa, 1960; Miki *et al*, 1978; Womble & Rownd, 1988). A feature of this r-determinant is that it is flanked by single copies of direct repeats of IS*1*, and is transposable as Tn*2670* (Iida *et al*, 1981; Hanni *et al*, 1982). In addition, the r-determinant contains a second transposon, Tn*21*, which is responsible for the streptomycin, sulphonamide and mercury resistance (de la Cruz & Grinsted, 1982; Zheng *et al*, 1982; Womble & Rownd, 1988). Thus, the r-determinant comprises a transposon within a transposon. As with the gene described in this study, the *cat* gene of Tn*2670* is located between IS*1*a and one of the IRs of Tn*21*. Furthermore, the region linking the *cat* gene to the IR contains a *Kpn*I site (Fig.17) (Womble & Rownd, 1988).

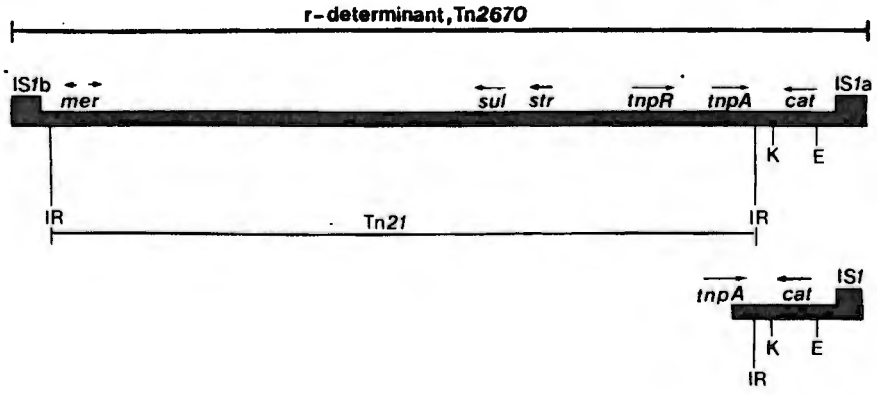


Fig.17. Physical maps of Tn*2670* and the 1.8kb *Pst*I fragment carrying the *cat* gene

Top: Genetic organization of Tn*2670* (Womble & Rownd, 1988).
Bottom: Genetic organization of the 1.8kb *Pst*I sequence cloned from the chromosomal DNA from strain SAK.
The location and the direction of transcription of the genes encoding resistance to sulphonamide (sul); streptomycin (str); chloramphenicol (cat) and mercury (mer) are indicated by arrows. The genes encoding resolvase (*tnpR*) and transposase (*tnpA*) are similarly indicated.
E, *Eco*RI; *K*, *Kpn*I.

Although we have no formal evidence that the IR and *tnpA* sequences of strain SAK are linked to the resistance markers streptomycin, sulphonamide and mercury, this may be the case as strain SAK is resistant to these compounds. If this linkage is the case, then it must also be assumed that strain SAK carries two mechanisms of resistance to streptomycin. The AAD(3")(9) activity encoded by the *aadA* gene of Tn*21* can be responsible for the resistance to streptomycin (and spectinomycin), and it will be recalled that AAD activity was not detected in lysates prepared from strain SAK [see 2.C.3.] but a phosphorylase with activity against streptomycin was demonstrated [see 2.C.3.]. It is possible that strain SAK contains two mechanisms of resistance to streptomycin, and that AAD(3")(9) is expressed at a low level. It seems likely that the *cat* gene in strain SAK is contained within a transposon that may be similar to Tn*2670*. This is the first time that this type of genetic element has been described in *Acinetobacter*.

The DNA sequencing data implies that the *cat* gene located on the plasmid is part of a similar structure. The data presented here show that the plasmid harboured by strain SAK is not NR1 (Womble & Rownd, 1988). Firstly, NR1 is a 94.5kb conjugative plasmid which is smaller than the 120kb estimated for the plasmid isolated from strain SAK. Secondly, NR1 contains more than 18 *Pst*I sites, whereas the 120kb plasmid has less than 10 (Fig.7), and thirdly, NR1 does not contain a 5.0kb *Hin*dIII fragment which contains a *cat* gene.

It is interesting to speculate on the origin of the putative transposon in *Acinetobacter*. Perhaps this organism acquired the transposon from a transient infection with an IncFII plasmid. Alternatively, it could have been infected with a plasmid from an incompatibility group which is stably maintained in this organism and which carried the r-determinant. The transfer of a variety of incompatibility group plasmids from *E.coli* to *Acinetobacter* has been demonstrated (Chopade *et al*, 1985). However, it was not possible to demonstrate the transfer of nine IncFII plasmids to an *Acinetobacter* recipient. This does not necessarily imply that IncFII plasmids such as

NR1 are excluded from *Acinetobacter*, it could be that these plasmids are not stably maintained in this organism. The plasmid may, however, be resident long enough for transposition events to occur.

A feature of resistance genes that are flanked by direct repeats of insertion sequences is that they can be amplified. This amplification is mediated by a recombination event between the IS elements. This process has been shown to occur in *Proteus mirabilis*. In the presence of the appropriate antibiotic, the r-determinant and the resistance transfer factor components of NR1 dissociate (Rownd *et al*, 1971) and the number of copies of the r-determinant is increased. The multiple, tandem copies of the r-determinant exist either autonomously or linked to the resistance transfer factor component of NR1 (Perlman & Rownd, 1975) and confer a high level of resistance to chloramphenicol, streptomycin and sulphonamide.

It might be argued that this putative transposon is of little clinical significance because it encodes resistance to antibiotics which are not generally used as first choice drugs. Two factors militate against this. Firstly, DNA insertions of additional antibiotic resistance genes into the r-determinant component of some IncFII plasmids have been described. R6 contains an insertion of a transposon carrying kanamycin resistance (Tn*903*) near the 3'end of the *tnpA* gene of Tn*21* (Sharp *et al*, 1973; Grindley & Joyce, 1980; Womble & Rownd, 1988). R1 contains two insertions: Tn*3*, which carries ampicillin resistance, has inserted into, and inactivated, the mercury resistance gene of the Tn*21* component. In R1 the equivalent of Tn*21* is called Tn*4* (Kopecko *et al*, 1976; Womble & Rownd, 1988). In addition, R1 contains an insertion of a transposon carrying kanamycin resistance (Tn*2350*) between IS1b and the IR component of Tn*21* (Clerget *et al*, 1981; Womble & Rownd, 1988). In this respect, as the *aacC* gene of strain SAK could be flanked by IS sequences [see 4.B.2.c.], it might have inserted into the putative transposon. Secondly, and perhaps more importantly, a mechanistically different form of insertion into Tn*21* has been described. There is a growing body of evidence which suggests that Tn*21* contains

"hot spots" into which additional antibiotic resistance genes can insert (Schmidt & Klopfer-Kaul, 1984; Cameron *et al*, 1986; Ouellette *et al*, 1987; Wohlleben *et al*, 1989; Lafond *et al*, 1989; Stokes & Hall, 1989; Mercier *et al*, 1990; Grinsted *et al*, 1990). Wohlleben *et al* (1989) have described Tn*21* as a "natural expression cassette" for antibiotic resistance genes. The insertion of additional antibiotic resistance genes into the Tn*21* component of the transposon described here, would be an effective method of generating new transposons and resistance plasmids [also see Chapter 8.].

CHAPTER 6

THE EXPRESSION OF THE AMINOGLYCOSIDE RESISTANCE GENES IN STRAIN SAK

A. INTRODUCTION

Two aminoglycoside resistance genes were cloned from the DNA prepared from strain SAK, namely, *aadB* and *aacC*. This suggests that two types of AME may be associated with the gentamicin/tobramycin resistance phenotype of this organism. However, in the absence of demonstrable AME activity in lysates prepared from strain SAK [see 2.C.3.], this evidence is equivocal. To ascertain whether both of the resistance genes contribute to the gentamicin/tobramycin resistance phenotype, it was necessary to demonstrate transcription of both genes by Northern hybridization.

B. DETECTION OF AMINOGLYCOSIDE MODIFYING ENZYME mRNA TRANSCRIPTS IN STRAIN SAK

B.1. Methods used in the preparation of RNA and the hybridization of Northern blots

B.1.a. Extraction and quantitation of RNA

Total cellular RNA was prepared as follows: strain SAK was grown in the absence or presence of gentamicin (5μg/ml) in 100ml of Luria broth at 37^{o}C with aeration. On reaching A_{600} 0.7, an aliquot (10ml) was withdrawn from each of the cultures and kept on ice. To inhibit further initiation of transcription, rifampicin (0.2mg/ml) was added to the cells grown in the presence of gentamicin. At selected time intervals, 10ml aliquots were withdrawn from the culture and chilled on ice. The cells were harvested by centrifugation and lysed at 65^{o}C for 5min in 0.15M sucrose, 0.01M sodium acetate,pH4.5, 1% SDS (Von Gabain *et al*, 1983). Total cellular RNA was recovered by extraction with hot phenol (equilibrated with 1M sodium acetate, pH4.5).

After centrifugation the aqueous phase was re-extracted with phenol (equilibrated with 1M Tris-Cl,pH8.0)/chloroform. The aqueous phase was then treated with RNAase-free DNAaseI (100 units/mg RNA) for 30min at room temperature. RNA was precipitated at -20^{o}C overnight after the addition of three volumes of alcohol. The RNA precipitate was collected by centrifugation and dissolved in 100μl of diethylpyrocarbonate treated distilled water.

The RNA concentration was determined by measuring the absorbance at 260nm and the purity of the preparation was estimated by determining the ratio of the reading at 260nm and at 280nm. All of the RNA preparations had an OD_{260}/OD_{280} of >2.0.

B.1.b. Preparation of Northern blots

Total RNA (10μg) was denatured and electrophoresed in formaldehyde agarose gels (Maniatis *et al*, 1982). The RNA was transferred to a nylon membrane using a vacu-blotter. After transfer, the filter was rinsed briefly in 2xSSPE and air dried. RNA was fixed to the filter with U.V light [see 2.E.1.].

B.1.c. DNA:RNA hybridization

The Northern blots were hybridized with the *aadB* probe [see 2.E.2.] or the *Sph*I/*Sal*I *aacC* probe [see 3.D.]. The conditions for prehybridization and hybridization were as for DNA:DNA hybridizations [see 2.E.1.]. After hybridization the filters were washed as previously described [see 2.E.1.]. The membranes were exposed to Agfa Curix X-ray film for 1-3 days at -70^{o}C.

B.2. Results

The *aacC* probe hybridized to RNA extracted from strain SAK (Fig.18). Furthermore, the RNA transcript remained present for more than 60min after rifampicin treatment (Fig.18). In contrast, no signal was obtained with the *aadB* probe (Fig.18). This probe did, however, hybridize to RNA prepared from *E.coli* DK1 harbouring the recombinant plasmid (pGSH108) which contains the *aadB* gene cloned from strain SAK (Fig.18).

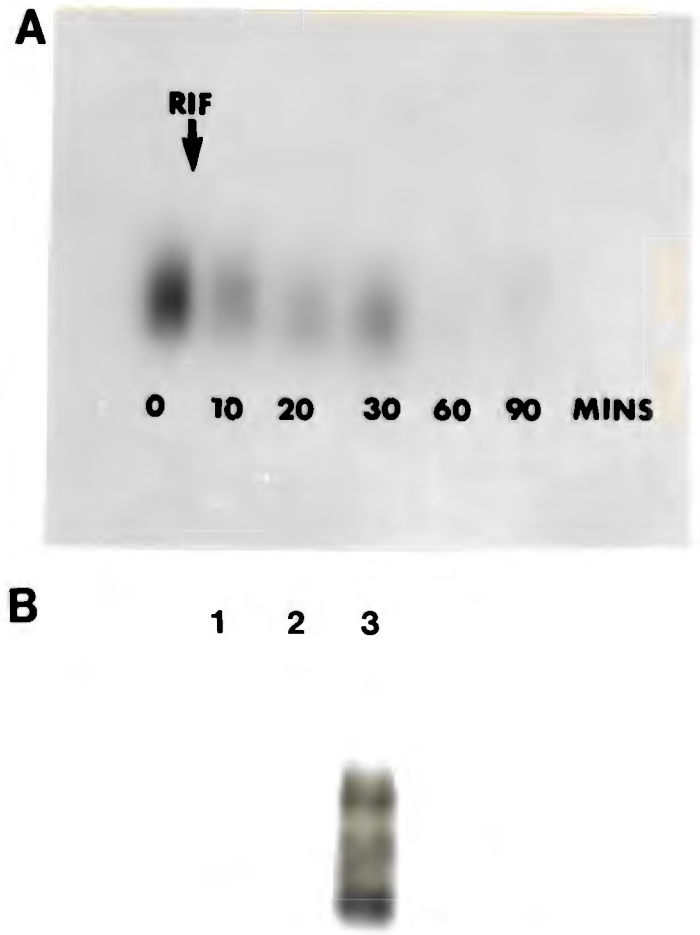


Fig.18. Hybridization of the Northern blots prepared from RNA extracted from strain SAK and E.coli(pGSH108) with the *aacC* and *aadB* gene probes

(A) Strain SAK was grown in the presence of gentamicin (5μg/ml) to an A_{600} of 0.7. At this point a 10ml aliquot was removed and RNA was extracted. After inhibition of transcription with rifampicin (0.2mg/ml), 10ml aliquots were withdrawn at either 10 or 30min intervals for up to 90mins. Total RNA was extracted from each of these aliquots. RNA (10μg) was electrophoresed in a formaldehyde gel. A Northern blot was prepared and probed with the *aacC* gene probe (801-bp *Sph*I/*Sal*I fragment from pGSH110). Autoradiography was for 24hrs.

(B) RNA was extracted from strain SAK grown in the absence and presence of gentamicin, and from *E.coli*(pGSH108). RNA(10μg) was electrophoresed in a formaldehyde gel. A Northern blot was prepared and probed with the *aadB* gene probe (1.1kb *Eco*RI/*Hin*dIII fragment from pGSH106). Lane 1, RNA from strain SAK grown in the absence of gentamicin; lane 2, RNA from strain SAK grown in the presence of gentamicin; lane 3, mRNA encoded by pGSH108 which contains the *aadB* gene cloned from strain SAK. Autoradiography was for 24hrs.

C. DISCUSSION

The RNA studies suggest that AAD(2") activity does not contribute to the gentamicin/tobramycin resistance phenotype of strain SAK. Furthermore, they illustrate a limitation in two of the approaches used in this study to investigate resistance mechanisms, namely, probing with an antibiotic resistance gene probe and cloning a functional resistance gene. Both techniques detect the potential for a bacterium to exhibit resistance, and not necessarily the mechanism of resistance. Recently, Vliegenthart *et al* (1990) have described the use of the polymerase chain reaction technique to identify aminoglycoside resistance genes. The same limitation applies to this approach: it only detects the potential for resistance. The gene must be shown to be functionally competent in the host strain before a role in the resistance phenotype can be ascribed to it.

Interestingly, the disc sensitivity pattern and MIC profile which has been used to infer mechanisms of resistance (Phillips *et al*, 1986) supports the finding that AAC(3) activity is responsible for the aminoglycoside resistance in strain SAK. Firstly, strain SAK is sensitive to kanamycin and secondly, the MIC of tobramycin for strain SAK was 16μg/ml. If AAD(2") activity was responsible for the resistance to the aminoglycoside, this figure would be more or less the same as the MIC of gentamicin (>128μg/ml) (Shannon & Phillips, 1982) as tobramycin is a better substrate for AAD(2") than for AAC(3) (Bongaerts & Vliegenthart, 1988).

It could be that the lack of expression of the AAD(2") is a function of the host strain. There are reports of the changeable phenotypic properties of plasmids and transposons in different genera of bacteria. For example, plasmids encoding resistance to gentamicin conferred effective resistance to this antibiotic on a *Pseudomonas* host, but not on *E.coli* (Kato *et al*, 1982). Hedges and Jacob (1975) have described a 98Mdal plasmid which conferred a high level of resistance to streptomycin and tetracycline but a low level of resistance to chloramphenicol (10μg/ml) on *Vibrio cholerae*. After

transfer to *E.coli* J62, the same plasmid conferred a high level of resistance to chloramphenicol (80μg/ml). Hedges and Jacob suggest that the reason for this is that the 98Mdal plasmid is imperfectly adapted to *Vibrio cholerae*.

An altered expression in different hosts has also been described with respect to transposons (Rubens *et al*, 1979). Tn*1699* carries resistance to ampicillin, carbenicillin, gentamicin and kanamycin; Tn*1700* encodes tobramycin resistance in addition to the markers carried by Tn*1699*. However, the kanamycin resistance phenotype was not apparent in their respective hosts, *Klebsiella pneumoniae* and *Serratia marcescens*. It was detected only after these sequences had transposed to pMB8 in *E.coli*.

Since the only enzyme with activity against gentamicin/tobramycin that we have previously demonstrated in local *Acinetobacter* isolates is AAD(2"), this study may reflect a change in the mechanism of resistance in this organism. What prompted this change is unknown. While both enzymes have activity against gentamicin/tobramycin, AAD(2") is not effective against netilmicin. Thus it is possible that the availability of netilmicin, coupled to a reduced usage of kanamycin, at Groote Schuur Hospital, facilitated the change. Studies on the changing patterns of mechanisms of resistance have been reported (Mayer, 1986; Phillips *et al*, 1986). Phillips *et al* (1986) reported that from 1974-1979, AAC(3)I activity accounted for most of the resistance in *Klebsiella* at St. Thomas' Hospital, London. By 1981, however, the picture had changed and AAC(3)II was the most commonly detected enzyme followed by AAD(2"). In a similar study, Witchitz (1981) reported that in 1969 the first aminoglycoside resistant Enterobacteriaceae were identified at the Claude-Bernard Hospital in France. The enzyme responsible for this resistance was AAD(2"), but subsequently, aminoglycoside resistance was due to AAC(3)I and AAC(3)II. In both of these studies there was no obvious reason, such as antibiotic selection pressure, for a change to have occurred. It is not known whether any of the organisms in these studies retained the former, albeit inactive resistance genes, or why the seemingly

redundant AAD(2") gene has been retained by strain SAK. If the *aadB* gene is truly redundant, it would be reasonable to assume that the gene will be lost to *Acinetobacter* isolates from a later period.

CHAPTER 7

THE DETECTION AND EXPRESSION OF AN *aadB* GENE IN A MORE RECENT *ACINETOBACTER* ISOLATE

The identification of an apparently redundant *aadB* gene in strain SAK was surprising. Moreover, it suggested the possibility that with time this gene might be lost to strains of *Acinetobacter*. To explore this, an *Acinetobacter* which was isolated in 1986, three years after strain SAK, was investigated. The investigation took a similar, although shortened route, to that adopted for strain SAK.

A. *ACINETOBACTER* STRAIN HAS: ANTIBIOGRAM AND INVESTIGATION OF AMINOGLYCOSIDE MODIFYING ENZYME ACTIVITY

The *Acinetobacter* strain identified in 1986 was isolated from a blood culture and was designated strain HAS.

Antibiotic susceptibility testing [see 2.B.1.] showed that strain HAS is resistant to chloramphenicol, gentamicin, tobramycin, netilmicin, neomycin, kanamycin and amikacin. Thus, unlike strain SAK, strain HAS is resistant to neomycin, kanamycin and amikacin.

To determine whether AME activity is present in strain HAS, cell free lysates were prepared from this strain, and assayed for the presence of AMEs [see 2.C.]. No acetylation of sisomicin (0.9-fold above background), and no adenylyation of sisomicin (0.8-fold above background) and streptomycin (0.9-fold above background) was detected. In the same extract, however, a phosphorylase with activity against neomycin (39-fold above background) was detected.

B. HYBRIDIZATION OF PLASMID AND CHROMOSOMAL DNA ISOLATED FROM STRAIN HAS WITH THE *aadB* PROBE

To ascertain whether the *aadB* gene is present in strain HAS, plasmid and chromosomal DNA were isolated [see 2.D.2.a.], DNA dot blots were prepared [see 2.E.1.] and hybridized with the *aadB* gene probe [see 2.E.2.]. The probe hybridized to JR66, the source of the *aadB* gene. A weak signal was generated by the plasmid preparation, but not the chromosomal preparation of strain HAS. To confirm that the weak signal was not a false positive, plasmid DNA isolated from strain HAS was separated by electrophoresis, transferred to a nylon membrane and hybridized to the *aadB* probe [see 2.E.1.]. The probe hybridized to two plasmid bands, one of which is probably the open circular form, harboured by strain HAS (Fig.19).

Fig.19. Plasmid DNA isolated from strain HAS and hybridized with the *aadB* gene probe

(A) Plasmid DNA was isolated from strain HAS and separated in a 0.8% agarose gel at 1V/cm for 16hrs.

(B) The autoradiograph of the Southern blot of the DNA shown in (A) hybridized with the *aadB* probe (1.1kb *Eco*RI/*Hin*dIII fragment from pGSH106) is shown. Autoradiography was for 18hrs.

C. CLONING AND CHARACTERIZATION OF THE *aadB* GENE FROM STRAIN HAS

To confirm that the sequences which hybridized to the *aadB* probe encode functional AAD(2") activity, the gene was cloned and expressed in *E.coli*. Total plasmid DNA prepared from strain HAS was digested with *Hin*dIII and cloned into the corresponding site in pUC18. *E.coli* DK1 transformants which were resistant to gentamicin/tobramycin were isolated. Recombinant plasmid DNA was isolated from twelve transformants and hybridized to the *aadB* probe. All of the recombinants contained an insert of 1.9kb which generated a signal with this probe. Furthermore, crude lysates prepared from *E.coli* DK1, containing a recombinant plasmid with an insert cloned from strain HAS, adenylylated sisomicin (20-fold above background).

D. DETECTION OF *aadB* mRNA TRANSCRIPTS IN STRAIN HAS

The DNA cloning experiments and the assays for AME activity suggest that strain HAS contains a structural *aadB* gene. The crucial question is whether the gene is expressed in strain HAS. To demonstrate RNA transcripts of the *aadB* gene, RNA was extracted from strain HAS, Northern blots were prepared and hybridized with the *aadB* probe [see 6.B.1.]. No signal was generated with the *aadB* probe, suggesting that this gene is not transcribed in strain HAS.

E. CONCLUSION

The objective of the studies described in this chapter was to determine whether the *aadB* gene had been lost to recent isolates of *Acinetobacter*. The data presented here suggest that a structural *aadB* gene is still present in *Acinetobacter* strain HAS. How, or why, it is "switched off" is not known.

CHAPTER 8

THE *aadB* GENES ISOLATED FROM STRAINS SAK AND HAS

Strains SAK and HAS which were isolated in 1983 and 1986, respectively, contain seemingly redundant *aadB* genes. This observation is based on two lines of evidence. Firstly, the DNA cloning experiments and the assays for AMEs in the *E.coli* hosts show that the coding sequences for AAD(2") are intact. In the case of the *aadB* from strain SAK, the promoter is functional, as the gentamicin/tobramycin resistant phenotype of the *E.coli* transformant is independent of the orientation of the insert in the recombinant plasmid. The *aadB* from strain HAS was cloned in one orientation only. Secondly, the RNA studies indicate that mRNA transcripts from the *aadB* genes are not made in the *Acinetobacter* strains. Why are the *aadB* genes expressed in *E.coli* but not in the *Acinetobacter* strains? To gain an insight into how the gene is regulated, and to determine whether there are any features at the DNA level that could account for this paradox, the *aadB* genes and their regulatory regions were sequenced as described in 4.B.1.

A. NUCLEOTIDE SEQUENCE ANALYSIS OF THE *aadB* GENES FROM STRAINS SAK AND HAS

A.1. DNA sequence of the *aadB* gene from strain SAK

The DNA sequencing strategy of the insert cloned from strain SAK is shown in Fig.20. Restriction fragments from the 1.9kb *Bam*HI/*Hin*dIII insert [see 3.B.1.] were subcloned in M13mp18 and 19 and sequenced [see 4.B.1.]. Occasionally band compressions occurred during gel electrophoresis which were resolved with formamide gels (Fig.21). The nucleotide sequence of the *aadB* of strain SAK is shown in Fig.22.

The DNA sequence of the *aadB* of strain SAK was compared with that of the gene described by Cameron *et al* (1986). They suggested that two potential initiation codons (ATG at nucleotides 945-947 and ATG at 1014-1016) could be functional. However, more recent information has shown that these codons are contained within a region which is common to many antibiotic resistance genes and they are, therefore, unlikely start codons. The ORF 1164-1695 is the most likely coding sequence for AAD(2") (Ouellette *et al*, 1987; Tenover *et al*, 1989). The DNA sequence of this region of the *aadB* of strain SAK is identical to the corresponding region of the *aadB* described by Cameron *et al* (1986). This ORF encodes a polypeptide of 19.8kDal which is at variance with the estimated M.W.(35kDal) of AAD(2") (Smith & Smith, 1974; Tenover *et al*, 1984). Thus, it is possible that the AAD(2") enzyme is a dimer.

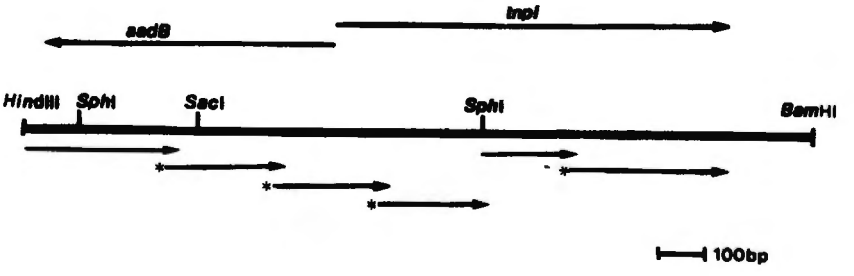


Fig.20. DNA sequencing strategy of the *aadB* gene and its flanking regions from strain SAK

The arrows below the bar indicate the direction and extent of the sequenced region.

◄——* , oligonucleotide primer. The location and the direction of the *aadB* and the integrase gene (*tnpI*) are indicated by arrows above the bar.

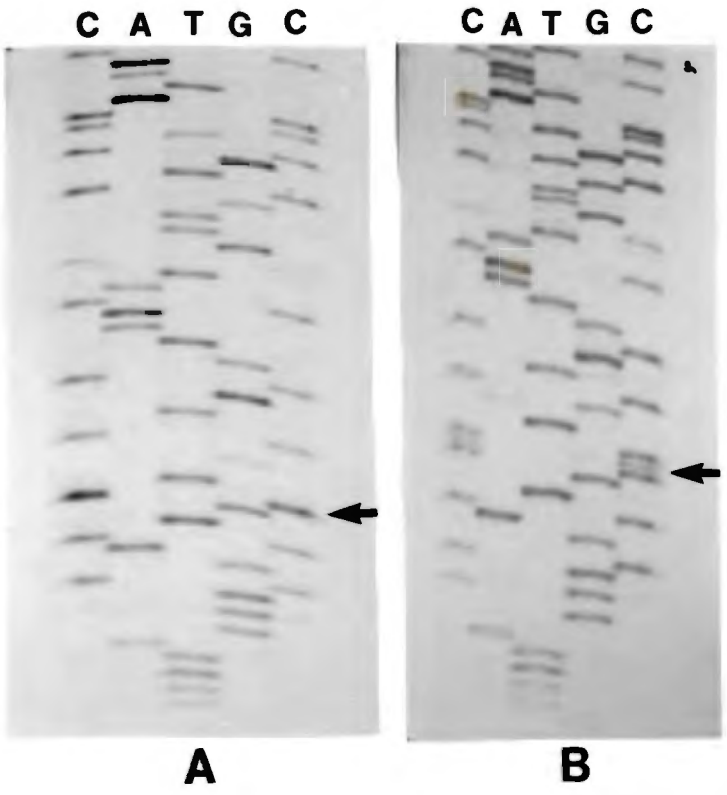
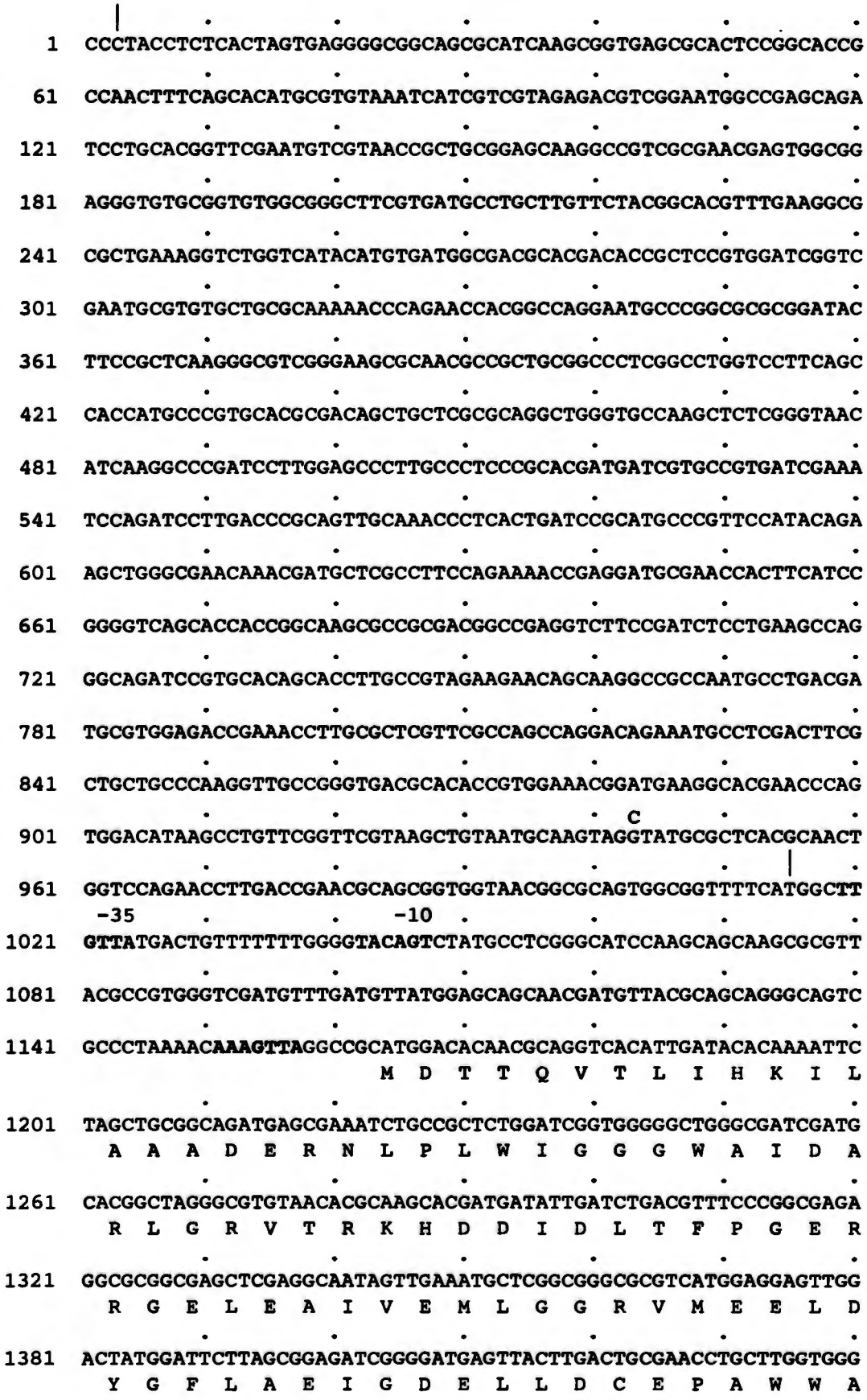


Fig.21. Autoradiographs of DNA sequencing gels showing band compressions and the resolution of these compressions with formamide gels

The sequence ladder illustrates the complementary strand of bases 1149-1103 in Fig.22.

(A) The sequencing reactions were separated by electrophoresis in a 6% polyacrylamide/urea gel. The compresed band is indicated with an arrow.

(B) The sequencing reactions were separated by electrophoresis in a 6% polyacrylamide and 40% formamide gel. The band indicated in (A) is no longer compressed and can be read as CCC (indicated with an arrow).


1 CCCTACCTCTCACTAGTGAGGGGCGGCAGCGCATCAAGCGGTGAGCGCACTCCGGCACCG
61 CCAACTTTCAGCACATGCGTGTAAATCATCGTCGTAGAGACGTCGGAATGGCCGAGCAGA
121 TCCTGCACGGTTCGAATGTCGTAACCGCTGCGGAGCAAGGCCGTCGCGAACGAGTGGCGG
181 AGGGTGTGCGGTGTGGCGGGCTTCGTGATGCCTGCTTGTTCTACGGCACGTTTGAAGGCG
241 CGCTGAAAGGTCTGGTCATACATGTGATGGCGACGCACGACACCGCTCCGTGGATCGGTC
301 GAATGCGTGTGCTGCGCAAAAACCCAGAACCACGGCCAGGAATGCCCGGCGCGCGGATAC
361 TTCCGCTCAAGGGCGTCGGGAAGCGCAACGCCGCTGCGGCCCTCGGCCTGGTCCTTCAGC
421 CACCATGCCCGTGCACGCGACAGCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAAC
481 ATCAAGGCCCGATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAA
541 TCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGTTCCATACAGA
601 AGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGAGGATGCGAACCACTTCATCC
661 GGGGTCAGCACCACCGGCAAGCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAG
721 GGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGA
781 TGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCG
841 CTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAG
901 TGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGGTATGCGCTCACGCAACT
961 GGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTT
-35 -10
1021 GTTATGACTGTTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTT
1081 ACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTC
1141 GCCCTAAAACAAAGTTAGGCCGCATGGACACAACGCAGGTCACATTGATACACAAAATTC
M D T T Q V T L I H K I L
1201 TAGCTGCGGCAGATGAGCGAAATCTGCCGCTCTGGATCGGTGGGGGCTGGGCGATCGATG
A A A D E R N L P L W I G G G W A I D A
1261 CACGGCTAGGGCGTGTAACACGCAAGCACGATGATATTGATCTGACGTTTCCCGGCGAGA
R L G R V T R K H D D I D L T F P G E R
1321 GGCGCGGCGAGCTCGAGGCAATAGTTGAAATGCTCGGCGGGCGCGTCATGGAGGAGTTGG
R G E L E A I V E M L G G R V M E E L D
1381 ACTATGGATTCTTAGCGGAGATCGGGGATGAGTTACTTGACTGCGAACCTGCTTGGTGGG
Y G F L A E I G D E L L D C E P A W W A

```
                .         .         .         .         .         .
1441  CAGACGAAGCGTATGAAATCGCGGAGGCTCCGCAGGGCTCGTGCCCAGAGGCGGCTGAGG
        D  E  A  Y  E  I  A  E  A  P  Q  G  S  C  P  E  A  A  E  G
                .         .         .         .         .         .
1501  GCGTCATCGCCGGGCGGCCAGTCCGTTGTAACAGCTGGGAGGCGATCATCTGGGATTACT
        V  I  A  G  R  P  V  R  C  N  S  W  E  A  I  I  W  D  Y  F
                .         .         .         .         .         .
1561  TTTACTATGCCGATGAAGTACCACCAGTGGACTGGCCTACAAAGCACATAGAGTCCTACA
        Y  Y  A  D  E  V  P  P  V  D  W  P  T  K  H  I  E  S  Y  R
                .         .         .         .         .         .
1621  GGCTCGCATGCACCTCACTCGGGGCGGAAAAGGTTGAGGTCTTGCGTGCCGCTTTCAGGT
        L  A  C  T  S  L  G  A  E  K  V  E  V  L  R  A  A  F  R  S
                .         .         .         .         .         .
1681  CGCGATATGCGGCCTAACAATTCGTCCAAGCCGACGGCTTCGCGGCGCGGCTTAACTCAG
        R  Y  A  A  *
                .         .         .
1741  GTGTTAGGCCGCATGGACACAGGCATGCAAG  1771
```

Fig.22. Nucleotide sequence and the derived amino acid sequence of the *aadB* gene from strain SAK

Dots above the sequence correspond to every 10 nucleotides. The sequence between nucleotides 1164-1695 contains the *aadB* structural gene. There is 100% sequence similarity between this sequence and the corresponding region of the *aadB* described by Cameron *et al* (1986). The putative promoter sequences, TTGTTA (-35) at nucleotides 1019-1024 and TACAGT (-10) at nucleotides 1042-1047, are indicated in bold type. Four direct repeats are underlined with arrows. The insertion site, AAAGTTA, at nucleotides 1151-1157, 5' of the *aadB* structural gene is indicated in bold type, so too is the 3' insertion site GTTA at nucleotides 1743-1746. The sequence 1015-3, between the two vertical lines, contains the *tnpI* structural gene. Although TAC and ATC are indicated as the start and termination codons, respectively, the actual codons are ATG and TAG, as the *tnpI* gene is transcribed from the opposite strand. There is a 99% sequence similarity between this region and the DNA sequence of the *tnpI* structural gene described by Mercier *et al* (1990). The sequence described by Mercier *et al* (1990) contains a C at position 943, which is indicated by a C above the sequence.

The region upstream of the start codon shares extensive homology, with not only the *aadB* described by Cameron *et al* (1986), but also with the 5'region of several, different resistance genes, all derived from Tn*21*-like transposons (Fig.23). These include: *aacC1* (Wohlleben *et al*, 1989; Tenover *et al*, 1989), *aacA1* (Tenover *et al*, 1988), ß-lactamase resistance genes (Ouellette *et al*, 1987; Levesque & Jacoby, 1988; Lafond *et al*, 1989); *dhfr* (Zolg & Hanggii, 1981; Swift *et al*, 1981; Flensburg & Steen, 1886) and *aadA* (Hollingshead & Vapnek, 1985).

Notwithstanding the DNA homology of the regulatory regions of the different resistance genes, there are a number of differences which need to be highlighted. Some of the resistance genes have one, strong promoter which consists of a -35 sequence, TTGACA, separated by 17-bp (18-bp in the case of the *aadA* gene) from a -10 sequence, TAAACT (Fig.23). However, in some of the antibiotic resistance genes, including the *aadB* of strain SAK, but not the *aadB* described by Cameron *et al* (1986), these promoter sequences contain two mutations, one in the -35 hexamer and one in the -10 hexamer (Fig.23). The mutation in the -35 region changes the second T of the hexamer to a G, which destroys the precise homology of this region to the consensus sequence (TTGACA). Alterations in the highly conserved TTG of the -35 sequence may cause a decrease in the promoter strength (McClure, 1985). The mutation in the -10 sequence affects one of the weakly conserved bases (McClure, 1985). It is, nevertheless, a down mutation (McClure, 1985). Interestingly, an additional mutation is found in the regulatory region of all the antibiotic resistance genes that have these mutations in the -35 and -10 sequences (Fig.23). This mutation consists of an insertion of the triplet, GGG. The importance of this insertion is that it alters the space between the sequences TTGTTA (-35) and TACAGT (-10) to 17-bp (18-bp in the *aadA* gene), thereby creating an alternative promoter (Fig.23). Relative to the start codon this could be termed the proximal promoter. Tenover *et al* (1989) suggest that sequences which contain the GGG insertion utilize the proximal promoter, while those lacking the insertion use the distal promoter. In which case, the proximal promoter is probably functional in the *aadB* gene cloned from strain SAK, while the distal promoter is used in the *aadB* described by Cameron *et al* (1986).

```
                -35                    -10
aacC1           TTGACATAAGCCT-GTTCGGTTCGTAAACTGTAATGCAAGTAGCGTA--TGC
aadB                        -                                    --
aadA            G           C          G                         AC
aacA1           G           -          G                         --
OXA-1           G           -          G                         --
aadB(SAK)       G           -          G                         --

aacC1           GCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGC
aadB
aadA
aacA1
OXA-1
aadB(SAK)

                                  -35                    -10
aacC1           AGTGGCGGTTTTCATGGCTTGTTATGAC-TGTTTTTTTG---TACAGTCTAT
aadB                                       -           ---
aadA                                       A           GGG
aacA1                                      -           GGG
OXA-1                                      -           GGG
aadB(SAK)                                  -           GGG

aacC1           GCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATG
aadB
aadA
aacA1
OXA-1
aadB(SAK)

aacC1           TTATGGAGCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGC
aadB                                       ------------------
aadA                                       ------------------
aacA1                                      ------------------
OXA-1                                      ------------------
aadB(SAK)                                  ------------------

aacC1           AGTCGCCCTAAAACAAAGTTAGGCCGCATG
aadB
aadA
aacA1
OXA-1
aadB(SAK)
```

Fig.23. The 5' region of some of the Tn*21*-like derived antibiotic resistance genes

The DNA sequence of the regulatory region of the *aacC1* gene (Wohlleben *et al*, 1989) is shown in full. The DNA sequences of the *aadB* (Cameron *et al*, 1986); *aadA* (Hollingshead & Vapnek, 1985); *aacA1* (Tenover *et al*, 1988) ; OXA-1 (Ouellette *et al*, 1987) and the *aadB* from strain SAK are identical to *aacC1* except where indicated. Nucleotides that differ from the *aacC1* are shown in bold type, while the absence of a nucleotide is indicated by a dash (-). The two putative promoters are shown. TTGACA (-35) and TAAACT (-10). The alternative promoter is TTGTTA (-35) and TACAGT (-10).

The DNA homology of the resistance genes derived from Tn*21*-like transposons is not confined to the 5' regulatory region. The genes share extensive DNA homology 3' of the structural gene as well (Fig.24). This homology extends over 59-bp (Cameron *et al*, 1986) and includes the TAA stop codon. A similar 59-bp element is found 3' of the *aadB* gene of strain SAK (Fig.24).

Early studies of the Tn*21* family of transposons revealed that the inserted resistance gene was always found in the vicinity of the gene encoding streptomycin/spectinomycin resistance (*aadA*) (Schmidt, 1984; Schmidt & Klopfer-Kaul, 1984; Wiedemann *et al*, 1985, 1986). Furthermore, these insertions always occurred either 5' or 3' of the *aadA* gene and in some cases replaced *aadA*. This led to the proposal of "hot spots" in the *aadA* gene region (Cameron *et al*, 1986; Ouellette *et al*, 1987; Schmidt *et al*, 1989). The "hot spot", AAAGTTA, 5' of the *aadA* gene was identified by Ouellette *et al* (1987). An identical sequence is present in the *aadB* of strain SAK at nucleotides 1151-1157 (Fig.22). The "hot spot", GTTA, 3' of the *aadA* gene is located in the common 59-bp element (Cameron *et al*, 1986; Ouellette *et al*, 1987; Wohlleben *et al*, 1989). This sequence is also present in the sequence of the *aadB* from strain SAK at nucleotides 1743-1746 (Fig.22).

Further observations were made by Sundstrom *et al* (1988). They suggested that the short GTTA sequence flanking the inserted gene (Fig.22) could be part of a target sequence that is duplicated at recombination. Furthermore, the four direct repeats (Fig.22) situated close to the putative target site suggested a DNA substrate that is efficient for an enzymatic recombination system. It has been demonstrated subsequently that Tn*21* encodes a *RecA* independent, site-specific integration system which may explain the recombination events adjacent to the *aadA* gene (Martinez & de la Cruz, 1988; Schmidt *et al*, 1989; Stokes & Hall, 1989). The site of recombination has been localized to GTTAG contained in the 59-bp element (Martinez & de la Cruz, 1990).

```
            C                                                    A
Consensus   G CTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCA GCGTTAGA
            T                                                    G
              *
aadB(SAK)   GCCTAACAATTCGTCCAAGCCGACG--GCTTCGCGGCGCGGCTTAACTCAGGTGTTAGG
```

Fig.24. A comparison of the region 3' of the *aadB* gene from strain SAK with the consensus sequence for this region

The 59-bp element which flanks the *aadB* gene from strain SAK is compared with the consensus sequence described by Cameron *et al* (1986). The stop codon for the *aadB* gene is contained within this element and is indicated by *. Nucleotides that differ from the consensus sequence are shown in bold type, while the absence of a nucleotide is indicated by a dash (-). The "hot spot" GTTA is underlined.

The gene for the enzyme that effects antibiotic resistance gene insertion, is adjacent to, and is transcribed in the opposite direction to the antibiotic resistance genes (Ouellette & Roy, 1987; Hall & Vockler, 1987; Martinez & de la Cruz, 1990; Mercier *et al*, 1990). The enzyme has been designated "integrase" (Martinez & de la Cruz, 1988, 1990; Mercier *et al*,1990) and it is the product of the *tnpI* gene. The sequence starting with an ATG at nucleotides 1015-1013 and terminating with the codon TAG at 5-3, which is adjacent to the *aadB* gene of strain SAK, was compared with the *tnpI* structural gene described by Mercier *et al* (1990). This region has a 99% DNA sequence homology with the *tnpI* gene. Similar DNA sequences, with varying degrees of homology to *tnpI*, have been found adjacent to antibiotic resistance gene insertions of other Tn*21*-like transposons (Cameron *et al*, 1986; Hall & Vockler, 1987; Ouellette *et al*, 1987; Tenover *et al*, 1989; Wohlleben *et al*, 1989).

A.2. DNA sequence of the *aadB* gene from strain HAS

To ascertain whether the *aadB* gene cloned from strain HAS is contained in a similar genetic structure, this gene was sequenced [see 4.B.1.]. Restriction fragments from the 1.9kb *Hin*dIII insert [see 7.D.] were subcloned in M13mp18 and 19 [see 4.B.1.]. Formamide gels were required to resolve areas of band compression. The nucleotide sequence of the *aadB* gene from strain HAS was compared with that of strain SAK. The two sequences are identical between nucleotides 781-1771 (Fig.22). This sequence includes the regulatory region and the 59-bp element 3' of the *aadB* gene. The *tnpI* gene of strain HAS has not been sequenced.

B. DISCUSSION

It is clear that Tn*21* is an acceptor which can receive and express new antibiotic resistance genes in response to changing patterns of antibiotic usage. This type of insertion event would explain the wide and rapid spread of antibiotic resistance genes. To substantiate this, Tn*21*-like transposons have been found on plasmids in different strains and genera of bacteria (Yamamoto *et al*, 1981; Schmidt, 1984; Levesque & Jacoby, 1988; Wohlleben *et al*, 1989; Grinsted *et al*, 1990). *Acinetobacter* can now be added to the list; this is the first time a Tn*21*-like transposon has been identified in this organism.

This finding raises important questions. Has the *aadB* gene of strain SAK inserted 5'of the *aadA*, or does it substitute for *aadA*? Since the sequencing data of the *aadB* from strain SAK stops shortly after the 59-bp element, this is not known. Another intriguing question is, has the *aadB* gene inserted into the Tn*21* component of the Tn*2670* described in Chapter 5? It is not known whether this gene has inserted into the chromosomally located putative Tn*2670*. It is unlikely to be part of the putative Tn*2670* which is located on the 120kb plasmid harboured by strain SAK, since this DNA did not generate a signal with the *aadB* probe [see 2.E.4.a.].

The *aadB* structural genes of strains SAK and HAS are identical. No feature of the DNA sequence can be used to explain why the genes are not transcribed. Two crucial questions remain. Why has the *aadB* gene been retained, and how is the *aadB* gene regulated ("switched off")?

With respect to the retention and stable inheritance of the *aadB* gene, Schmidt and Klopfer-Kaul (1984) have identified a transposition defective Tn*21*-like transposon, Tn*4000*, and suggest that deletion of the transposition functions could facilitate the stable inheritance of transposon acquired resistance genes. Interestingly, the gene acquired by Tn*4000* is *aadB*. It will be important to determine if the *aadB* genes of strains SAK and HAS are contained in defective transposons.

How is the *aadB* gene regulated? The simplest explanation would be that the promoter sequences are not recognised by the RNA polymerase of *Acinetobacter*. The possibility that *Acinetobacter* contains an RNA polymerase that recognises -35 and -10 sequences with a spacer of less than 17-bp has already been discussed [see 4.C.]. Curiously, deletion of the GGG triplet would result in a 14-bp spacer. However, this does not appear to be the answer. Preliminary studies suggest that the cloned *aadB* gene from strain SAK is expressed in *Acinetobacter* C91 BD413. It must be pointed out that it is not known whether this is in response to mutations that may have occurred in the regulatory region following transformation.

It is tempting to propose that the presence of the *aacC* gene influences transcription from the *aadB* promoter. How might this be? The hybridization studies suggest that there may be more copies of the *aacC* gene [see Fig.6] than the *aadB* gene [see Fig.3]. This, combined with the possibility that the *aacC* gene has a CAP sensitive promoter may facilitate its preferential expression. The intracellular concentration of AAC(3) may then act as a regulatory factor. On the other hand, it may be that CAP perturbs the DNA topology by its ability to bend DNA (McClure, 1985; Travers, 1989; Goodman & Nash, 1989). If *aadB* is a near neighbour of *aacC*, this alteration in DNA topology may make it impossible for the RNA polymerase to form a closed complex with the *aadB* promoter.

CHAPTER 9

AN ATTEMPT TO ACTIVATE THE *aadB* GENE IN STRAIN SAK

The presence of an intact structural *aadB* gene in strain SAK raises the question, can the *aadB* gene be "switched on"? To explore this possibility, strain SAK was grown in the presence of kanamycin. Kanamycin was chosen for two reasons. Firstly, and importantly, the kanamycin resistance phenotype of *E.coli* DK1(pGSH108), which carries the *aadB* gene cloned from strain SAK, demonstrates that this genotype is associated with kanamycin resistance [see 3.B.]. Secondly, the product of the *aacC* gene, which is expressed in strain SAK [see 6.B.2.], does not have activity against kanamycin. The evidence for this is two fold. Strain SAK is sensitive to kanamycin [see 2.B.1.], and *E.coli* HB101(pGSH110), which contains the *aacC* gene cloned from strain SAK, is also sensitive to this antibiotic [see 3.B.].

A. THE ISOLATION OF KANAMYCIN RESISTANT DERIVATIVES OF STRAIN SAK

The MIC of kanamycin for strain SAK was 8μg/ml [see 2.B.1.]. In an attempt to activate the *aadB* gene, strain SAK was cultured in the presence of kanamycin, the concentration of which was increased in 2-fold increments until a final concentration of 120μg/ml was reached. Initially, strain SAK was grown at 37^{o}C in 10ml of Luria broth containing 5μg/ml kanamycin. From this culture 0.04ml was removed and inoculated into 10ml of fresh Luria broth containing 10μg/ml kanamycin and incubated at 37^{o}C. This serial transfer was continued until strain SAK was resistant to 120μg/ml. This strain was designated strain SAK(Km^{r}). It was not possible to achieve adaption to a high level of resistance to kanamycin by a single transfer.

Antibiotic disc sensitivity testing [see 2.B.1.] showed that strain SAK(Km^{r}) is sensitive to neomycin and amikacin, however, it is less sensitive to tobramycin than the parent strain. MIC determination confirmed this observation; the MIC of tobramycin for strain SAK was 16μg/ml [see 2.B.1.], while that for strain SAK(Km^{r}) was 128μg/ml.

B. THE EXPRESSION OF THE *aadB* AND *aacC* GENES IN STRAIN SAK(Km^r)

The kanamycin resistant phenotype suggested that the *aadB* gene had been activated in strain SAK(Km^r). Indeed, the high MIC of tobramycin for strain SAK(Km^r) added weight to this interpretation, since tobramycin is a better substrate for AAD(2") than for AAC(3) (Bongaerts & Vliegenthart, 1988). To determine whether these phenotypic changes were due to AAD(2") activity, it was necessary to demonstrate the presence of this enzyme, or *aadB* transcripts in strain SAK(Km^r).

Cell free lysates were prepared from strain SAK, and from strain SAK(Km^r) and assays for AAC and AAD activity were performed [see 2.C.]. In these assays kanamycin, and not sisomicin, was used as the substrate. The lysates prepared from strain SAK did not contain detectable AAC activity (0.6-fold above background), or AAD activity (0.8-fold above background) against kanamycin (Table VI). This finding was expected, since a similar lysate prepared earlier from strain SAK did not acetylate, or adenylylate sisomicin (Table III). However, it was surprising to discover that the lysate prepared from strain SAK(Km^r) did not contain detectable AAD activity against kanamycin (0.8-fold above background). In the same lysate, however, AAC activity against kanamycin was detected (142-fold above background) (Table VI).

TABLE VI

ASSAY OF AMINOGLYCOSIDE MODIFYING ENZYMES IN STRAIN SAK (Km^r)

(i) Assay of acetyltransferase activity

	Substrate	
	None	Kanamycin
Strain SAK	1567	1078
Strain SAK(Km^r)	343	48867

(ii) Assay of adenylyltransferase activity

	Substrate	
	None	Kanamycin
Strain SAK	161	139
Strain SAK (Km^r)	151	130

Results are expressed as cpm bound to the phosphocellulose paper.
Aminoglycoside modification was assumed to have occurred if the cpm obtained in the presence of the substrate were at least twice the cpm obtained in its absence.

This result implies that the kanamycin resistant phenotype is not due to AAD(2") activity. Rather, it suggests that AAC activity is responsible for the kanamycin resistance. However, since the enzyme assay might not be sensitive enough to detect a low level of AAD(2") activity [see 1.C.3.b.ii.], RNA:DNA studies for the detection of aadB transcripts were done [see 6.B.1.].

Total cellular RNA was extracted from cells grown in the presence of 120μg/ml kanamycin. Northern blots were prepared and probed with the *aadB* and *aacC* probes. The RNA prepared from strain SAK(Km^r) did not generate a signal with the *aadB* probe, suggesting that transcripts from the *aadB* gene are not made in this strain. This result, in conjunction with the enzyme assays, implies that AAD(2") activity is not the mechanism of resistance to kanamycin in strain SAK(Km^r). However, the RNA prepared from strain SAK(Km^r) generated a signal with the *aacC* probe, indicating that this gene is transcribed in strain SAK(Km^r).

Swiatlo and Kocka (1987), and Hawkey and Constable (1988) have described similar findings. Swiatlo and Kocka described two strains of *Providencia stuartii* that were induced to grow in increasing concentrations of gentamicin and netilmicin. An increase in the MIC of gentamicin and netilmicin was parallelled by an increase in AAC(2') activity. In the study described by Hawkey and Constable, the MIC of netilmicin for *Serratia* increased after exposure to this antibiotic. This increase in MIC was coupled to an increase in AAC(6') activity. In both of the studies, growing the bacteria in antibiotic free media reversed this effect.

Strain SAK(Km^r) was grown in kanamycin free media. After 10 transfers, the activity of AAC returned to the original level (0.8-fold above background). The kanamycin resistant phenotype was also reversed.

C. DISCUSSION

It was possible to induce strain SAK to grow in the presence of 120μg/ml of kanamycin, but this was not associated with an increase in detectable AAD(2") activity. Instead, there was an increase in AAC activity. Interesting questions arise: is the AAC different from the AAC(3) already described in strain SAK? Is the increased production of the enzyme due to gene amplification, or to a regulatory effect? And finally, is the increased enzyme activity exclusively responsible for the kanamycin resistant phenotype?

The enzyme assays indicate that acetylation, and not adenylation, is responsible for the modification of kanamycin in strain SAK(Km^r). DNA cloning and preliminary sequencing data indicate that the enzyme is AAC(3). Furthermore, these data suggest that the AAC(3) is the same as that already described in strain SAK. It has been suggested that DNA mutations in the structural gene of an AME, may alter the substrate affinity profile of the enzyme (Bryan, 1984). Preliminary sequencing data suggest that mutations have not occurred in the structural *aacC* gene. Thus a variant of AAC(3), capable of efficiently recognizing and acetylating kanamycin, is probably not the mechanism of resistance to this antibiotic.

The increased AAC(3) activity is, if not wholly, certainly in part, responsible for the kanamycin resistance in strain SAK(Km^r). It is interesting to speculate on how this increase in production is achieved. It may be due to the activation of pre-existing *aacC* genes, or it may be that the *aacC* gene is amplified. In this context, there is strong evidence that strain SAK contains a plasmid which carries an *aacC* gene [see 3.D.], and a r-determinant which is similar to Tn*2670* [see 5.B.2.]. One of the features of r-determinants, such as Tn*2670*, is their ability to form, either autonomous poly r-determinants, or poly r-determinant R-plasmids [see 5.C.]. If the *aacC* gene, which may be linked to an IS*981*-like element [see 4.B.2.c.], has inserted into the putative Tn*2670*, it may be amplified in this way.

Alternatively, the increased production of AAC(3) may be due to an alteration in the regulation of the gene. Preliminary sequencing data have not identified any nucleotide changes in the regulatory region of the *aacC* gene. It is, however, possible that the activity of CAP regulates the *aacC* gene. There is evidence to suggest that the *aacC* gene has a catabolite sensitive promoter [see 4.C.]. If this is so, then it may be that releasing the *aacC* gene from catabolite repression, results in a considerably higher level of expression of AAC(3).

Whatever the mechanism of increased production, an important question remains: is the increase in AAC(3) activity exclusively responsible for the kanamycin resistant phenotype of strain SAK(Km^r)? There is evidence which suggests that it might not be. Consider the high copy number plasmid, pGSH110, which encodes AAC(3) activity, in *E.coli* HB101 [see 3.C.]. Lysates prepared from this transformant contained AAC activity against sisomicin (107-fold above background) (Table IV). If resistance to kanamycin depends on the amount of AAC(3) activity alone, then this transformant would be expected to be resistant to kanamycin. However, the kanamycin sensitive phenotype of *E.coli* HB101(pGSH110) is inconsistant with this prediction, and militates against elevated AAC(3) activity being the sole mechanism of resistance to kanamycin in strain SAK. A similar finding has been described by Perlin and Lerner (1986). They have demonstrated that an increase in AME activity alone, did not effect a large increase in the MIC of aminoglycosides for which the enzyme has a high K_m. Kanamycin is a poor substrate for AAC(3)II (Bongaerts & Vliegenthart, 1988). Interestingly, exposure to kanamycin induced *E.coli* HB101(pGSH110) to a high level of kanamycin resistance (120μg/ml); all of which suggests that another mechanism acts in concert with the increased AAC(3) activity to effect resistance to kanamycin.

To understand the nature of this additional mechanism, it is necessary to recall some of the details concerning AME mediated aminoglycoside resistance [see 1.C.3.c.]. Aminoglycoside resistance is dependent on the outcome of a competition between the

rate of uptake of drug into the bacterial cell and the rate of enzymatic modification of the aminoglycoside (Bryan & Van Den Elzen, 1977; Dickie & Bryan, 1978; Kagan & Davies, 1980). Thus, the rate of aminoglycoside uptake and its subsequent enzymatic modification interact to effect aminoglycoside resistance. It follows that an alteration in one, or both of these parameters, could affect the level of resistance. Perlin and Lerner (1986) have shown that the MIC of kanamycin and amikacin for *E.coli* was dependent on both the amount of APH(3')II and the reduced uptake of the antibiotics. It was also shown that exposure to kanamycin and amikacin raised the MICs (2-8 fold) of a variety of aminoglycosides for *E.coli* lacking APH(3')II. This increase in MIC was shown to be due to a diminished uptake of the aminoglycoside. Further studies on the affect of exposure to aminoglycosides, on the uptake of these antibiotics, have been described by Daikos *et al* (1990). They have demonstrated that growing *Pseudomonas aeruginosa* and some strains of *Enterobacteriaceae* in the presence of netilmicin induced an "adaptive resistance". This resistance was unstable, and was shown to correlate with a temporary down regulation of the enhanced aminoglycoside uptake phase (EDPII) [see 1.C.3.c.].

Thus, an increase in AAC(3) activity, together with a diminished uptake of the kanamycin, may be the mechanism of resistance to this antibiotic in strain SAK(Km^r). However, it is not known if kanamycin induces these changes, or whether exposure to kanamycin selects bacteria with an increased AAC(3) activity and an impaired uptake of the antibiotic.

Growing strain SAK in the presence of kanamycin was an attempt to activate the *aadB* gene. It was unsuccessful. If the presence of the *aacC* gene somehow or other influences the transcription of the *aadB* gene, it may be that inactivation of the former gene will effect transcription of the *aadB* gene. This still has to be investigated.

CHAPTER 10

SUMMARY AND GENERAL CONCLUSIONS

A clinical isolate of *Acinetobacter* (strain SAK) was screened for the presence of the antibiotic resistance genes, *aadB* and *cat*, by DNA:DNA hybridization with specific DNA probes. The *aadB* probe hybridized to chromosomal DNA, while the *cat* probe hybridized to plasmid and chromosomal DNA.

Chromosomal DNA fragments of strain SAK were cloned in pUC18 and transformed *E.coli* to gentamicin and tobramycin resistance, or to chloramphenicol resistance. Plasmid DNA fragments cloned in pUC18 conferred effective resistance to chloramphenicol on *E.coli*.

Screening of the recombinants encoding gentamicin and tobramycin resistance by DNA hybridization with the *aadB* probe, demonstrated that strain SAK contains at least two aminoglycoside resistance genes; one that hybridized to the *aadB* probe, and one that did not. Enzyme assays indicated that the hybridization positive insert encodes AAD(2") activity, while the recombinant that did not hybridize to the *aadB* probe encodes AAC(3) activity. Neither of these enzymes was demonstrated in lysates prepared from strain SAK.

If further studies had not been carried out, it would have been presumed that both genes are functional in strain SAK. However, RNA studies indicated that only the AAC(3) contributes to the gentamicin and tobramycin resistant phenotype. Transcripts from the *aadB* gene were not detected in total RNA prepared from strain SAK. Thus, antibiotic resistance gene probes and molecular cloning are useful tools in the investigation of resistance mechanisms, only if they are supplemented with studies to determine whether the gene is expressed in the host strain. On their own, they only detect the potential for resistance.

The AAC(3) group of enzymes comprise a number of isoenzymes which can be differentiated according to their antibiotic substrate profile (Vliegenthart *et al*, 1989). Based on the kanamycin sensitive phenotype of strain SAK, the AAC(3) enzyme in this strain would have been identified as AAC(3)V (Gomez-Lus *et al*, 1980). However, the DNA sequence of the *aacC* stuctural gene from strain SAK shows a 99% similarity with the DNA sequence of *the aacC2* gene [AAC(3)II] (Vliegenthart *et al*, 1989) also designated *aacC3* [AAC(3)III] (Allmansberger *et al*, 1985). Kanamycin is a poor substrate for AAC(3)II but a very good substrate for AAC(3)III (Table V). The complete DNA sequence similarity between two structural genes encoding supposedly different enzymes, is but one example of the confusion that surrounds the AAC(3) group of enzymes. The DNA sequence encoding AAC(3)V has been shown to hybridize to sequences encoding AAC(3)III activity (Barg, 1988). To avoid this sort of confusion, a careful and complete characterization of an enzyme is required before it can be classified as "new".

An interesting feature of the *aacC* structural gene from strain SAK is that it may have a catabolite sensitive promoter. Furthermore, it is possibly linked to an IS and, therefore, part of a class I transposon.

The *aadB* structural gene from strain SAK is flanked by sequences which share extensive homology with sequences 5' and 3' of antibiotic resistance genes that originate in Tn*21*-like transposons. This is the first time that a Tn*21*-like transposon has been identified in *Acinetobacter*.

No feature of the DNA sequence explains why the *aadB* gene is not transcribed in strain SAK. It may be that the presence of the *aacC* gene influences transcription of the *aadB* gene. The DNA hybridization studies indicate that there may be more copies of the *aacC* gene than the *aadB* gene; this could explain the preferential transcription from the *aacC* promoter. Alternatively, the putative CAP sensitive promoter of the *aacC* gene may affect transcription from the *aadB* promoter. Firstly, CAP facilitates

the productive interaction of RNA polymerase (McClure, 1985); it may be that enhanced transcription from the *aacC* promoter results in a low occupancy of the *aadB* promoter. Secondly, CAP is known to bend DNA (Travers, 1989), such a perturbance in the DNA topology may make it impossible for the RNA polymerase to interact with the *aadB* promoter, particularly if this gene is a near neighbour of *aacC*.

It was not possible to activate the *aadB* gene. Growing strain SAK in the presence of kanamycin, which is a substrate for AAD(2"), did not effect transcription. It did, however, affect transcription of the *aacC* gene; the quantity of AAC(3) produced by strain SAK grown in the presence of 120μg/ml of kanamycin was greatly increased. Moreover, enzyme assays showed that it has activity against kanamycin. The affinity of the AAC(3) for kanamycin is not explained by a mutation in the structural gene which results in an altered enzyme. The data suggest that the increased quantity of AAC(3), combined with a reduced uptake of kanamycin is the mechanism of resistance to this antibiotic. This is an important finding; as suggested by Martinez and Baquero (1990), antibiotic selective pressure may result in the emergence of a new resistance phenotype which is due to an increase in the production of an existing AME, rather than to the acquisition of a gene encoding a "new" AME. This finding should also caution against the hasty identification of "new" enzymes.

The mechanism of the increased production of AAC(3) is not known. It may be due to an altered regulation, or to an amplification of the gene.

Resistance to chloramphenicol in strain SAK is mediated by CAT. The gene for this enzyme has a plasmid and a chromosomal locus. Irrespective of the location, the 5'end of the *cat* gene is linked to IS*1*, whereas the 3'end is linked to sequences with 100% homology to the IR and the *tnpA* gene of Tn21. A similar linkage has been described for the *cat* gene in Tn*2670* (Womble & Rownd, 1988). A feature of Tn*2670* is that it is flanked by IS*1* sequences and is transposable (Iida *et al*, 1981; Hanni *et al*, 1982); moreover, it contains a second transposon, Tn*21* (Womble & Rownd, 1988).

It is instructive to note that Tn*2670*, contained on plasmid NRI was responsible for the resistance encountered in strains of *Shigella* in 1959 (Nakaya *et al*, 1960; Womble & Rownd, 1988). Nakaya *et al* (1960) demonstrated that this plasmid could be transferred by conjugation between bacteria. This was the first demonstration of plasmid associated antibiotic resistance, and offered an explanation as to how antibiotic resistance genes are disseminated. It is interesting to consider that 24yrs later a similar element is present in a strain of *Acinetobacter* isolated from a patient in South Africa.

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APPENDIX 1

Bacterial strains used in this study

Bacterial strain	Relevant characteristics	Reference/Source
Acinetobacter		
Strain SAK	Gm^R Tob^R Net^R $Chlor^R$ Str^R $Sulph^R$ Hg^R Neo^S Km^S $Amik^S$	Elisha & Steyn (1991a.b)
Strain HAS	Gm^R Tob^R Net^R Neo^R Km^R $Amik^R$ $Chlor^R$	This study
Strain YON	Gm^S Tob^S Net^S $Chlor^S$ Neo^S Km^S $Amik^S$	Elisha & Steyn (1991b)
C91 BD413	Str^R Rif^R	Dr A Vivian Bristol Polytechnic Bristol U.K.
E. coli		
J53	Nal^R	Prof J N Coetzee University of Pretoria R S Africa
HB101	F^- Str^R Rec^-	Boyer & Roulland-Dussoix (1969)
DK-1	del (*ara*, *leu*)7697 del (*srl* - *recA*)306	Willis *et al* (1981)
LK111	derivative of K514, *recA*, *lac* 2 Δ M15	Zabeau & Stanley (1982)

Amik, amikacin; Chlor, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nal, nalidixic acid; Neo, neomycin; Net, netilmicin; Rif, rifampicin; Str, streptomycin; Sulph, sulphonomides; Tob, tobramycin; Hg, mercury.

APPENDIX 2

Plasmids and phage DNA used in this study

	Relevant Characteristics	Reference/Source
Plasmids		
pBR322	Amp^R Tet^R	Bolivar *et al* (1977)
pUC18	Amp^R	Norrander *et al* (1983)
pMTL23	Amp^R	Chambers *et al* (1988)
pAT15 containing a *Hin*dIII fragment of pox DNA	$Chlor^R$. Used as a size standard for restriction analysis	K.R. Dumbell, University of Cape Town R.S. Africa
JR66	Gm^R Tob^R.$Chlor^R$. Used in the construction of the *aadB* probe	J. Davies, Institut Pasteur, Paris, France
pA_{10} cat_2	$Chlor^R$. Used in the construction of the *cat* probe.	G.K. Khoury Molecular Virology National Cancer Institute, Bethesda, Maryland, U.S.A.
Phage DNA		
M13mp 18 & 19		Norrander *et al* (1983)
λ	Used as size standard for restriction analysis	Daniels *et al* (1980)

Amp, ampicillin; Chlor, chloramphenicol; Gm, gentamicin; Tet, tetracycline; Tob, tobramycin.

APPENDIX 3

Plasmids constructed in this study

Recombinant	Origin of insert	Vector	Antibiotic modifying enzyme	Chapter reference
pGSH101	JR66	pBR322	AAD(2")	2.E.2
pGSH102	pGSH101	pBR322	AAD(2")	2.E.2
pGSH103	pGSH102	pUC18	AAD(2")	2.E.2
pGSH104	pGSH103	pUC18	AAD(2")	2.E.2
pGSH105	pGSH104	pUC18	AAD(2")	2.E.2
pGSH106	pGSH105	pUC18	AAD(2")	2.E.2
pGSH108	Strain SAK: chromosomal DNA	pUC18	AAD(2")	3.B.
pGSH110	Strain SAK: chromosomal DNA	pMTL23	AAC(3)	3.B.
pGSH201	Strain SAK: chromosomal DNA	pUC18	CAT	5.A.

AAD(2") 2" - *O* - adenylyltransferase
AAC(3) 3 - *N* - acetyltransferase
CAT Chloramphenicol acetyltransferase

APPENDIX 4

Materials, media, buffers and solutions

4.A Materials

4.A.1 DNA modifying enzymes were obtained from Boehringer Mannheim, BRL or Amersham International. Nick translation kits, ^{32}P-dCTP, ^{35}S-ATP & Hybond-N were all obtained from Amersham International. Sequenase kits were purchased from U.S. Biochemicals. All of the reagents were analytical grade or higher.

4.B. Media

4.B.1. Luria broth

For 1 litre :		
	Yeast extract	5g
	Tryptone	10g
	NaCl	5g

4.B.2. Luria agar

For 1 litre :		
	Yeast extract	5g
	Tryptone	10g
	NaCl	5g
	Agar	12g

4.B.3. Nutrient broth No. 1

For 1 litre :		
	Lab-Lemco powder	10g
	Peptone	10g
	NaCl	5g
	pH 7.5	

4.B.4. Mueller-Hinton broth

For 1 litre :		
	Beef, infusion from	300g
	Casein hydrolysate	17.5g
	Starch	1.5g
	pH 7.4	

4.C. Buffers and solutions

4.C.1. Agarose gel running buffer

0.4M Tris-acetate
0.001M EDTA
pH 8.0

4.C.2. Sequencing gel running buffer

0.133M Tris
0.04M Boric acid
0.0025M EDTA

4.C.3. RNA gel running buffer

0.04M MOPS
0.10m Sodium acetate
0.001M EDTA

4.C.4. 1 x SSC

0.15M NaCl
0.15M Sodium citrate
pH 7.0

4.C.5. 1 x SSPE

0.18M NaCl
0.01M Sodium phosphate
0.001M EDTA
pH 7.7