PURIFICATION, CHARACTERISATION AND MUTAGENESIS OF THE AMINOGLYCOSIDE (3')(9) NUCLEOTIDYLTRANSFERASE, ANT(3")-I

BY

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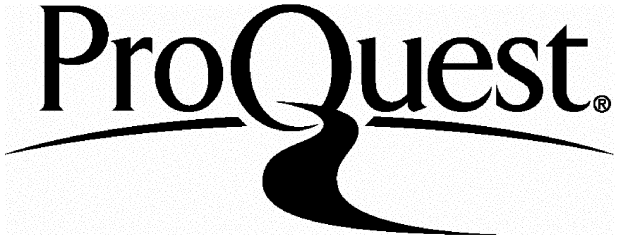

ABSTRACT

The antibiotic resistance gene, aminoglycoside(3")nucleotidyltransferase (*ant(3")-I*), which confers resistance to spectinomycin and streptomycin was isolated on a 1.95Kb *Hind*III restriction fragment from plasmid pIJ4642 (Davey, Ph.D. thesis 1992) and cloned into pBluescriptKS$^-$ vector in both orientations to form pQR601 and pQR602. Plasmid pQR601 was found to contain the gene in the same orientation as the *lac* promoter, whereas pQR602 was in the opposite orientation to the lac promoter of pBluscriptKS$^-$. To enhance expression of *ant(3")-Ia*, the gene was further cloned into other expression vectors namely, pTTQ19 and pUC19. This gave rise to pQR603 and pQR604 respectively upon transformation into *E.coli* JM107. To further increase expression levels, PCR was used to remove the large non-coding sequences flanking the gene and introduce two restriction sites at either end of the gene.

The PCR product was further cloned into two other expression vectors, pMTL2023 containing the *trp* promoter and pMTL1005 containing the *mdh* (malate dehydrogenase) promoter. this gave rise to recombinant plasmids pQR606 and pQR609 respectively. Expression studies indicated a two fold increase in expression from plasmid pQR606 and a 22 fold increase in expression from plasmid pQR609 with respect to pQR601.

The enzyme has subsequently been purified to homogeneity by a combination of ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography and gel filtration steps. Kinetic and molecular characteristics of the enzyme have been determined.

Comparative amino acid sequence homology studies have been performed with other members of ANT(3")-I enzymes and nucleotidyltransferases. This has

revealed extensive homology throughout, although certain regions of the gene appear to be more highly conserved than others. In order to test the biological importance of these regions, site-directed mutagenesis has been carried out to alter some highly conserved aspartic acid residues. The ability of mutant enzymes to confer resistance to antibiotic subtrates was evaluted.

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I DEDICATE THIS WORK TO MY FAMILY.

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ABBREVIATIONS

A	Adenine
AAC	Aminoglycoside Acetyltransferase
AAD	aminoglycoside adenylyltransferase
Amk	Amikacin
Amp	Ampicillin
ANT	Aminoglycoside nucleotidyltransferase
APH	Aminoglycoside Phosphotransferase
ATP	Adenosine 5'-triphosphate
bp	Base pair
Bis	N'N-methylenebis-acrylamide
C	Cytosine
Ci	Curie
cm	centimetre
Cm	Chloramphenical
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytosine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	DL-Dithiothreitol
EDTA	Ethylenediamineteraacetic acid
EtBr	Ethidium bromide
fplc	Fast performance liquid chromatography
Frac	Fraction
g	Centripetal force equal to gravitational acceleration
g	Gram
G	Guanine
IEF	Isoelectric focusing
IPTG	Isopropylthio-β-galactiside
Kb	Kilobase
kDa	Kilodaltons
Kg	Kilogram
Km	Kanamicin
KNT	Kanamycin nucleotidyltransferase enzyme

l	litre
lacZ	β-galactosidase gene
LNT	Lincosaminide nucleotidyltransferase enzyme
M	Molar
mA	Milliamperes
mdh	Malate dehydrogenase promoter
mg	Milligrams
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitres
mM	Millimolar
mol	Mole
M_r	Relative molecular mass
Neo	Neomycin
OD_n	Optical density at wavelength n
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PP	Pyrophosphate
p.s.i.	Pounds per square inch
Rnase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Sec	Second
Sm	Streptomycin
Spcm	Spectinomycin
T	Thymine
TBE	Tris borate tris buffer
TCA	Tricarboxylic acid
TE	Tris EDTA buffer
TEMED	NNN'N'-tetramethylethylenediamine
Tn	Transposon
Tob	Tobramycin
Trp	Tryptophan promoter
U	Uracil
UV	Ultraviolet light
V	Volts

Vol.	Volume
(v/v)	Volume to volume ratio
(w/v)	Weight to volume ratio
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microlitre
℃	Degrees Centigrade

E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
K. pneumoniae	*Klebsiella pneumoniae*
P. aeruginosa	*Pseudomonas aeruginosa*
P. fluorescens	*Pseudomonas fluorescens*
S. aureus	*Staphylococcal aureus*
S. choleraesuis	*Salmonella choleraesuis*
A. tumefaciens	*Agrobacterium tumefaciens*

AMINO ACIDS

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Aspartate	Asp	D
Asparagine	Asn	N
Cystine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Valine	Val	V

CHAPTER ONE

INTRODUCTION

1.1 AMINOGLYCOSIDE ANTIBIOTICS

1.1.1 Mode of Action

Aminoglycoside antibiotics are a large and important group of antimicrobial agents used particularly for serious, often life threatening episodes of infection. Table 1.1 summarises some of the more common clinical uses of this class of antibiotics. The mode of action of aminoglycosides has been extensively studied using streptomycin (or dihydrostreptomycin). Streptomycin binds irreversibly to a single site in the bacterial 30S ribosomal subunit (Bryan, 1984). Binding of streptomycin to free 30S ribosomal subunits about to initiate protein synthesis blocks their further progress and the intitiation complexes which form with these ribosomes are non-productive. Amino acyl-tRNA cannot bind to this distorted acceptor site and f-Met-tRNAfMet is released. Binding of streptomycin to ribosomes already engaged in protein synthesis slows the chain elongation process and can cause misreading of the genetic code to produce misread or nonsense proteins. Misreading results from indirect distortion of 16S ribosomal RNA at a critical point in its structure (the so called '530 loop'). However, misreading effects are not thought to contribute directly to the bactericidal action of the drug and the killing action results from the irreversible block on protein synthesis mediated at the level of protein initiation. Protein S12 of the 30S ribosomal subunit influences the binding of streptomycin to ribosomes, but the anti-intiation and misreading activities of streptomycin relate to perturbation of ribosomal RNA structure following binding of the antibiotic to its single site in the 30S ribosomal subunit (Cundliffe, 1987). Streptomycin therefore binds to ribosomal RNA, but its affinity for the site in 16S RNA is influenced by protein S12 (Gorini, 1974). The interaction of streptomycin with the 30S subunit involves at least four other ribosomal proteins S3 S5, S9 and S14. Since a change in the S12 protein alone causes streptomycin-resistance and a concomitant loss of the binding affinity of the 30S subunit for the drug, this protein must also have a role in the binding (Franklin & Snow, 1981). Therefore the streptomycin binding site includes residues in the 16S RNA which also bind protein S12.

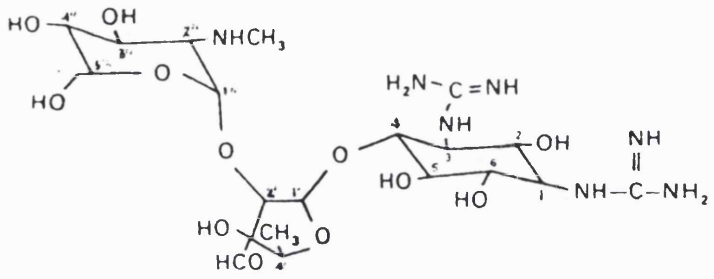
1.1.2 Classification and Structure

Strictly speaking, most of these antibiotics should be called aminoglycosidic aminocyclitols as their common structure consists of cyclic alcohols (aminocyclitols) in glycosidic linkage with amino-substituted sugars. The aminoglycosides may be divided into two main groups on the basis of whether they contain streptidine or 2-deoxystreptamine, although a few compounds (notably fortimicin) contain neither of these aminocyclitols (Price *et al.*, 1977).

Aminoglycoside Antibiotic	Clinical Use	Resistance Mechanism
Gentamicin	Septicaemia, Neonatal sepsis, Meningitis, Eye infections, Pneumonia, CNS infections	AAC (2), AAC(3), AAC(6), APH(2), APH(3), ANT(2),
Tobramycin	Acute pyelonephritis, Endocarditis, Pneumonia, serious eye infections, CNS infections	AAC(3), AAC(6), AAC(2), APH(2), ANT(2), ANT(4)
Neomycin	Infections of the skin or mucus membranes	AAC(1), AAC(3), APH(2), APH(3)
Amikacin and Netilomicin	Serious Gram negative infections	AAC(3), AAC(6), AAC(2), APH(2), APH(3), ANT(4)
Streptomycin	Tuberculosis (in conjunction with other drugs due to resistance)	APH(3), APH(6), ANT(3), ANT(6)
Spectinomycin	Gram negative infections including Gonorrhoeae	ANT(3), ANT(9)

Table 1.1-Table to show the clinical use of aminoglycoside antibiotics. The classes of enzymes able to inactivate the antibiotics are shown in the right hand column. Information on clinical usage gathered from the British National Formulary (1994).

STREPTOMYCIN



SPECTINOMYCIN

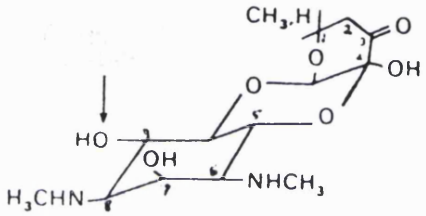


Fig. 1.1-The chemical structures of streptomycin A and spectinomycin. From Gale *et al.*, 1981 in Molecular Basis of Antibiotic action.

Spectinomycin, a pure aminocyclitol which lacks an amino sugar, is usually included among the aminoglycosides for convenience. It is also transported into bacteria in a fashion different from most "true" aminoglycosides.

The most common aminocyclitol appearing in aminoglycoside-amino-cyclitol antibiotics is deoxystreptamine. Other important aminocyclitols are streptidine (streptomycin) and actinamine (spectinomycin). The structures of these two antibiotics are shown in fig.1.1

1.1.3 Uptake of Aminoglycosides into cells

The mechanisms of uptake of aminoglycosides into bacterial cells has been extensively investigated (Dickie *et al.*, 1978; Holtje, 1979; Bryan and Kwan 1983). It has been shown that uptake of aminoglycoside in sensitive strains of both gram-positive and gram-negative bacteria occurs in three stages:

The first phase of uptake is rapid energy-independent (EIP) binding to the cell surface. Aminoglycosides are polycations with positive charges and are able to bind to anionic sites distributed on cell surfaces. Binding sites are mainly lipopolysaccharide, polar

heads of phospholipids, and possibly outer membrane proteins in gram-negative bacteria and teichoic acids and phospholipids in gram-positive bacteria. In gram-negative bacteria, a hydrophilic diffusion pathway through pores formed by outer membrane porin proteins is the major mechanism by which aminoglycosides reach the periplasm and ultimately, the cytoplasm (Bryan, 1984). In gram-positive bacteria, diffusion is through large, water filled interstices formed in peptidoglycan (Bryan and Kwan, 1983).

The second stage of uptake is the slow energy-dependent phase I (EDPI), which represents the initial passage of the drug across the cytoplasmic membrane. It requires the membrane to be sufficiently energised and uses the electrical potential of the proton motive force. The duration of EDPI depends on the concentrations of the aminoglycoside in the medium and the susceptibility of the ribosomes.

The third phase of aminoglycoside transport is called the energy-dependent phase II (EDPII). It is initiated when sufficient drug is present in the cytoplasm to bind to all of the ribosome particles. It signals the onset of lethality and the cessation of protein synthesis. The initiation of this phase requires susceptible ribosomes in the cell to be actively engaged in protein synthesis.

1.2 RESISTANCE TO AMINOGLYCOSIDES

Resistance to aminoglycosides can be divided into three categories; ribosomal resistance (Funatsu & Wittman, 1972; LaCosta *et al.,* 1977), modification of antibiotic by modifying enzymes and reduced uptake of antibiotic (Miller *et al.,* 1980).

Ribosomal resistance does not seem to be an important mechanism of resistance in clinical isolates. High level single step resistance to streptomycin results from mutation affecting the 30S ribosome subunit in bacteria (Nomura, 1970). This resistance results from a single amino acid change in the S12 protein of the small subunit (Funatsu & Wittmann, 1972). The ribosomes of the streptomycin resistant mutants that have been analysed do not bind streptomycin (Chang & Flaks, 1972). The alteration in 30S protein S12 is sufficient to prevent binding of the drug to the ribosomes. Analysis of streptomycin resistant mutants of *E.coli* has identified four different allelic sub-classes (*strA1, strA2, strA40 and strA60*) based on their influences on phenotypic suppression of nonsense mutations (Berckenridge & Gorini, 1970); each of these classes has been identified with substitutions at different amino acid positions in ribosomal protein S12 (Funatsu & Wittmann, 1972). Other target mutations causing resistance to aminoglycosides are *rpsE* (*spcA*), which results in an altered S5 protein and high level spectinomycin resistance; *neaB*, resulting in neamine resistance; *rpsQ* affecting the S17 protein; *ksgA*, which modifies the S-adenosylmethionine-6-N,N'-adenosyl(rRNA)-dimethyltrnsferase for 16-S rRNA; *ksgC*, which alters ribosomal protein S2 resulting in

kasugamycin resistance; and *ksgB* which again causes kasugamycin resistance (Bryan, 1984). Target resistance has been mainly described as a mechanism of resistance of aminoglycoside producing bacteria rather than the main mechanism of resistance in pathogens (Hotta *et al.*, 1981, Piendl & Bock, 1982). This is due to the fact that fast growing bacteria have multiple copies of the rRNA genes, and because resistance is genetically recessive to antibiotic sensitivity, only rare mutations in the gene for protein S12 are isolated. However, because the slow growing mycobacteria possess only single copies of the rRNA genes, streptomycin resistance can arise by mutational alteration of either 16S rRNA or ribosomal protein S12. Both types of mutation have been identified in *M.tuberculosis* (Finken *et al.*, 1993). As a result this type of resistance seems to be an important mechanism of resistance in this pathogenic species.

The other two mechanisms of resistance, presence of aminoglycoside modifying enzymes, and reduced uptake of antibiotic are of more clinical significance and have been studied in detail.

A number of bacterial strains have shown resistance to aminoglycosides as a result of mutations affecting membrane-energy metabolism or prevention of access of drug to bacterial targets by low-permeability barriers and active efflux (Nikaido, 1994). Whereas the other two mechanisms of resistance (target alteration and modification of antibiotic) are quite specific for a single drug or a single class of drugs, this seems to be a more general mechanism of drug resistance. Mutants have been isolated which show a decreased accumulation of aminoglycosides due to impaired transport across the membrane, resulting from a defect in membrane energisation (Ahmad *et al.*, 1980; Bryan and Van den Elzen, 1977; Miller *et al.*, 1980). Mutations producing resistance include ones affecting the synthesis of heme (Tien and White, 1968), menaquinone (Taber and Halfenger, 1976), cytochromes (Bryan *et al.*, 1980, Taber & Halfenger, 1976), and the proton-translocating adenosine triosphosphatase (ATPase) (Kanner & Gutnick, 1972). Mutations which produce resistance reduce or abolish the conversion of energy from oxidation-reduction reactions in the membrane to the synthesis of ATP or other uses. As this type of resistance affects all aminoglycoside antibiotics regardless of structure, some relative nonspecific property of aminoglycosides must be involved. One common property of the aminoglycosides is a large net positive charge at physiological pH due to multiple amino groups. Any positively charged molecule experiences a strong driving force for the entry into bacteria which maintain a large electrical potential, interior negative, across their membrane (Padan *et al.*, 1976). A reduction in electrical potential reduces this driving force and could decrease the rate of aminoglycoside uptake to make cells more resistant (Damper and Epstein, 1981).

Additionally there are other mechanisms of antibiotic resistance that are non-specific and of clinical significance. These mechanisms of resistance include: Prevention of

access of the antibiotic into the cell by means of a permeability barrier, mutations affecting the outer membrane porins (OmpF and OmpC), and active efflux of the antibiotic from the cell. These mechanisms can produce clinically significant resistance, for example, the intrinsic resistance of *P.aeruginosa* to a wide variety of antibiotics is due to a combination of a multidrug efflux transporter and an effective permeability barrier (Nikaido, 1994).

The genetic basis of resistance to aminoglycosides may be divided into two categories; intrinsic and acquired. Intrinsic resistance to aminoglycosides has been observed among anaerobic and fermentative bacteria. The genetic basis of most such resistance is chromosomal as it results from the metabolic patterns of the organisms.

Acquired resistance often results from plasmid-specified gene products. This type of resistance is wide spread and in a majority of cases, a result of aminoglycoside-modifying enzymes. Plasmid specified aminoglycoside resistance not associated with modifying enzymes has been reported in a small number of cases (Sagai *et al.*, 1975; Kono and O'Hara, 1976, 1977). However, very low levels of modifying enzymes may result in resistance and be difficult to detect (Prince and Jacoby, 1982).

Resistance to aminoglycosides due to the presence of modifying enzymes only occur in the EIP and EDPI phases of transport as the drug is inactivated during the EDPI phase and fails to induce EDPII as it cannot bind the ribosomes and inhibit protein synthesis. However, if the rate of drug transport exceeds that of modification, then significant amounts of unmodified antibiotic are able to bind to ribosomes. Resistance to a particular aminoglycoside is hence determined by the outcome of drug accumulation and drug modification. Thus, the most important factor in this process is the affinity of the modifying enzyme for its substrate. If the enzyme has a high affinity, the aminoglycoside is modified at the lowest concentration at which it is accumulated. Fig. 1.2 illustrates the concept of this rate competition, modification versus accumulation.

The transporter indicated in Fig1.2 is thought to be an inducible transport system for polyamines. There are two main reasons for this: firstly, cells which have been pretreated with low concentrations of streptomycin show a greatly enhanced uptake rate not only for streptomycin but also for spermidine and putrescine. The uptake of other nutrients into the cell under the same conditions was not increased. Secondly, streptomycin inhibits the uptake of spermidine as well as the uptake of putrescine in a competitive manner in induced cells. Conversley, polyamines compete with streptomycin for the same uptake system (Holtje, 1978).

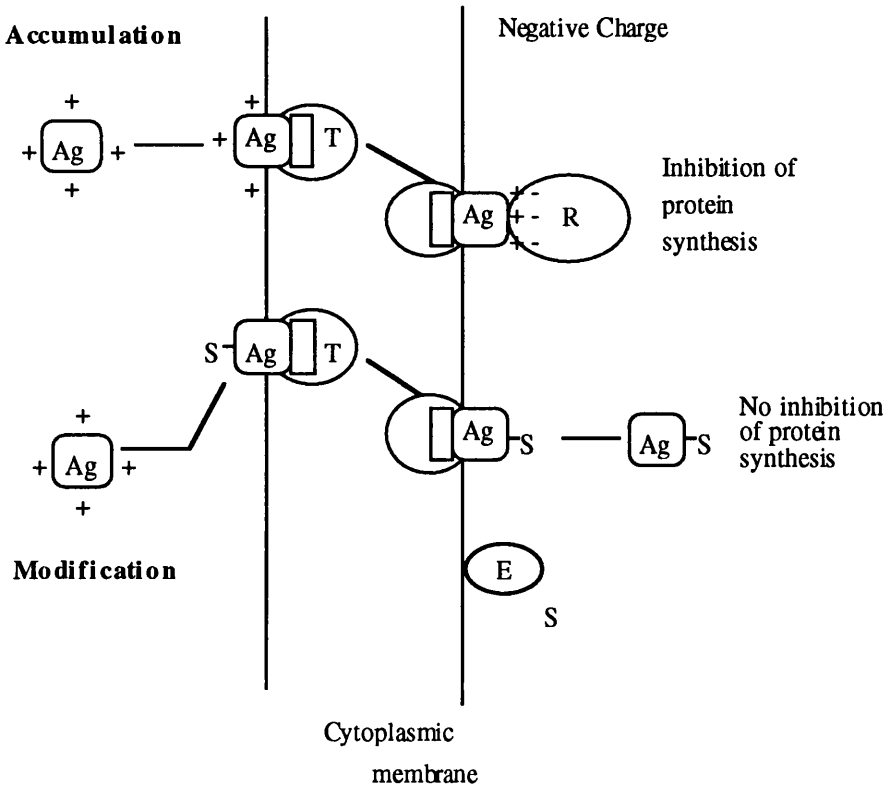


Fig. 1. Ag, aminoglycoside; S-Ag, modified aminoglycoside; E, modfying enzyme T, transporter; R, ribosome.

1.3 AMINOGLYCOSIDE-MODIFYING ENZYMES

In bacteria, resistance to aminoglycosides is often due to enzymatic inactivation of the antibiotic. There are three classes of aminoglycoside-modifying enzymes. The acetyltransferases, nucleotidyltransferase (adenylyltransferases), and phosphotransferases (Benveniste and Davies, 1973; Foster, 1983). The genes encoding these enzymes are often carried on plasmids or are associated with transposons, which aid the rapid spread of drug resistance across species boundaries. The reactions that they catalyse are N-acetylation, O-nucleotidylation, and O-phosphorylation respectively (fig 1.3). Within each of these reaction classes there are several groups of enzymes that attack a specific amino or hydroxyl group. In turn, a specific hydroxyl or amino group may be modified by more than one class of enzyme. Enzymes may differ by the spectrum of substrates that are modified.

A rational nomenclature for these enzymes was first proposed by Mitsuhashi, (1975) and has been amended since due to the discovery of new modifying enzymes. Nomenclature has followed the pattern: aminoglycoside acetyltransferase (AAC),

aminoglycoside nucleotidyl- or adenylyltransferase (ANT or AAD) and aminoglycoside phosphotransferase (APH). The site of the modification is provided by the number of the position modified given in the parentheses following the enzyme designation. In general, the positions of the cyclitol moiety are numbered 1-6, those of the moiety substituted at the 4-position of deoxystreptomine 1'-6', and those of the substitution at the 5 or 6 position of deoxystreptamine 1"-6". Enzymes with different substrate profiles are numbered by Roman numerals following the designation of the type of enzyme activity and site of modification. Finally, a, b, c, etc., is used for unique protein designations. For example, AAC(6')-Ia and AAC(6')-Ib are acetyltransferases that modify the antibiotic at the same position. However they are two unique proteins that confer identical resistance profiles. The genes encoding these proteins (*aac(6')-Ia* and *aac(6')-Ib* respectively) are unique genes with the same resistance profile.

There has been some confusion as to the location of these enzymes within the cells. Originally it was suggested that they were located in the periplasmic space of gram-negative bacteria as significant amounts were released by osmotic shock (Davies and Benveniste, 1974; Goldman and Northrop, 1975, 1976). However, the sequences of these enzymes do not show the existence of a signal sequence for secretion and the enzymes may be located at sites in the cell which are cytoplasmic but can be released by osmotic shock. Therefore, it is assumed that any membrane attachment of enzymes must be loose and easily disrupted (Foster, 1983).

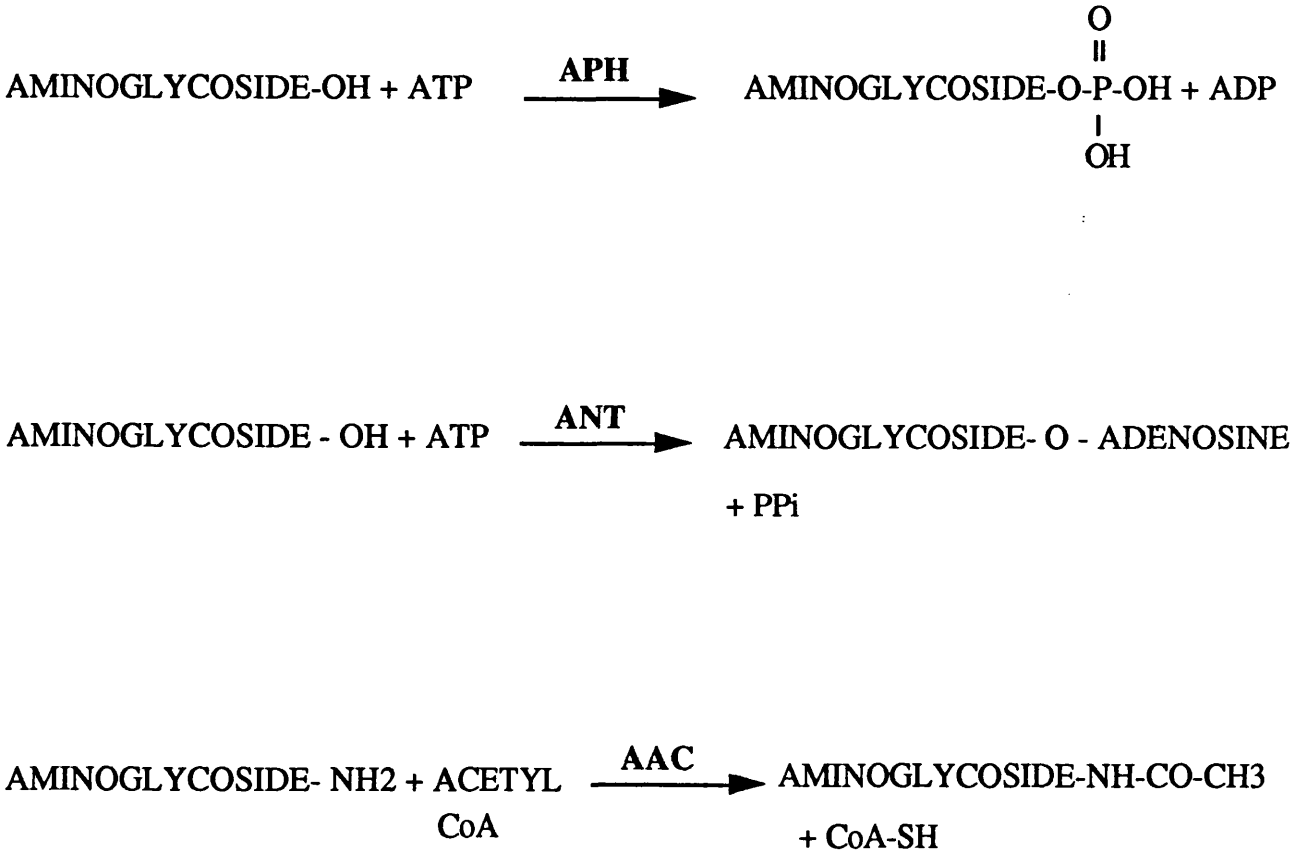


Fig.1.3 Biochemical mechanisms of aminoglycoside modification. The mechanisms of modification of aminoglycoside antibiotics by phosphotransferases (APH), nucleotidyltransferases (ANT), and acetyltransfearses (AAC).

1.3.1 Origin and spread of genes encoding aminoglycoside modifying enzymes

It is generally assumed that the aminoglycoside resistance genes were derived from organisms producing the aminoglycosides (Benveniste and Davies, 1973, Dowding and Davies, 1975, Davies *et al.,* 1977). Therefore, the *Streptomycetes* could have provided the initial gene pool from which some of the present aminoglycoside resistance genes were derived. The presence of these enzymes in aminoglycoside producing strains could provide a method of self protection against the lethal effect of the antibiotic produced. Support for this hypothesis has been provided by nucleic acid and protein sequence comparisons of aminoglycoside resistance determinants from producing organisms and clinical isolate sources (Shaw, 1984, Davies, 1992). However, producing organisms are not the only potential source of antibiotic resistance mechanisms. "Housekeeping" genes such as the sugar kinases and acyltransferases may have evolved to modify aminoglycoside antibiotics (Udou *et al.,* 1989, Shaw *et al.,* 1992, Rather *et al.,* 1993).

These genes may have been transferred to other bacteria as plasmid- or transposon-encoded drug resistance or both. Also there is evidence that soil bacteria commonly harbor antibiotic resistance plasmids (Bingham *et al.,* 1979, Docherty *et al.,* 1981, Falkow, 1975) persumably to help them combat antibiotics released into their environment by competitors. These genes could have transferred to clinically important bacteria either directly or via a number of intermediate hosts and plasmid vectors. Indeed, several genes encoding these modifying enzymes have been cloned from aminoglycoside producing organisms, and some have been shown to be genetically linked to the genes encoding enzymes involved in aminoglycoside production. Investigations carried out by Walker and Skorvaga (1973) and Nimi and collaborators (1971), have shown that streptomycin producing strains of *Streptomyces* possess enzymes that synthesise various phosphorylated streptomycin derivatives. In addition, DNA hybridization studies have indicated the presence of a cryptic chromosomal gene for an aminoglycoside acetyltransferase in many enterobacteria (Shaw *et al.,* 1993). Although experiments have indicated the presence of acetyltransferases and phosphotransferases in *Streptomyces*, no nucleotidyltransferase enzymes have so far been isolated from any strain. The evidence so far suggests that the gene for protein kinases and protein acetyltransferases were the ancestral sources of some classes of aminoglycoside modifying enzymes (Piepersberg *et al.,* 1988; Martin *et al.,* 1988). Even though *Streptomyces* may have provided the gene pool for

acetyltransferases and phosphotransferases, this does not seem to extend to the nucleotidyltransferase family of enzymes indicating that these genes may have arisen from a different source.

A second theory is that aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism (Piepersberg *et al.*, 1988). According to this theory, the selective pressure of aminoglycoside usage selects for mutations which alter the action of these enzymes, resulting in the ability to modify aminoglycosides.

The mechanisms of dissemination of aminoglycoside resistance genes has also been investigated. Many of the genes encoding resistance enzymes are associated with transposable genetic elements such as Tn21 (Hollingshead and Vapnek, 1985, Ouellette *et al.*, 1987, Stokes and Hall, 1989). In Tn21 there seems to be a specific region, the integron, into which many different resistance genes have inserted and although this region is most often associated with this transposon, there are a few other examples of integron sequences found independently (Hall *et al.*, 1991, Ouellette *et al.*, 1987, Shaw *et al.*, 1989). The ability of resistance genes to move to and from various replicons, some with a very broad host range, has allowed the rapid dissemination of these genes within bacteria.

Furthermore, the emergence of modern resistance genes such as those encoding gentamicin-tobramycin resistance may be attributed to the insertion of genes into pre-existing transposable elements to provide multi-resistance transposons. In addition, it has now become apparent that transposons and plasmids are not the only means by which genes encoding resistance are distributed; they may be picked up, transferred, and integrated by bacterial viruses using a non-homologous recombination system known as co-integration (Piepersberg *et al.*, 1984).

1.3.2 Aminoglycoside Acetyltransferases (AAC) and Phosphotransferases (APH)

1.3.2a The AAC Family of Enzymes

The aminoglycoside acetylating enzymes catalyse the transfer of acetate from acetyl-CoA to an amino group on the antibiotic (see fig. 1.3). Four classes of N-acetyltransferases, which modify aminoglycosides in the 1-, 3-, 6'-, and 2'- amino groups, have been identified (Benveniste and Davies, 1973; Foster, 1983; Lovering *et al.,* 1987; Mitsuhashi, 1975).

1.3.2b APH Family of Modifying Enzymes

Aminoglycoside phosphotransferases are probably the most widely distributed of all aminoglycoside-aminocyclitol modifying enzymes. All phosphorylating enzymes inactivate the antibiotics by catalysing the transfer of the terminal phosphate of ATP to a hydroxyl group of the drug (fig. 1.3). There are three groups of phosphotransferases which modify the aminoglycosides in the 2", 3, 4, and 6-hydroxyl position (Courvalin & Davies, 1977; Oka *et al.,* 1981; Beck *et al.,* 1982; Gray & Fitch, 1983; Herbert *et al.,* 1986; Heinzel *et al.,* 1988; Martin *et al.,* 1988)

1.4 AMINOGLYCOSIDE NUCLEOTIDYLTRANSFERASES

The nucleotidylating enzymes utilise ATP or other nucleotides as substrates in the enzymatic modification of a hydroxyl group on an aminoglycoside-aminocyclitol antibiotic. These enzymes form an important group of resistance determinants and are found in both gram-negative and gram-positive organisms.

Five classes of aminoglycoside nucleotidyltransferase enzymes have been identified to date (Hollingshead and Vapnek, 1985; Jacoby *et al.,* 1990; Lee *et al.,* 1987; Matsumura *et al.,* 1984; Murphy, 1985; Ounissi and Courvalin, 1987). The classification of these enzymes, as already mentioned, is based upon the site of adenylation on the antibiotic. They are further classified by their substrate specificities. Table 1.2 shows a summary of the classification and characterisation of the nucleotidyltransferase enzymes which have so far been identified.

ANT(2")- This enzyme catalyses the nucleotidylation of the susceptible antibiotic on the 2"-hydroxyl group of amino-hexose III. Substrates for this enzyme include gentamicin, tobramycin, dibekacin, sisomicin, and kanamycin. ANT(2") is widespread among many gram-negative bacteria. Three genes encoding 2"-O-adenylyltransferase activity have been reported (Lee *et al.*, 1987). However, DNA sequence analysis has determined that one of these genes is identical to an acetyltransferase aminoglycoside modifying enzyme, and hence probably misclassified.

ANT(3")- This enzyme is characterised by resistance to streptomycin and spectinomycin (Davies and Smith, 1978, Hollingshead and Vapnek, 1985). It modifies the 3"-hydroxyl group of streptomycin or the 9-hydroxyl group of spectinomycin. The *ant(3")* gene has been cloned in association with several transposons (Hollingshead and Vapnek, 1985, Schmidt *et al.*, 1988) and it is

widespread among gram-negative bacteria. Adenylation of streptomycin by ANT(3")-I enzyme is illustrated in fig. 1.4.

ANT(4')-The ANT(4') aminoglycoside adenylyltransferases confer resistance to tobramycin, amikacin, isoepamicin, and other aminoglycoside antibiotics with 4'-hydroxyl groups by adenylating this position on the drug (Jacoby *et al.*, 1990). The ANT(4') group of enzymes have further been sub-classified into distinct groups. ANT(4')(4")-I has been shown to modify aminoglycosides at both the 4'- and 4"-hydroxyl groups, and hence, confer resistance to dibekacin (Santanam and Kayser, 1978, Schwotzer *et al.*, 1978). However, this mechanism of resistance is only found in gram-positive bacteria (Miller *et al.*, 1980, Schwotzer *et al.*, 1978). ANT(4')-II has been found in gram-negative bacteria and results of hybridisation studies carried out on clinical isolates suggest that it is a different enzyme to that found in gram-positive bacteria (Jacoby *et al.*, 1990).

ANT(6) group of aminoglycoside nucleotidyltransferases are characterised by resistance to streptomycin and are found to be unique to gram-positive organisms, in particular, enterococci and streptococci (Ounissi *et al.*, 1990).

ANT(9)- This enzyme confers resistance to spectinomycin only by adenylating the 9-hydroxyl group of this antibiotic. The gene encoding this enzyme was isolated from *Staphylococcus aureus* transposon Tn554 and has not been found in *Enterococcus* species (Davies and Smith, 1978; Murphy, 1985). Although the resistance profile of the ANT(9) and ANT(3") enzymes differ (spectinomycin only as opposed to spectinomycin and streptomycin), the two proteins show 61% sequence similarity and 34% sequence identity, suggesting a common origin.

A number of other aminoglycoside nucleotidyltransferases have been isolated which up to now, have not been assigined an "ANT" nomenclature. These include the AADS, isolated from *Bacteroides* compound transposon Tn4551 (Smith *et al.*, 1992), and AADK from *Bacillus subtilis* (Ohmiya *et al.*, 1989).

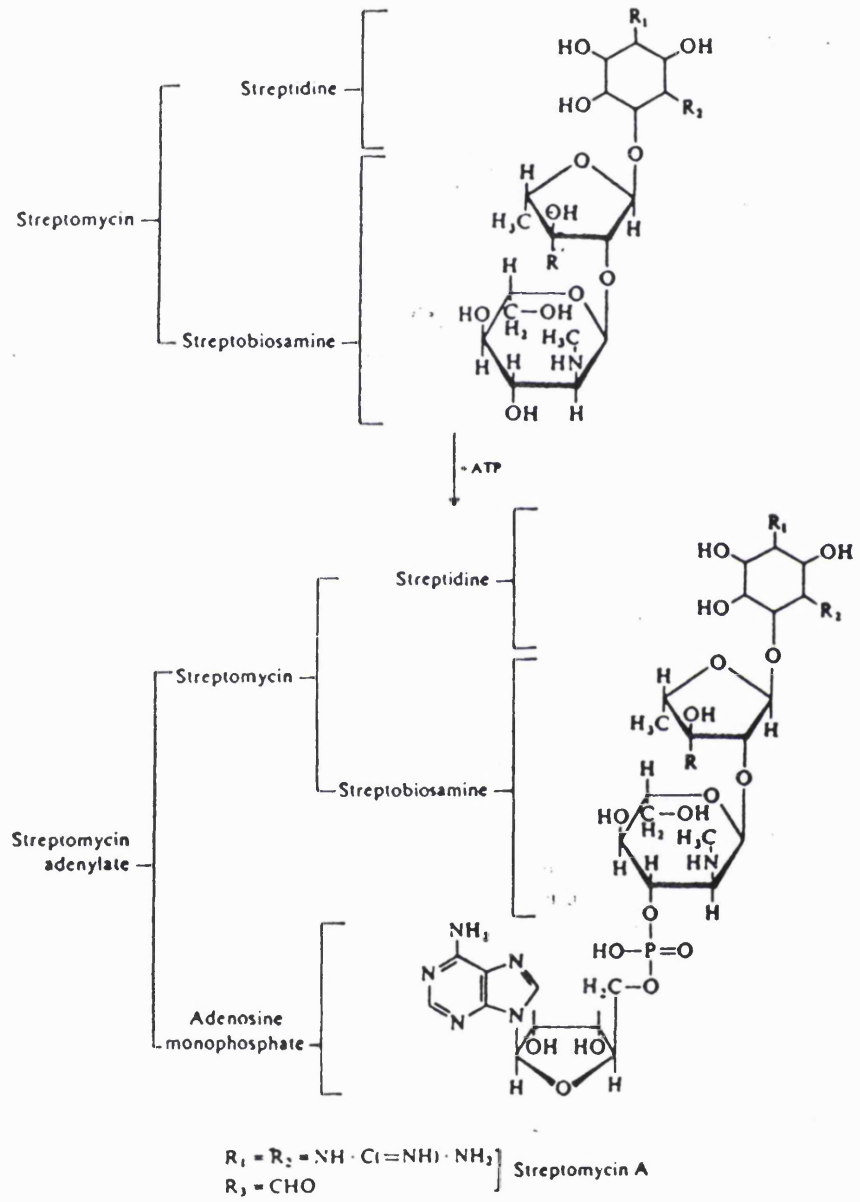


Fig. 1.4-The adenylation of streptomycin by ANT(3")-I enzyme. Gale *et al.* (1981) in Molecular basis of antibiotic action.

Resistance Mechanism	Cloned Genes	Alternative Nomenclature	Resistance Profile	Molecular Mass (kDa) (D/P)	Isolated From	Plasmid	Reference
ANT(2")	*ant(2")-Ia* *ant(2")-Ib* *ant(2")-Ic*	*aadB* *aac(3)-IIIc*	Gm, Tob, Dbk, Siso, Km	28.0 P 29.6 P	*Enterobacteriaceae* *K.oxytoca*, strain 80 *E. cloacae*, strain 178	pSCL14 pSCL29 pSCL35	Hollingshead & Vapnek (1985); Lee *et al.* (1987)
ANT(3")-I	*ant(3")-Ia*	*aadA,* *aad(3")(9)*	Sm, Spcm	31.6 D/33.2 P	*Enterobacteriaceae*	R 538-1	Hollingshead & Vapnek (1985)
ANT(4')-I	*ant(4')-Ia*		Tob, Amk, Isp, Dbk	34 D/29.1 P	*S. aureus*		Matsumura *et al.* (1984)
ANT(4")-II	*ant(4")-IIa*		Tob, Amk, Isp	29.2 P	*P. aeruginosa*	pMG77	Jacoby *et al.* (1990)
ANT(6)-I	*ant(6)-Ia*	*ant6*	Sm	36.1 P	*E. faecalis*	pJH1	Ounissi *et al.* (1987)
ANT(9)-I	*ant(9)-Ia*	*aad(9), spc*	Spcm	29.0 P	*S. aureus*	Tn554	Murphy (1985)

Table 1.2-Characteristics of aminoglycoside nucleotidyltransferase enzymes. Abbreviations: Amk, amikacin; Dbk, dibekacin; Gm, gentamicin; Isp, isepamicin; Km, kanamycin; Sm, streptomycin; Spcm, spectinomycin; Tob, tobramycin.
D, determined molecular mass
P, Molecular mass predicted from putative protein sequence data.

1.5 Structure-Function Relationships Among Aminoglycoside-Modifying Enzymes

There is significant protein sequence homology among some of the aminoglycoside modifying enzymes, as well as homology to other proteins with related functions (Allmansberger *et al.,* 1985; Martin *et al.,* 1988; Piepersberg *et al.,* 1988; Salauze *et al.,* 1991). Shaw *et al.* (1993) reported the relatedness of aminoglycoside resistance proteins by comparing a large number of resistant strains of bacteria with all of the known genes coding for aminoglycoside resistance enzymes. Fig.1.5 shows the results obtained where clustering of similar sequences into families can be seen. Several distinct subfamilies could be identified; (i) APH, including all of the 3'-phosphorylating enzymes, (ii) AAC(6)-Ib group of enzymes; (iii) ANT(9) and ANT(3"); (iv) AAC(3)-I group; (v) APH(6) enzymes; (vi) AAC(6)-Ic; and (vii) AAC(3). Some of the aminoglycoside modifying enzymes, however, do not fall into any distinct family. These include most of the nucleotidyltransferases (ANT(6)-Ia, ANT(4')-Ia, ANT(4")-IIa, and ANT(2")-Ia), two groups of acetyltransferases (AAC(6')-Ia and AAC(2')-Ia), and one phosphotransferase group, APH(4)-Ib.

Multiple sequence alignment analysis carried out on amino acid sequences coding for aminoglycoside phosphotransferases and aminoglycoside acetyltransferase enzymes has shown that the presence of common motifs within members of the same class (Shaw *et al.,* 1993). Even though members of phosphotransferases and acetyltransferases show extensive homology at the amino acid level to other members in their class, the same is not true of nucleotidyltransferase enzymes.

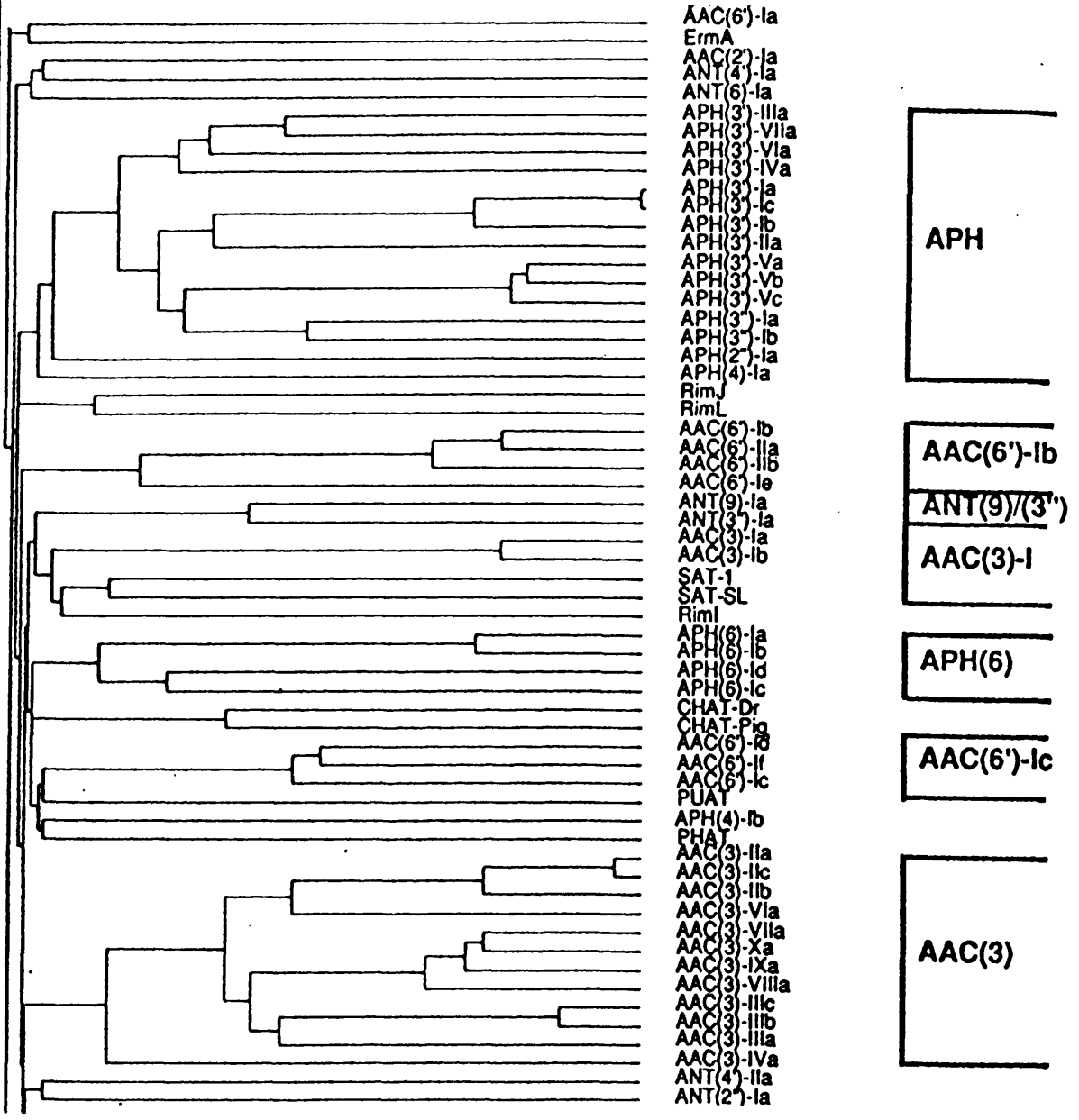


Fig. 1.5-Comparison of aminoglycoside modifying enzymes and other acetyltransferase enzymes. The bold type represents the main groups of the modifying enzymes. Figure taken from Shaw *et al.* (1993).

1.6 Nucleotide sequence analysis of nucleotidyltransferases

Comparison of amino acid sequences from various members of the nucleotidyltransferase family has revealed no significant homology between the different classes of these enzymes (Shaw *et al.,* 1993). Moreover, there is also little protein sequence homology within members of the same class of ANTs. For instance, when comparing ANT(4')-I and ANT(4')-II, only 45% of their protein sequences were similar (Matsumura *et al.*, 1984). This limited similarity and lack of conservation may be due to the evolution of this class of enzymes.

Hypothetical genealogy of aminoglycoside nucleotidyltransferase genes is represented in (fig. 1.6). The lower half of the chart represents the long-term micro-evolution of the *ant* genes where they may have arisen from chromosomally encoded functions and developed into aminoglycoside resistance genes. Their divergent evolution leading to ANT(3'), ANT(9), ANT(2"), and ANT(4',4") types of enzymes also seems to have occurred during this long-term evolution. Macro-evolutionary changes, such as the 'hot spot' insertion of the *ant* genes into transposons are thought to be events independent of their short-term evolution. This also seems to be true for the alterations of adjacent sequences and transposition functions (Schmit, 1984). The *ant(2)* gene types I and II (Coombe and George, 1981) are thought to be separated only by short term changes from an ANT(4', 4") encoding gene, which is thought to represent a more original state.

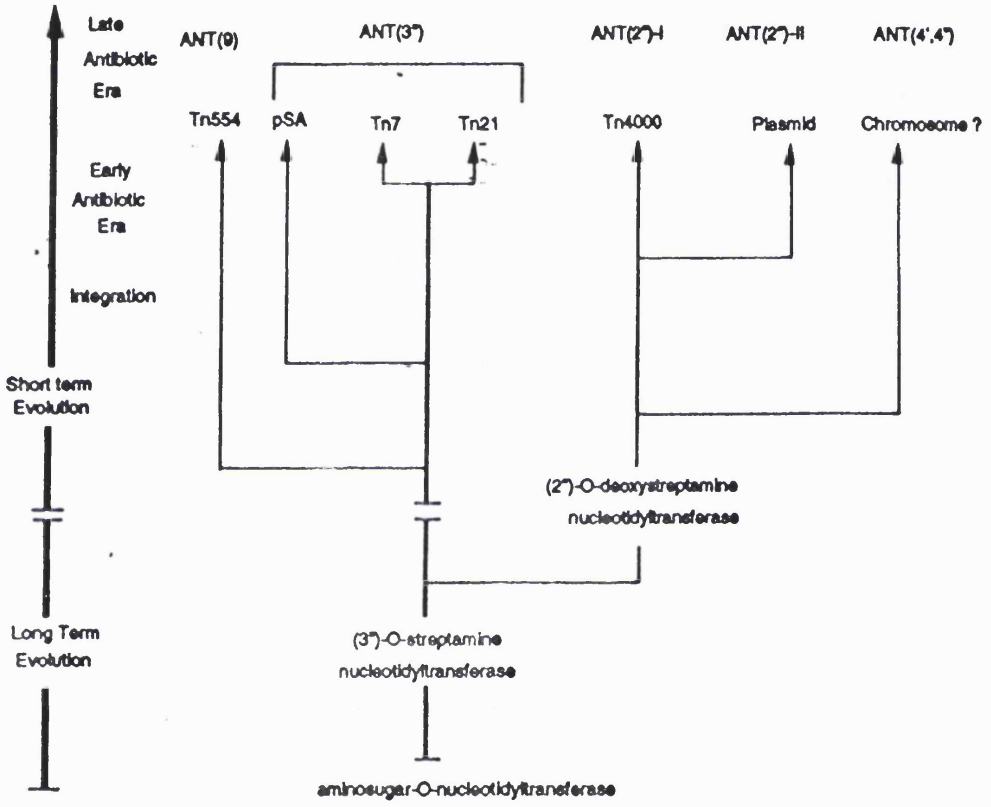


Fig. 1.6-Hypothetical genealogy of the aminoglycoside nucleotidyltransferase family of enzymes. The tree shows the possible ancestral origin of these genes through their short and long term evolution. Figure from Schmidt et al. (1988).

The therapeutic use of aminoglycoside antibiotics has decreased in recent years, largely because of the resistance confered to these antibiotics by the presence of modifying enzymes in clinical isolates (Davies, 1994). Due to the introduction of the less toxic broad spectrum β-lactams, there has been a relatively limited effort to seek specific inhibitors of the aminoglycoside modifying enzymes, which might have offered therapeutic potential to extend the effective range of this class of antibiotics. Although some active inhibitors have been identified, none of them has been deemed fit for introduction into clinical practice (Davies, 1983).

Knowledge of the molecular biology and structure-function relationships of these enzymes would expand our understanding of the origin, evolution and dissemination of these genes. In addition, they would provide insight into which new aminoglycoside resistance mechanism may arise in the future, how fast they can be disseminated, and potentially, how aminoglycoside usage can overcome some of the problems.

The understanding of the biological activity of enzymes in molecular terms generally requires an understanding of the roles of the specific amino acids in the catalytic activity or the substrate binding specificity of proteins. To investigate the molecular biology of these enzymes, site-specific mutations need to carried out on conserved residues in the protein. Previous studies have indicated that single-point mutations carried out on the conserved residues in acetyltransferases and phosphotransferases can dramatically alter the substrate profile of an enzyme (Rather *et al.*, 1992; Kocabiyik and Perlin, 1991; Blazquez *et al.*, 1991).

Comparison of amino acid sequences of nucleotidyltransferse proteins isolated from different species can identify conserved (and potentially) essential amino acid residues which may be involved in catalysis.

Lastly, X-ray crystallisation studies of the structures of these enzymes could reveal precisely where the substrate interacts and what changes in the enzymes may potentially allow the emergence of new resistance profiles.

Very little research has been carried out on the aminoglycoside nucleotidyltransferase class of enzymes as compared to the phosphotransferase and acetyltransferase modifying enzymes. The ANT(3") family of enzymes has been the least investigated of the nucleotidyltransferase modifying enzymes. This is probably due to the introduction and clinical use of semi-synthetic derivatives of aminoglycoside antibiotics which offer more resistance to modifying enzymes. The study of ANT(3)-I enzyme was of particular interest as these enzymes are the main class of modifying enzymes responsible for the inactivation of streptomycin and the only group capable of adenylating spectinomycin. As a result of their action these once useful antibiotics are now considered clinically redundant. A better knowledge of their actions may provide useful inhibitors of these enzymes.

1.7 Objectives of this study

The objectives of this project were two fold:

1) To purify and characterise the ANT(3")-I aminoglycoside nucleotidyltransferase enzyme and carry out physical and chemical characterisation of the protein. As the genes coding for aminoglycoside modifying enzymes are expressed at a very low level, the *ant(3")-Ia* gene needs to be over expressed.

2) To identify important amino acid residues within the ANT(3")-I protein that may be implicated in binding of either, the aminoglycoside substrate or the co-substrate ATP. This was to be achieved by performing site directed mutagenesis on conserved residues present in the protein. Conserved residues will be identified

by sequence analysis studies on members of the aminoglycoside nucleotidyltransferase family.

CHAPTER TWO

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. CHEMICALS AND MATERIALS

All chemical reagents were of analytical reagent grade (Analar) where possible and were obtained from the following companies unless stated otherwise in the text:

Aldrich Chemical Company, Dorset, UK

Amersham International, Bucks, England

Amicon Ltd., Gloucestershire, England

BDH Chemicals Ltd, Poole, Dorset, England

Bethesda Research Laboratories (BRL/GIBCO), Paisley, Scotland

Biorad Laboratories, Caxton way, Watford, Herts

Difco Laboratories, West Molesley, Surrey, England

Oxoid Ltd, Basingstoke, Hants, England

Pharmacia (LKB) Ltd, St. Albans, Herts, England

Promega Ltd., Southampton, England

Sigma Chemical Company (UK) Ltd., Poole, Dorset, England

Stratagene Ltd., Cambridge, England

2.1.2 Bacterial Strains

The bacterial strains used in this study are listed below:

Strain	Genotype	Reference
E.coli JM107	F'*traD36,lacI*^{q}Δ*(lacZ) M15, proAB/e*14$^-$(mcrA$^-$) Δ*(lac$^-$ proAB),thi,gyrA96,* (Nal^r)*end*A1,*hsd*R17(r_K^- m_K^+),*rel*A1,*Sup*E44,*mcr*A	Yanish-Perron *et al.* (1985)
E.coli BMH71-18 *mut* S	*thi, sup*E, Δ*(lac-pro*AB), [*mut*S::Tn10][F',*pro*A$^+$B$^+$, *laq*I^{q}ZΔM15]	Zell & Fritz (1987)
E.coli BL21 (DE3)	F$^-$, *ompT, hsdS$_B$*, (r_B^- m_B^-) *gal, dcm* (DE3)	Studier & Moffat (1986)

2.1.3 Plasmids and Phagemids

The plasmids and phagemids used in this study are tabulated below:

Plasmid/Phagemid	Marker	Reference
pIJ4642	Sm^r/Spm^r	Davey (1992)
pBluescript KS^-	Ap^r	Stratagene
pUC19	Ap^r	Yanisch-Perron *et al.* (1985)
pTTQ19	Ap^r	Sprat, M.J. (1987)
pMTL2023	Cm^r	Chambers *et al.* (1988)
pMTL1005	Ap^r	Alldread *et al.* (submitted)

2.1.4 Media

All media listed below were prepared in distilled, de-ionised water and autoclaved at 15 p.s.i (121°C) for 20 min. unless otherwise stated.

2.1.4.1 Growth Media

Nutrient broth: 25 gl^{-1} nutrient broth No.2 (oxoid) was used for routine growth of cultures.

M9 Salts (10x Stock): This defined minimal media was used for the cultivation of auxotropic *E.coli* strains requiring thiamine.

	gl^{-1}
Na_2HPO_4	152
KH_2PO_4	15
NaCl	5
NH_4Cl	10

The pH was adjusted to 7.4 with 10M NaOH, and the mixture autoclaved, 10ml of this 10x stock was added to 180ml molten 2% agar/water (w/v) prior to the addition of the following constituents:

1ml 100mM $MgSO_4$	(autoclaved separately)
1ml 10mM $CaCl_2$	(autoclaved separately)
1ml 20%(w/v) Glucose	(filter sterilised)
0.1ml 2mg ml^{-1} Thiamine	(filter sterilised)

2.1.4.2 Buffers and Solutions

All solutions listed below were prepared in double distilled , de-ionised water and autoclaved unless otherwise stated.

TE buffer (1x)

Tris-HCl	10mM
Na_2EDTA	1mM

pH 8.0

TBE Buffer (10x)

Tris base	0.9M
Boric acid	0.9M
Na_2EDTA	0.03M

The Na_2EDTA was added as a made up solution at pH 8.0

Stop Mixture

Sucrose	40% (w/v)
Na_2EDTA	0.1mM
Bromophenol blue	0.015mgml^{-1}

Restriction Buffer (10x)

Tris-HCl	500mM
$MgCl_2$	100mM
Dithiothreitol	10mM

pH 7.5

Ligation buffer (10x)

Tris-HCl	660mM
$MgCl_2$	100mM
Dithiothreitol	100mM
ATP	1mM

pH 7.5

Birnboim solution

Tris-HCl	25mM
Na_2EDTA	10mM
Glucose	0.9% (w/v)
Lysozyme	2mg ml^{-1}

pH 7.5

SDS-NaOH solution

SDS	1% (w/v)
NaOH	0.2N

Solution III

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

SDS-PAGE resolving gel buffer

Tris-HCl	0.75M
SDS	0.2% (w/v)

pH 8.8

SDS-PAGE stacking gel buffer

Tris-HCl	0.125M
SDS	0.1% (w/v)

pH 6.8

SDS-PAGE electrophoresis buffer

Tris-HCl	50mM
Glycine	0.38M
SDS	0.1% (w/v)

pH 8.3

SDS-PAGE sample loading buffer

Tris-HCl	0.200M
Glycerol	10% (v/v)
β-mercapoethanol	5% (v/v)
SDS	2% (v/v)
Bromophenol blue	0.005%(w/v)

pH 6.8

SDS-PAGE gel staining solution

Coomassie brilliant blue R250	0.05%(w/v)
Glacial acetic acid	100 ml
Methanol	450 ml

SDS-PAGE gel clearing solution

Methanol	40%(v/v)
Glacial acetic acid	10%(v/v)

SDS acrylamide stock solution

	$g\ l^{-1}$
Acrylamide	300
Bis-acrylamide	8

Made up to 1 l with distilled water, de-ionised with amberlite resin, and filtered.

Western blot transfer buffer

Tris-HCl	25mM
Glycine	190mM
methanol	20%(v/v)

pH 8.3

TBS

Tris-HCl	20mM
NaCl	500mM

pH 7.4

Nucleotidyltransferase Assay Buffer

Tris-HCl	1.3mM
$MgCl_2$	0.81mM
NH_4Cl	7.8mM
DTT	30μM
Spectinomycin	0.25mM
ATP	0.5mM
[8^{-14}C] ATP	0.1μCi

pH 7.4

2.1.4.3 Antibiotics

Antibiotics were incorporated in both solid and liquid media for the selection of resistant bacterial clones.

(i) Ampicillin

Stock solution: 50mg ml^{-1} of the sodium salt of ampicillin in distilled water. This was sterilised by filtration (0.22μm Millipore disposable filter) and stored at -20°C.

(ii) Streptomycin and Spectinomycin

Stock solution: 20mg ml^{-1} of streptomycin sulphate in distilled water, sterilised by filtration (0.22μm Millipore disposable filter) and stored at -20°C.

(iii) Other antibiotics

Stock solutions of 20mg ml^{-1} of antibiotics were made in distilled water, and sterilised by filtration as before. Antibiotics not soluble in water, such as chloramphenicol, were dissolved in ethanol. No filter sterilisation was necessary.

2.2 METHODS

2.2.1 Growth and preservation of bacterial strains

E.coli strains were routinely grown aerobically at 37°C in nutrient broth. These strains were also cultivated on this broth solidified with agar (2% w/v). Where necessary, the medium was supplemented with the appropriate antibiotic. When required, the plate medium was supplemented with X-gal (stock 40mg ml^{-1} in dimethylformamide) to a final concentration of 0.08mg ml^{-1} and with IPTG (stock 20mg ml^{-1} in distilled water)

to a final concentration of 0.05mg ml^{-1}. Cultures were maintained for the short term at 4°C on nutrient agar plates. Longer term storage was at -70°C as broth cultures supplemented with 20% (v/v) sterile glycerol.

2.2.2 Transformation of *E.coli*

Strains of *E.coli* were transformed essentially as described by Cohen *et al* (1972)

2.2.2.1 Preparation of competent cells

An overnight culture of the desired *E.coli* strain, grown in 5ml nutrient broth (NB) supplemented with 20mM $MgSO_4$, was used to inoculate 50 to 100ml prewarmed NB, containing 20 mM $MgSO_4$, in a 250ml conical flask. The culture was incubated at 37°C with vigorous aeration and the OD_{450} of the culture monitored at 15 to 30min intervals. When the OD_{450} reached 0.6 (approximately 3-4 hours) the culture was centrifuged (Sorval RC5B, SS34 rotor, 15000g for 10min at 4°C) and the bacterial pellet washed in 25ml of ice cold 75mM $CaCl_2$ containing 15% (v/v) glycerol. Following centrifugation, the pellet was resuspended in 2.5ml ice cold sterile 15% (v/v) glycerol, 75mM $CaCl_2$. The competent cells were either used immediatly or stored as 200µl aliquots at -70°C.

2.2.2.2 Transformation with plasmid DNA

Transformation was achieved by incubation of the transforming DNA (typically 50 to 500 ng of DNA in a maximum volume of 10µl) with 200µl of competent cells on ice for 30-60min, followed by a 10min heat shock at 37°C. The cells were added to 5ml nutrient broth and grown at 37°C with vigorous aeration for 2-3 hours before plating out on selective agar.

2.2.2.3 Determination of Minimum Inibitory Concentration (MIC)

Minimum inhibitory concentration were determined in universal bottles containing 5ml nutrient broth and the desired concentration of the aminoglycoside antibiotic. An overnight culture of *E.coli* carrying the desired plasmid, was used as the inoculum. The bottles were then incubated overnight at 37°C with aeration.

2.3 TREATMENT OF DNA WITH ENZYMES

2.3.1 Restriction Endonucleases

The reaction conditions were in accordance with the manufacturer's instructions. The digestion products were analysed directly by agarose gel electrophoresis. If the restricted DNA was to be ligated, the remaining restriction enzyme activity was destroyed by either heat inactivation at 70°C for 10min (for heat labile enzymes), or by phenol extraction.

2.3.2 T4 polynucleotide Ligase

DNA fragments were typically ligated at a final concentration of 15 to 30 $\mu g\ ml^{-1}$ in 1x ligation buffer which was added as a tenfold concentrate to the reaction mixture. T4 DNA ligase was added at a final concentration of 0.1 to 1.0 units μg^{-1} of DNA and the reaction mixture was either incubated overnight at 15°C or for 48 hours at 4°C.

2.3.3 DNase free RNase

DNases free RNase was used for the selective removal of RNA in DNA samples. Stock solutions of RNase A (10mg ml^{-1} in 10mM Tris-HCl, pH 7.5) were boiled (100°C) for 15 min prior to use or storage at -20°C.

DNA samples were typically treated with RNases at a final concentration of 25-50 μg ml^{-1} by incubation at 37°C for 1 hour.

2.4 ISOLATION OF DNA

2.4.1 Small scale isolation of Plasmid DNA from *E.coli*

Small scale plasmid isolations were carried out using the method of Birnboim and Doly (1979).

A 1.5ml sample of overnight nutrient broth culture was centrifuged in microcentrifuge (MSE microcentaur, 13000g for 2min). The bacterial pellet was resuspended in 100μl of Birnbom solution (see section 2.1.4.2) and incubated at room temperature for 5 minutes. To this 200μl of SDS-NaOH solution (see section 2.1.4.2) was added and mixed by inversion. 150μl of 4M sodium acetate pH 6.0 was then added, briefly vortexed to mix the contents and incubated on ice for 5 minutes. Following this incubation, the mixture was centrifuged (13000g for 2min) and the supernatant decanted into a fresh Eppendorf tube. An equal volume of phenol:chloroform (see section 2.5.1) was added to the supernatant and mixed by vortexing. The mixture was then centrifuged for a further 5min (13000g) and the top layer containing the plasmid, carefully removed to another fresh Eppendorf tube. To precipitate the plasmid DNA, 1ml of ethanol was added. The tube contents were mixed by vortexing and allowed to stand on ice for 10 minutes. The plasmid DNA present was recovered from the precipitate by centrifugation for 10 minutes. The supernatant was carefully decanted away and the DNA pellet immediately washed by the addition of 1ml of ethanol followed by a final centrifugation for 2 minutes. Again, the supernatant was discarded and the pellet dried in a 50°C oven. The dried DNA pellet was resuspended in 20-30 μl

of TE containing 1µl of Dnase free Rnase (see section 2.3.3). The plasmid DNA was stored at -20°C until required.

2.4.2 Large scale isolation of plasmid DNA from *E.coli*

Large scale isolation of plasmid DNA from *E.coli* strains was carried out by the method of Sambrook *et al.* (1989).

One litre nutrient broth supplemented with the appropriate antibiotic was inoculated with 1ml of an overnight culture and incubated overnight at 37°C with aeration. The cells were harvested by centrifugation (Sorval RC5B, GSA rotor) for 20 minutes and the pellet resuspended in 25ml of solution I (50mM glucose, 25mM Tris-HCl (pH 8.0) 10mM Na_2EDTA). Lysozyme, 1ml of a 10mg ml^{-1} solution was added and the mixture incubated at 37°C for 30min, after which time 50ml of SDS-NaOH solution (see section 2.1.4.2) was added. The mixture was mixed by gentle invertion and incubated on ice for 20min prior to the addition of 37.5mls of *solution III* (see section 2.1.4.2). After 10min on ice, the mixture was cetrifuged for 30min to remove cell debris. The supernatant was collected and placed in a fresh tube. The plasmid DNA was precipitated by the addition of 2 volumes of ethanol and incubation of the tube at -20°C for 20 minutes. The precipitate was recovered by centrifugation for 20 min at 4°C. The pellet was then washed in 70% ethanol, resuspended in TE and subjected to isopycnic centrifugation.

2.4.3 Isopycnic centrifugation of plasmid DNA

Plasmid DNA was separated from chromosomal DNA by centrifugation to equilibrium in caesium chloride-ethidium bromide density gradients (isopycnic centrifugation) as follows:

One gram of solid caesium chloride was added to each 1ml volume of cleared lysate containing 0.1ml of ethidium bromide solution (10mg ml^{-1} in distilled water). After gentle mixing by inversion, the contents were transfered to a Du Pont/sorval 'quick-seal crimp tube and the tube completely filled and sealed by crimping with a Du Pont/Sorval tube crimping system. If insufficient gradient mix was available the 'quick-seal' tubes were topped up with paraffin oil and balanced appropriately. The tubes were centrifuged in a Sorval Ti75 fixed angle rotor at 160000g (10ml tubes) for 36 to 48 hours (20°C). After centrifugation, the plasmid (lower) and chromosomal (upper) DNA bands were visualised with a UV long wave transilluminator. The lower band was removed by piercing the side of the tube with an 18-gauge hypodermic needle 0.5cm below the band and withdrawing it into a 2ml syringe; an additional needle was used to pierce the top of the tube thereby facilitating a smooth removal of the desired band. Ethidium bromide was removed by extracting the sample 3 or 4 times with isopropanol which had been equilibrated with caesium chloride.

2.4.4 Recovery of DNA from agarose gels

DNA was extracted from agarose gels using Geneclean II kit (BIO 101 Inc.) and following the manufacturer's instructions.

2.5 SOLVENT EXTRACTION AND ETHANOL PRECIPITATION

2.5.1 Phenol:Chloroform Extraction of DNA

The phenol:chloroform mixture was prepared by the addition of 50 ml of 1M Tris-HCl pH 7.5, 0.2M Na2EDTA pH 8.0 and 200ml of distilled water to 500g of phenol (BDH). The mixture was shaken to dissolve the phenol crystals and then allowed to

stand until it separated into two layers. The lower layer (phenol) was removed and placed in a fresh bottle. To this an equal volume of chloroform was added and stored at 4°C until required.

Protein was removed from DNA samples by extraction with phenol (pre-equilibrated with TE buffer, see above): chloroform. An equal volume of phenol:chloroform was mixed with the aqueous DNA sample, the emulsion was separated by centrifugation (MSE Microcentaur, 13000g for 1min). The aqueous phase was removed (the top layer) and transferred to a fresh tube. The purified DNA sample was then ethanol precipitated.

2.5.2 Ethanol Precipitation

DNA was routinely precipitated from solution by the addition of one-tenth volume of either 5M sodium chloride or 3M sodium acetate (pH 6.5) and two volumes of chilled absolute ethanol (-20°C), followed by incubation at -20°C for at least 1 hour. Alternatively, incubation was at -70°C for 15 min. After precipitation, the DNA was recovered by centrifugation (13000g for 10 min at 4°C). The supernatant was carefully decanted and discarded. The DNA pellet was either vacum dried or washed in an appropriate volume of 70% (v/v) ethanol, prior to vacum drying. The dessicated DNA pellet was resuspended in an appropriate volume of 1xTE buffer.

2.6 ELECTROPHORESIS OF DNA

2.6.1 Agarose Gel Electrophoresis

This was perfomed with a horizontal slab gel electrophoresis tank system (BioRad). The gel system employed was of the Schaffer type (Maniatis et al., 1982) in which the gel is poured into a portable gel casting tray , or of the 'mini-gel' type where the gel is

cast directly into the electrophoresis apparatus. The gel consisted of agarose (Sigma, ultra-pure) at concentrations between 0.7 and 1.5% (w/v) containing ethidium bromide at 0.5µg ml^{-1} in 1x TBE buffer. The gel was formed by boiling together (in a microwave oven) appropriate amounts of agarose, 1x TBE buffer, and ethidium bromide solution (10mg ml^{-1} in distilled water).

Samples to be loaded were mixed with a one-tenth volume of stop mixture (section 2.1.4.2) and loaded into the wells of the gel submersed in 1x TBE gel running buffer. The gel was then subjected to electrophoresis to give a voltage across the gel between 1 to 5 V cm^{-1}. After electrophoresis the DNA was visualised by UV illumination and photographed with Kodak black and white negative film using a red filter.

2.7 DNA SEQUENCING

The method employed was the chain termination procedure first described by Sanger *et al.* (1977).

2.7.1 Preparation of Template DNA

To a sterile Eppendorf, 20µl of double stranded plasmid DNA was added. This was denatured with the addition of 40µl of a freshly prepared solution of 0.2M NaOH/0.2mM EDTA, vortexed and incubated at 37°C. After 5 min, the Eppendorf was placed on ice and neutralised with 4µl of 2M ammonium acetate, pH 4.5. Immediately, 130µl of 100% ethanol was added, vortexed and placed at -20°C. After 1 hour, the Eppendorf was spun for 20 min in an Eppendorf microfuge (MSE Microcentaur, 13000g), the pellet washed with 100µl 80% ethanol, and the pellet dessicated. The dessicated pellet was dissolved in 10µl sterile double distilled water and used directly for primer annealing.

2.7.2 Annealing of Template and Primer

Templates to be sequenced first required annealing with the appropriate oligonucleotide primer. This was carried out in capped 1.5ml Eppendorf tubes. Template DNA (10µl) was mixed with 2µl annealing buffer, and 2µl primer (5 pmol/µl). The contents were annealed by incubation of the tube in a 65°C water bath for 5min, after which the tube was quickly transfered to a 37°C water bath and incubated for 10min. Following this, the tube was placed at room temperature for at least 5mins before a brief centrifugation to ensure recovery of the contents in the bottom of the tubes.

2.7.3 Sequencing reactions

Sequencing of DNA was carried out using a T7 sequencing kit (Pharmacia Biotech.) according to the manufacturer's instructions for double stranded sequencing.

2.8 POLYMERASE CHAIN REACTION

DNA fragments were amplified using a Vent DNA Polymerase amplification kit (New England Biolabs) and a programmable thermal controller. The general method followed is described below.

Between 30ng and 1.5µg of template DNA was used in a PCR reaction, the primers were used at concentrations of 100 picomoles each per reaction. The initial template denaturation was programmed for 5 min. Thereafter, the cycle profile was programmed as follows; 3min at 58°C (annealing), 4 min at 72°C (extension), and 2 min at 95°C (denaturation). This profile was repeated for 25 cycles

2.9 SITE-DIRECTED MUTAGENESIS

Site-specific mutations were carried out using a Clontech Transformer mutagenesis kit, following the manufacturer's instructions. The Transformer mutagenesis kit is based on

the method of Deng and Nickoloff (1992). The method works by simultaneously annealing two oligonucleotide primers to one strand of a denatured double stranded plasmid. One primer introduces the desired mutation (mutagenic primer), whilst the second primer mutates a restriction site unique to the plasmid for the purpose of selection (selection primer). The two oligonucleotides are annealed to one strand of the denatured double stranded plasmid under conditions favouring the formation of hybrids between the primers and the DNA template. After DNA elongation and ligation, a primary selection of mutated DNA is carried out by digesting with a specific restriction enzyme. The mixture of mutated and unmutated DNAs are transformed into a *mutS* *E.coli* strain defective in mismatch repair. The transformants are pooled and plasmid DNA is prepared from the mixed bacterial population. The isolated DNA is then subjected to a selective restriction digest. Since the mutated DNA should lack the restriction enzyme recognition site, it is resistant to digestion. The parental DNA, however, is sensitive to digestion and will be linearised, rendering it at least 100 times less efficient in the transformation of bacterial cells (Cohen *et al.*, 1972, Conley and Saunders, 1984). A final transformation using the digested DNA is then carried out, which results in the highly efficient recovery of the mutated plasmid.

The selective primer used for the mutagenesis study was one recommended by the manufacturer (Clontech Laboratories Inc.) for the pBluescript vector. The primer altered a unique *Sca*I restriction site to a *Stu*I site on the plasmid. The mutagenic primers were obtained, phosphorylated, from Pharmacia.

2.10 PROTEIN ISOLATION

2.10.1 Preparation of Cell Lysate

Routinely, 3l of *E coli* cells carrying the construct pQR606 were grown overnight in NB at 37°C supplemented with streptomycin and spectinomycin (both at 80 µg/ml). Cells were harvested by centrifugation and resuspended in 5mM Tris-HCl, pH 7.4, 1mM DTT. To reduce proteolytic degradation by proteases present in the cell, protease inhibitors (antipain, bestatin, leupeptin, pepstatin A, at a concentration of 10 µg/ml) and PMSF at 0.1% (w/v) were also included. Lysozyme was added to a final concentration of 10 µg/ml.

2.10.2 Sonication of cells and isolation of lysate

Sonication of cell suspensions was carried out on ice at an amplitude of 10 microns, 10x10 sec bursts were used with 20 sec intervals using an MSE Soniprep 150 Sonicator.

Following sonication, cell debris was removed by centrifugation at 20,000x g for 20 min. and the supernatant containing the enzyme was collected.

2.11 NUCLEOTIDYLTRANSFERASE ASSAY

2.11.1 Phosphocellulose binding assay

The activity of the enzyme was determined by the cellulose phosphate binding assay (Ozanne *et al.*, 1969) as modified by Werner *et al.*(1982). Reactions were carried out in a final volume of 50µl in nucleotidyltransferase asssay buffer. The reaction was initiated by the addition of enzyme and incubated at 37°C for 20min. Following this incubation, the reaction was terminated by incubation of the reactions at 100°C for

5min. 10µl aliquots were subsequently removed and spotted onto Whatman p81 ion exchange phosphocellulose paper squares, immersed in boiling distilled water for 5min and washed five times in 500ml of distilled water. After allowing the paper squares to air-dry briefly, they were added to 5mls of Ecoscint A (National Diagnostics, Georgia, USA) for scintillation counting.

2.11.2 Liquid scintillation counting

Samples were counted in 5ml of Ecoscint A in disposable plastic vial inserts in a Packard CA1500 liquid scintillation counter. Vials were counted for a minimum of 10min/vial or until 40,000 cpm had registered.

2.12 PROTEIN ASSAYS

Protein concentrations were determined by the Bradford assay (Bradford, 1976) and method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

2.12.1 Bradford protein assay

Bradford assay reagent was obtained from BioRad. The method was performed as per manufacturer's instruction.

2.12.2 Lowry protein assay

Samples and bovine serum albumin standards were made up to 0.2ml with 0.1ml NaOH. 2ml of 0.05% (w/v) sodium potassium tartrate, 0.01% (w/v) copper II sulphate, 2% (w/v) sodium carbonate, 0.1M NaOH was added to each tube, mixed well and allowed to stand at room temperature for 5-10 minutes. 50µl of undiluted Folin-Ciocalteu's phenol reagent (Sigma) was then added to each sample and standard,

mixed immediately and incubated at room temperature to allow colour production to proceed. The absorbance at 660nm was then measured against a reagent blank on a spectrophotometer. Protein concentrations of the samples were estimated from the standard curve, obtained after subjecting the standard values to a linear regression analysis.

2.13 ELECTROPHORESIS OF PROTEINS

2.13.1 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out essentially by the method of Laemmli (1970).

Proteins were separated by electrophoresis using polyacrylamide gels under denaturing conditions. The proteins were denatured by heating to 95°C with SDS and the reducing agent β-mercaptoethanol before they were loaded onto the gels.

SDS-polyacrylamide gel electrophoresis was performed with 15% (w/v) slab gels run on a Bio-Rad mini-gel system. The gel was poured in two stages, the stacking gel being cast after the resolving gel had polymerised. Just prior to casting, the polymerisation was initiated by the addition of N, N, N', N'-tetramethylenediamine (TEMED) to a final concentration of 0.1% (v/v) and ammonium persulphate to a final concentration of 0.1% (w/v). The mixture was quickly stirred and poured into the glass cassette leaving sufficient room at the top for the stacking gel. By overlaying the top of the gel with butanol saturated water, it was possible to form a perfectly flat gel top thus ensuring proteins would enter the gel at a perfect right angle. The gel was then left to set for at least 30 minutes with mini-gels and 1 hour for large gels. The water/butanol was washed off the gel top prior to the application of the stacking gel. The gel was

polymerised in the same manner as before prior to casting. After applying the stacking gel, a well forming comb (containing 10 well spaces for large gels and 12 well spaces for mini-gels) was inserted and again the apparatus was left for a minimum of 1 hour to allow polymerisation to take place. The combs were subsequently removed for the application of the samples.

2.13.2 Preparation of samples for SDS-PAGE

Samples to undergo SDS-PAGE were mixed with sample loading buffer (see section 2.1.4.2) which acted as tracking dye and boiled for 3 min. Tubes containing samples were then allowed to cool prior to being loaded onto the gel.

2.13.3 Pharmacia Phast Gel System and Determination of pI

The Phast Gel system (Pharmacia Ltd.) is an automated system for the rapid electrophoresis and development of protein gels. Prepacked SDS and isoelectric focusing (IEF) gels were run according to the manufacturer's instructions.

The pI was determined using broad-pH-range gels (pH 3.5-9.5), as described by the manufacturer.

2.14 PROTEIN CONCENTRATION

Protein samples were concentrated by ultrafiltration using Amicon Centricon-10 and Centriprep-10 concentrators and used as per the manufacturer's instructions.

The molecular weight cut-off point for these filtration columns was 10kDa. The samples were centrifuged for 1 hour at room temperature with a maximum g-force of 5000.

2.15 PROTEIN PRECIPITATION

2.15.1 Precipitation by Ammonium Sulphate

Ammonium sulphate precipitation was carried out to fractionate the protein. Chilled saturated ammonium sulphate solution was added to the cell lysate to give the desired % saturation. Precipitation was allowed to occur over a 30min period at 4°C. The precipitated proteins were subsequently removed by cetrifugation at 16,000g for 20min (Sorvel RC5B) and resuspended in 1ml of 50mM Tris, 1mM DTT, pH 7.4. The supernatant was then brought to a new % saturation by further addition of saturated ammonium sulphate solution and the mixing and spinning steps repeated. This procedure yielded distinct ranges of ammonium sulphate precipitated proteins.

2.15.2 Precipitation by Polyethylene Glycol (PEG)

Precipitation using polyethylene glycol was carried out using a 50% stock solution of PEG 6000. The appopriate amount of the stock solution of PEG was added to cell lysate to give a % saturation of 0-10% and the precipitation was allowed to occur over a 30min period at 4°C. After which time the precipitated proteins were removed by centrifugation and the pellet resuspended in 2.0ml of 50mM Tris, 1mM DTT, pH 7.4. The supernatant was brought to the desired % saturation (10-20%) and the previous steps repeated. A final 'cut' of 20-30% saturation with PEG was carried out before the solution became too viscous.

2.16 ANTIBODY PRODUCTION AND WESTERN BLOTTING

2.16.1 Preparation of Alum Adjuvent

To a 50ml flask containing 10 ml 10% (w/v) ammonium potassium sulphate (Potassium Alum) solution 22.8 ml of 0.25N NaOH was added dropwise. The flask was stirred vigorously and incubated at room temprature for 10 minutes. Following this incubation, the mixture was centrifuged (Sorval RC5B, 1000g for 10min) and the pellet resuspended in 50 ml of distilled water. The mixture was again centrifuged, the pellet recovered and used for the administration of protein into rabbits.

2.16.2 Polyclonal Antibody Production

Polyclonal antibodies were raised against the purified ANT(3")-I protein. The purified protein (50μg) in a total volume of 0.1ml was mixed well with an equal volume of Alum Adjuvent (see section 2.16.1) until a smooth emulsion was formed. It was consequently administered in five subcutaneous injections into two New Zealand rabbits. Three booster injections of the same dose were adminstered by the same route over an eight week period. Blood was collected from the ear marginal vein 10 days following the final injection and allowed to clot prior to centrifugation to collect the serum.

2.16.3 Western Blotting

The electrophoretic transfer of protein polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin *et al.* (1979) in a Bio-Rad western blot apparatus as follows:

Two sheets of filter paper were wetted with transfer buffer and layered on a Scotch-Brite pad which was supported on a rigid plastic grid. A sheet of nitrocellulose

(0.45μm pore size) was then wetted with the same buffer and layered on top of this. The gel to be blotted was placed onto the nitrocellulose sheet and all the air bubbles removed from the interface. After trimming the nitrocellulose to the same size as the gel, two further pre-wetted pieces of filter paper were overlayed followed by a second Scotch-Brite pad. The top plastic grid was placed in position and the whole assembly secured together with a plastic band. This ensured that the gel was both firmly and evenly pressed against the nitrocellulose sheet. The assembly was placed in the blotting chamber containing Western Blot transfer buffer (see section 2.1.4.2) with the gel facing the anode electrode. A 50V supply was connected between the plates for one hour to ensure maximum transfer of the resolved proteins to the nitrocellulose membrane.

2.16.4 Immunological Staining of Western Blots

Proteins were resolved by SDS-PAGE as described in section 2.13.1 and electrophoretically transferred to nitrocellulose sheets as described in section 2.16.3

Prior to probing with the primary antisera, it was first necessary to 'block' unoccupied sites on the nitrocellulose with an irrelevant protein. This was achieved by immersing the sheets in TBS buffer (see 2.1.4.2) containing 3% (w/v) gelatin at 37°C for a minimum of 90 min. Following this incubation, the gelatin was poured off and replaced typically with a 1:100 dilution of primary antiserum in 1% (w/v) gelatin in TBS, and incubated overnight at 25°C. The primary antiserum was then removed and the blots extensively washed with water followed by two 15min washes with TBS containing 0.5ml/l Tween 20 (TTBS). After a further two x 15min washes with TBS, a 1:50 dilution of second antibody in 1%(w/v) gelatin in TBS was added and allowed to incubate at 25°C for 2-3 hours. The second antibody consisted of horseradish peroxidase conjugated anti-rabbit IgG. The second antibody was poured off and the

previous washing cycle repeated. The blots were developed with 2.8mM 4-chloro-1-naphtol, 0.5% (v/v) hydrogen peroxide in TBS as a substrate for the peroxidase to make cross reacting bands visible.

2.17 CHROMATOGRAPHY

A Pharmacia Fast Protein Liquid Chromatography (FPLC) system was used to purify the enzyme to homogeneity. The fractions collected from each column were assayed for enzyme activity using the phosphocellulose binding assay for nucleotidyltransferase enzymes. The following columns were used in order:

2.17.1 Gel Filtration Chromatography-Superdex 200 column

Protein extracts resuspended in 50mM Tris-HCl, pH 7.4, 0.15M NaCl were applied to a Superdex 200 column pre-equilibrated with the same buffer. Two ml fractions were collected at a flow rate of 1ml/min.

2.17.2 Ion Exchange Chromatography-Mono Q

Anion Exchange chromatography was performed on a Mono Q column. It involved the ionic interaction of charged species at pH 7.4 with the quaternary nitrogen functional group of the matrix. The samples were typically applied to a 1ml Mono Q HR5/5 column which had been previously equilibrated in 20mM Tris-HCl, pH 7.4, at a flow rate of 0.5ml/min. After unbound proteins were washed from the column, a linear gradient of the same buffer to 0.5M NaCl in 20mM Tris-HCl, pH 7.4 was run over 30 column volumes.

2.17.3 Hydrophobic Interaction Chromatography- PhenylSuperose

Samples were brought to a final concentration of 1.7M ammonium sulphate and applied to a 1ml PhenylSuperose HR 5/5 hydrophobic interaction column which had been pre-equilibrated with 50mM Tris-HCl, pH 7.4 containing a final concentration of 1.7M ammonium sulphate. Excluded proteins were washed from the column and desorption of the bound proteins was initiated with a linear gradient of 50mM Tris-HCl, pH 7.4/ 1.7M ammonium sulphate to buffer alone over 30 column volumes.

2.17.4 Gel Filtration Chromatography- Superdex 75 column

Fractions were applied to a Pharmacia Superdex 75 HR10/30 column which had been pre-equilibrated in Tris-HCl, pH 7.4, 0.15M NaCl. Fractions of 0.5ml were collected.

CHAPTER THREE

OVER EXPRESSION OF

ant(3")-Ia

SUMMARY

The aim of the work presented in this chapter was to optimise the expression of the aminoglycoside (3") nucleotidyltransferase gene (*ant(3")-Ia)* and increase the production of the enzyme to aid purification. The *ant(3")-Ia* gene has been sub-cloned into various expression vectors and the level of expression from the different constructs compared.

3.1 Isolation and sub-cloning of *ant(3")-Ia*

The source of the aminoglycoside (3") nucleotidyltransferase gene (*ant(3")-Ia*) used in this study was pIJ4642 (Davey, 1992). This plasmid was constructed as a cloning vector and carries the streptomycin/spectinomycin resistance gene *ant(3")-Ia* gene as a selectable marker. This gene was originally isolated from the R-plasmid R538-1 of *E.coli* (Hollingshead&Vapnek, 1985).

Plasmid pIJ4642 was digested to completion with *Hin*dIII and electrophoresed. A band of 1.95Kb (fig 3.1) corresponding to *ant(3")-Ia*, was excised from the gel and recovered using the Geneclean II kit (see section 2.9 in methods). The fragment was then ligated into *Hin*dIII digested pBluescriptKS$^-$. Streptomycin resistance (20μg/ml) was used to select for the recombinant plasmid. Colonies were picked onto agar plates containing the selection antibiotics (ampicillin and streptomycin).

Plasmid DNA was isolated from these colonies, single restriction enzyme digests confirming the presence of the *ant(3")-Ia* gene, on a 1.95Kb *Hin*dIII fragment, in all isolates. Double restriction enzyme digests were also carried out using *Sph*I and *Eco*RI to identify the orientation of the insert. By analysis of the restriction endonuclease fragments generated from the double digests, it could be determined

that the *ant(3")-Ia* gene was present in both orientations (Fig. 3.2 A&B). Recombinant plasmid pQR601 was found to have its *ant(3")-Ia* gene insert in the same orientation as the *lac* promoter, whereas in pQR602 this fragment was in the opposite orientation to the *lac* promoter of the pBluescriptKS$^-$ vector.

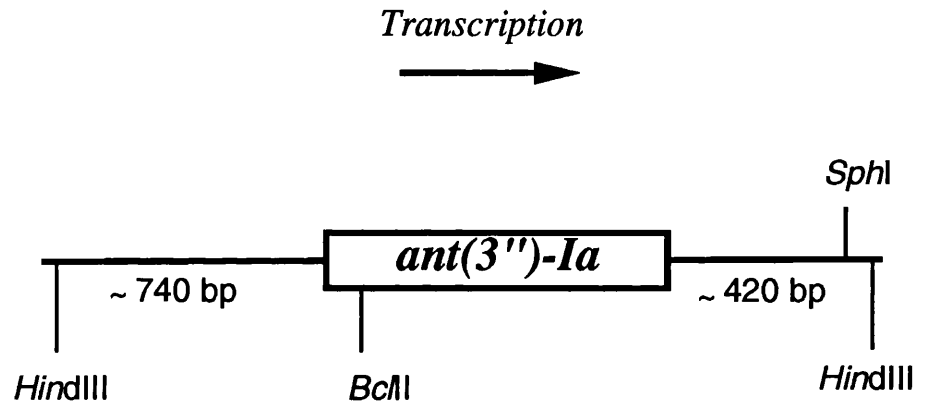


Fig.3.1- Diagramatic representation of the 1.95Kb *Hin*dIII fragment isolated from plasmid pIJ4642. The arrow indicates the direction of transcription. The approximate length of the non coding sequences flanking the gene are shown .

To enhance expression of *ant(3")-Ia*, the 1.95Kb *Hin*dIII fragment was further cloned into other expression vectors namely, pTTQ19 and pUC19. This gave rise to pQR603

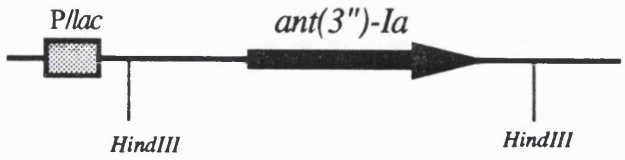
and pQR604 respectively, upon transformation into *E.coli* JM107 (fig. 3.2 C&D). Studies were carried out to compare expression from each construct.

3.2 Over Expression of *ant(3")-Ia*

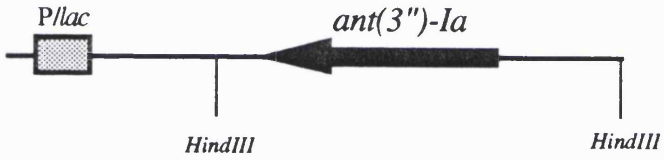
Enzyme assays on total cell lysates were carried out to determine the level of expression of the gene from each construct. Low expression levels from pQR601, pQR603, and pQR604 (data shown in fig 3.6) were thought to be as a consequence of the large non-coding sequences flanking the gene. Addition of the inducer IPTG had no significant effect on the expression of the gene (results not shown). Enhanced expression would, therefore, require the removal of these regions and transcription from an efficient promoter.

In order to remove these large non-coding sequences, PCR was used to introduce two restriction sites at either end of the gene (fig 3.3). The initiation ATG triplet was incorporated into an *Nde*I restriction site (CATATG) by use of the PCR primer. Scharf *et al.* (1986) first showed that it was simple to introduce restriction site sequences into DNA fragments produced by PCR merely by attaching these sequences to the 5' ends of the oligonucleotides used as primers. Higuchi (1989) recommended that at least two more bases should be added beyond the restriction site sequence in case the restriction enzyme is less able to cut at an absolutely terminal sequence. Although these sequences at the 5' terminus are mismatched to the template DNA, in most cases they have little effect on the specificity or efficiency of the amplification. Two synthetic oligonucleotides were, therefore, constructed to introduce *Eco*RI and *Nde*I upstream of the start codon and *Bam*HI and *Hin*dIII at the end of the gene (fig 3.4)

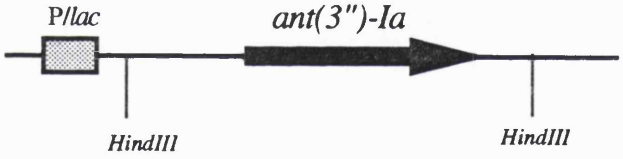
A: pQR601



B: pQR602



C: pQR603



D: pQR604

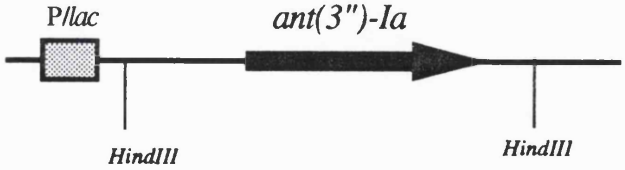
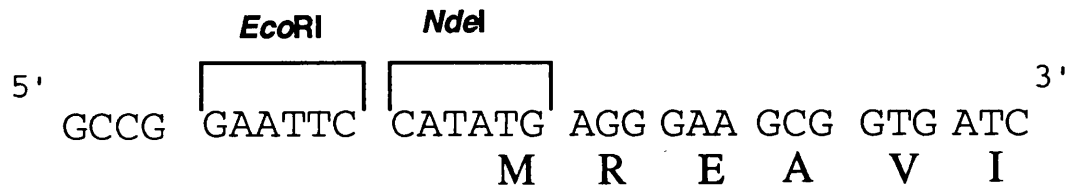


Fig. 3.2-Schematic representation of constructs carrying the 1.95Kb *Hin*dIII fragment isolated from pIJ4642. The direction of transcription is indicated by the arrow. P/*lac* represents the *lac* promoter

Oligonucleotide 1:



Oligonucleotide 2:

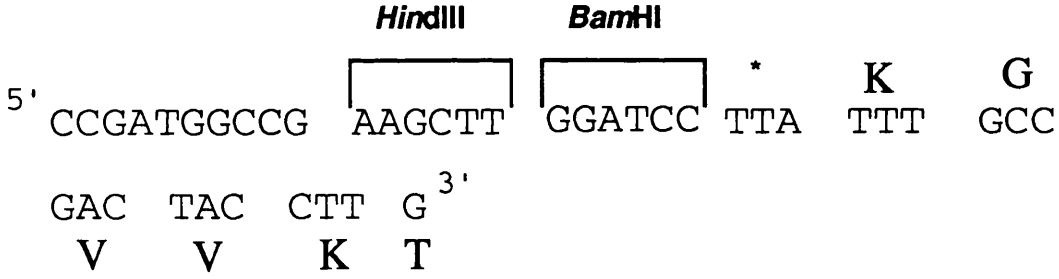


Fig. 3.3- Synthetic oligonucleotides used to create two restriction sites at either end of the gene using PCR. The bold letters indicate the one letter code of the amino acid sequences present in the gene. Oligonucleotide 1 was annealed to the N-terminal end of the gene and oligonucleotide 2 to the C-terminus. The restriction sites introduced are indicated. * indicates the stop codon.

Following PCR, the products were digested with *Nde*I and *Bam*HI restriction endonucleases and electrophoresed. A band of approximately 800bp (corresponding to the *ant(3")-Ia* gene plus the added restriction sites) was excised from the gel and the DNA recovered (using Geneclean). The fragment was then ligated into an

expression vector, pMTL2023 (Chambers *et al.*, 1988). This is a medium copy number plasmid (150 copies/cell) containing a *trp* promoter and a selectable chloramphenicol resistance gene.

A very low transformation frequency was observed upon transformation into *E.coli* JM107 and a number of attempts were necessary to clone the gene fragment. The insertion of the fragment into the transformants was confirmed by restriction of plasmid DNA isolated from colonies with *Nde*I and *Bam*HI. Large scale caesium chloride prepared plasmid DNA was isolated from the positive transformant in pMTL2023, labeled pQR606 (fig 3.5).

Comparison studies were carried out to elucidate the level of expression between the various constructs.

3.3 Hyper-Expression of *ant(3")-Ia*

During the course of this study, a new vector for high level expression of proteins was reported. The expression system for malate dehydrogenase in *E.coli* has been shown to be extremely efficient for the expression of not only *E.coli* genes, but also for several heterologous genes (Alldread *et al.*, 1995). (For the nucleotide sequence of the *mdh* promoter see appendix 2).

The malate dehydrogenase promoter (*mdh*) was carried on a similar type of vector as the *trp* promoter in pMTL2023. This plasmid (pMTL1005) is a medium copy number plasmid (same copy number as pMTL2023; 150copies/cell) and contains an ampicillin resistance marker, furthermore, it possess the same multiple cloning site as pMTL2023, which made it ideal for use in this study.

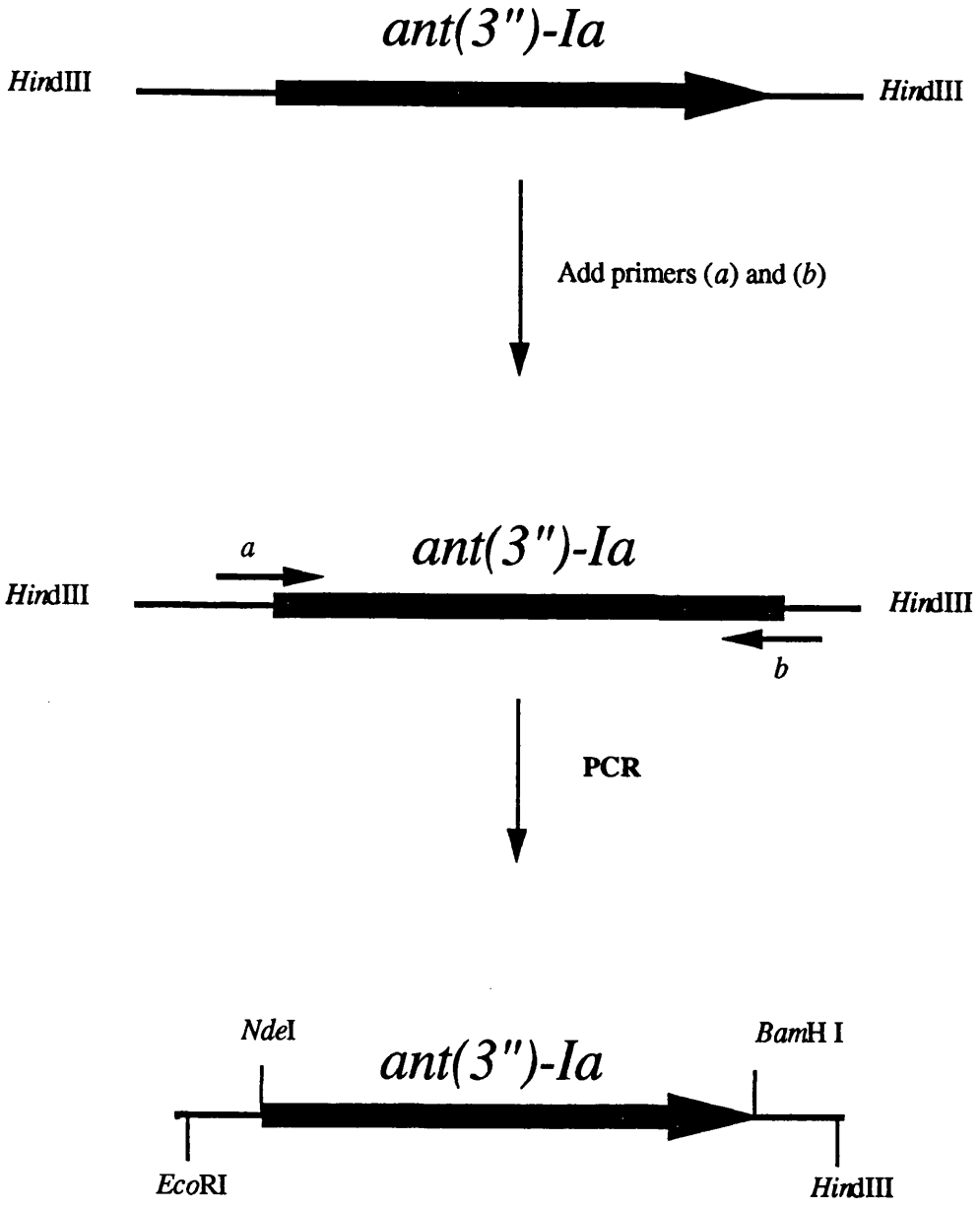
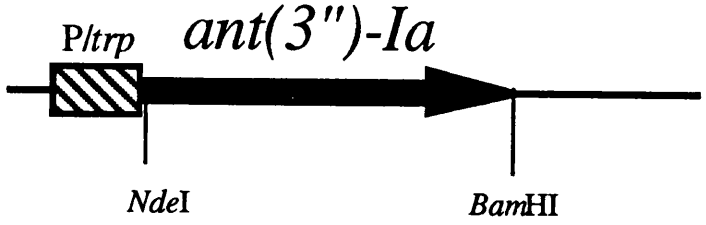
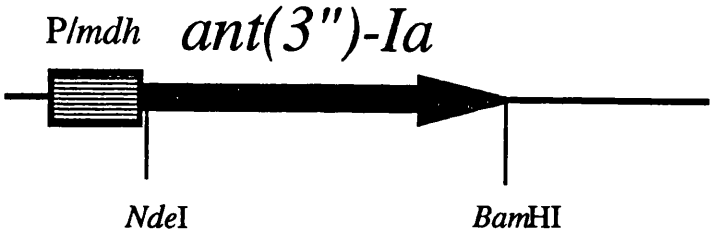


Fig. 3.4- Diagramatic representation of the PCR procedure carried out on the 1.95Kb *Hin*dIII fragment to remove the non-coding sequences flanking the gene. The new restriction sites are indicated. The arrow represents the direction of transcription.



pQR 606



pQR 609

Fig. 3.5- representation of the high expression constructs pQR606 and pQR609. P/*trp* and P/*mdh* represents the tryptophan and the malate dehydrgenase promoters, respectively.

The *ant(3")-Ia* gene was isolated from pQR606 on an *NdeI*/*Bam*HI fragment and ligated into pMTL1005, giving rise to pQR609 (fig. 3.5 B). Again, several attempts were necessary to clone the gene into this plasmid. This was thought to be due to the

hyper expression of the gene and possible toxic effects on cells. To partially repress the gene, glucose was added to the growth media to induce catabolite repression, which is known to give some repression of the *mdh* promoter (Alldread *et al.*, 1995).

3.4 Determination of the level of expression of the *ant(3")-Ia* gene from constructs

Cultures of *E.coli* JM107 containing each recombinant plasmid were grown to stationary phase at 37℃ in nutrient broth supplemented with the appropriate selection antibiotic (Ampicillin at a final concentration of 20μg/ml for cells carrying constructs pQR601 and pQR609 and chloramphenicol also at 20μg/ml for cells carrying pQR606) together with streptomycin and spectinomycin (each 80μg/ml). Total cell lysates were prepared (see section 2.10.1) and the supernatant of the extracts were assayed for aminoglycoside nucleotidyltransferase activity (see section 2.11). The results are presented in fig 3.6 and 3.7.

3.5 Minimum Inhibitory Concentration studies

To investigate the effect of high expression of the gene on resistance conferred by the enzyme, minimum inhibitory concentration studies (section 2.2.2.3) were carried out. *E.coli* cells carrying the recombinant plasmids pQR601 (*lac* promoter), pQR606 (*trp* promoter) and pQR609 (*mdh* promoter) were used to determine MICs for streptomycin and spectinomycin. The results obtained are shown in table 3.1

JM107 carrying Constructs	MIC (μg/ml)	
	Streptomycin	Spectinomycin
pQR601	120	450
pQR606	200	1200
pQR609	370	above 2000
Control	4	18

Table 3.1-The minimum inhibitory concentrations (MIC) of streptomycin and spectinomycin required to inhibit the growth of *E.coli* JM107 containing plasmids pQR601, pQR606, and pQR609. The control was performed on JM107 cells carrying the native plasmid (pBlueScript KS$^-$) not expressing *ant(3")*.

3.6 Results and Discussion

The comparison studies carried out on the constructs prior to PCR and insertion of the restriction sites, indicated that the level of expression from construct pQR601 was actually higher than the other three constructs (pQR602, pQR603, and pQR604) even when the latter were induced with IPTG (results not shown). Although the expression of the gene was initiated from the *lac* promoter in all cases, very low levels of nucleotidyltransferase activity were observed from the other constructs (see fig. 3.6). In pQR602, the gene was inserted in the opposite orientation to the *lac* promoter such that expression was initiated from its own promoter. In this construct, the level of expression was only slightly higher than that observed in pQR603 and pQR604.

Higher levels of expression was observed from pQR606 and on comparison of activity on total cell lysates from *E.coli* JM107 carrying pQR601 and pQR606, a two fold increase in specific activity of the enzyme was seen.

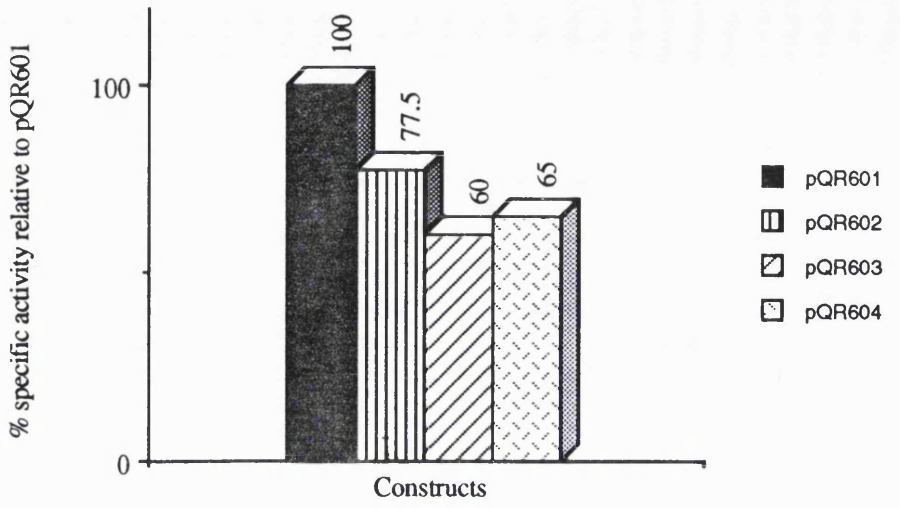


Fig. 3.6-Comparison of expression of *ant(3")-Ia* gene from pQR601, pQR602, pQR603 and pQR604. The results are represented as % specific activity in relation to the specific activity obtained for pQR601. The 100% value represents 0.525nmol/min/mg of enzyme.

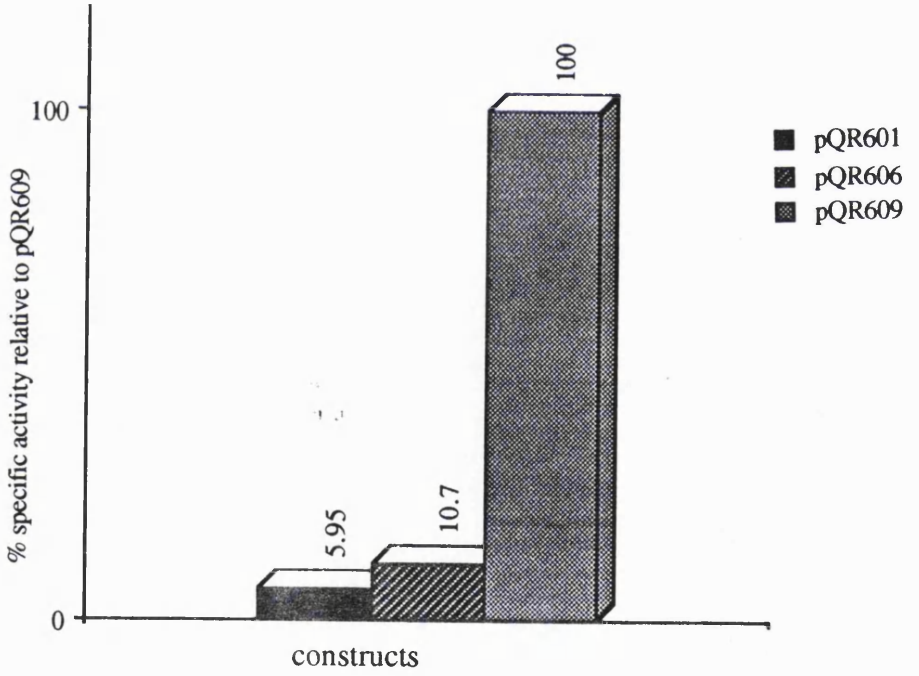


Fig.3.7- Comparison of expression of *ant(3")-Ia* gene from the high expression constructs. The values are represented as % specific activity relative to pQR609. The 100% value represents 11.94 nmol/min/mg of enzyme.

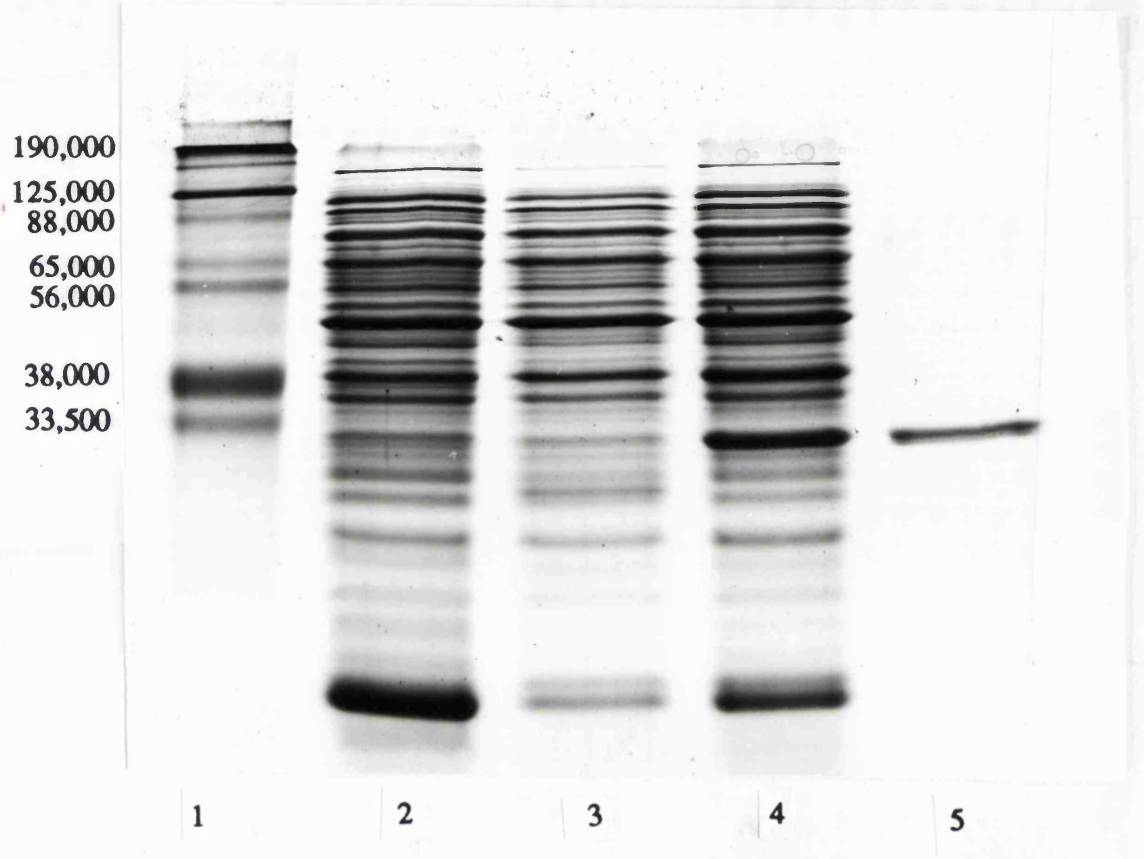


Fig. 3.8-SDS polyacrylamide gel of total cell lysates from JM107 cells carrying constructs: 1, Molecular weight marker, 2, pQR606; 3, pQR601; 4, pQR609. Track 5 shows the pure enzyme.

Experiments previously performed indicated that induction of *ant(3")-Ia* from the *trp* promoter with indolacrylic acid does not increase expression levels beyond those obtained by tryptophan starvation (Murphy, personal communication). Because of the copy number of these plasmids (150 copies/cell), repression of the *trp* promoter by

tryptophan in unlikely, and a high percentage of constitutive expression probably occurs. As a result the *trp* promoter was not induced by additional factors.

The level of expression from the recombinant plasmid pQR609 was found to be 10 fold higher than that produced from pQR606 and 22 fold higher than pQR601 as measured by the specific activity of total lysates (fig. 3.7).

Furthermore, SDS polyacrylamide gel electrophoresis of total cell extracts from *E.coli* JM107 cells carrying the recombinant plasmids (see fig. 3.8) confirmed the more efficient synthesis of the enzyme in this construct by showing a considerably more intense protein band in the 29-30 kDa position (consistent with the size of the enzyme).

Comparison of the MIC values obtained (table 3.1) from each construct revealed that the higher expression of the gene is directly proportional to the level of resistance conferred to the antibiotic substrate. This further verified expression of the gene from the construct pQR609.

CHAPTER FOUR

PURIFICATION AND CHARACTERISATION OF ANT(3")-I

SUMMARY

The enzyme has been purified to homogeneity by a combination of ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. Chemical and physical properties of the enzyme have been investigated.

4.1 PURIFICATION

Several aminoglycoside nucleotidyltransferase enzymes have been partially purified and characterised (Smith and Smith, 1974; Coombe and George, 1981; Santanam and Kayser, 1976). A number have even been purified to homogeneity by preparative gel electrophoresis (Chinault *et al.,* 1986) using SDS-polyacrylamide gels to aid in physical characterisation of the enzymes, however, as this method often results in denaturation of the protein no precise kinetic characterisation have been performed on these preparations. Attempts at purifying these enzymes to homogeneity in an active state have so far been unsuccessful, possibly due to low expression levels and instability of these enzymes in the producing strain.

During the course of this study, the *ant(3")-Ia* gene was successfully cloned into two expression vectors (see chapter 3), resulting in constructs pQR606 (carrying the *trp* promoter) and pQR609 (carrying the novel *mdh* promoter). The first construct obtained was pQR606, the purification protocol outlined in this chapter was elucidated using *E. coli* cells carrying this construct. Expression vector pMTL1005

(carrying the *mdh* promoter) was not available at the onset of purification procedures and the purification protocol was completed prior to the construction of pQR609. Although the expression of the gene from the *trp* promoter was found to be approximately ten fold lower than that from the *mdh* promoter carried by pQR609, purification of the enzyme from this construct has not been performed due to time constraints.

4.1.1 FRACTIONATION OF CELL LYSATE

4.1.1.1 Ammonium Sulphate Precipitation

In order to fractionate the enzyme using ammonium sulphate, initial studies were carried out to determine the appropriate percent saturation range required to precipitate ANT(3")-I. This was done by the addition of ammonium sulphate to the cell lysate to give the desired % saturation and allowing precipitation to occur over a 30 min period at 4℃. The precipitated proteins were subsequently removed by centrifugation (see 2.15.1). The supernatant was then brought to a new % saturation by the further addition of ammonium sulphate and the mixing and spinning steps repeated. This procedure yielded distinct ranges of ammonium sulphate precipitated proteins, each of which were assayed for enzyme activity (see 2.11.1).

The results (table 4.1) indicated that the enzyme fractionated within the range 40-60% ammonium sulphate saturation, this was subsequently used for bulk purification of the enzyme.

% Saturation	Total Activity (nmoles/min)	% Yield
0% (cell lysate)	9.84	100
0-20%	0.46	2.34
20-40%	0.10	0.51
40-60%	17.11	86.94
60-80%	0.1	0.51
Supernatant	2.9	14.7

Table 4.1-Ammonium Sulphate Fractionation range of ANT(3")-I enzyme

4.1.1.2 PEG Precipitation

Precipitation using Polyethylene glycol (PEG) was also carried out (see section 2.15.2). However, as table 4.2 indicates, the enzyme did not precipitate even at 30% PEG, the maximum concentration that can be used for this procedure. Higher PEG concentrations increase the viscosity to such an extent that recovery of the precipitate would be difficult.

Fraction	Total Activity (nmoles/min)	% Yield
Lysate	124.02	100
0-10% pellet	0.48	0.4
10-20% pellet	1.03	0.8%
20-30% pellet	3.03	2.44
30% supernatant	86.85	70

Table 4.2-PEG precipitation profile of ANT(3")-I enzyme

4.1.2 Gel Filtration Chromatography- Superdex 200

The precipitate from the ammonium sulphate fractionation was resuspended in 50mM Tris-HCl, pH 7.4, 0.15M NaCl (Buffer A) and applied to a Superdex 200 pg column, pre-equilibrated with the same buffer (see 2.17.1). Two ml fractions were collected at a flow rate of 1ml/min and assayed for enzyme activity (fig.4.1). A cocktail of protease inhibitors was added to the active fractions collected from the column to minimise degradation of the protein by proteases.

4.1.3 Ion Exchange Chromatography- Mono Q

Fractions containing the highest activity were pooled and loaded onto a 1ml Mono Q HR5/5 column which had been pre-equilibrated in 20mM Tris-HCl, pH7.4 (Buffer B) at 0.5ml/min (section 2.17.2). After unbound proteins were washed from the column, a linear gradient of buffer B to 500mM NaCl in buffer B was run over 30 column volumes. ANT (3")-I eluted between 300-360mM NaCl (fig.4.2).

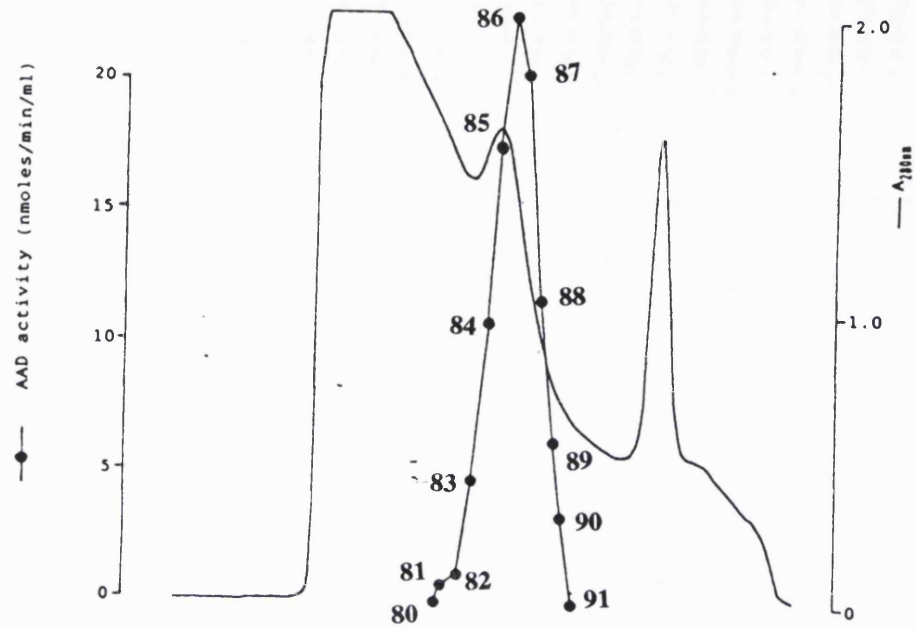


Fig. 4.1-Elution profile of ANT(3")-I from Superdex 200 column at a flow rate of 1ml/min. The fractions collected for further purification are numbered.

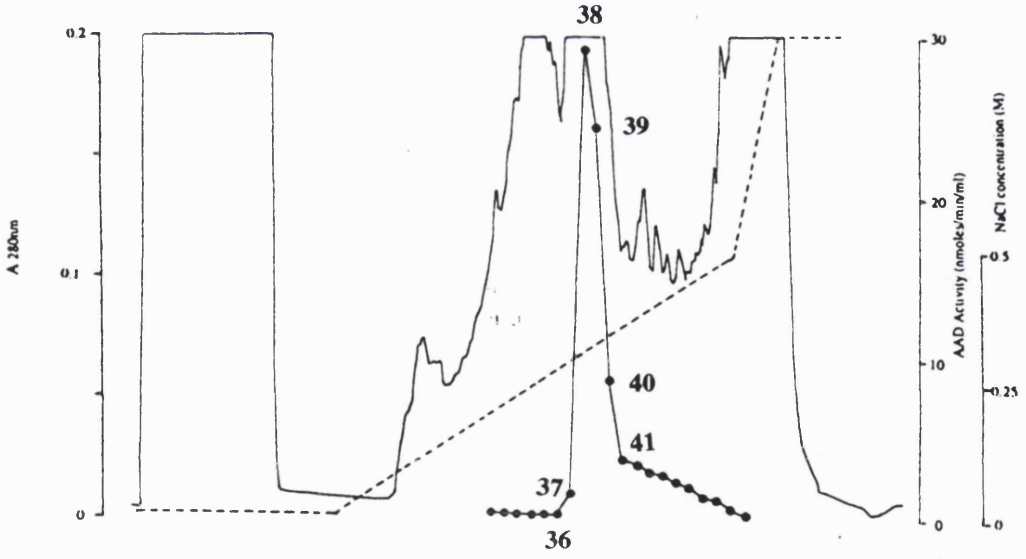


Fig.4.2-Elution profile of ANT(3")-I from Mono Q column at a flow rate of 0.5ml/min. The collected fractions are numbered.

4.1.4 Hydrophobic Interaction Chromatography- PhenylSuperose

Fractions containing enzyme activity were pooled and brought to a final concentration of 1.7M ammonium sulphate and applied to a 1ml PhenylSuperose HR5/5 hydrophobic interaction column which had been pre-equilibrated with 50mM Tris-HCl, pH7.4 (Buffer C) containing a final concentration of 1.7M ammonium sulphate (see 2.17.3). Excluded proteins were washed from the matrix and desorption of the enzyme was initiated with a linear gradient of buffer C/ 1.7M ammonium sulphate to buffer C over 30 column volumes. ANT(3")-I eluted at the very end of the gradient where ammonium sulphate was essentially absent from the elution buffer (fig.4.3).

4.1.5 Gel Filtration Chromatography-Superdex 75

Active fractions were pooled, concentrated to 200µl in a centricon micro-concentrator and applied to a Superdex 75 HR10/30 column which had been pre-equilibrated in buffer A (see 2.17.4). Fractions (0.5ml) were collected and assayed for enzyme activity. ANT(3")-I eluted with a retention time consistent with a molecular weight of approximately 30 kDa (fig.4.4).

The purity of the final preparation was assessed by SDS-PAGE in 15 % gels according to the method of Laemmli (1970), and stained with coomassie blue. It showed a single migrating band at approximately 29-30 kDa in relation to standards of known molecular weight (fig.4.5).

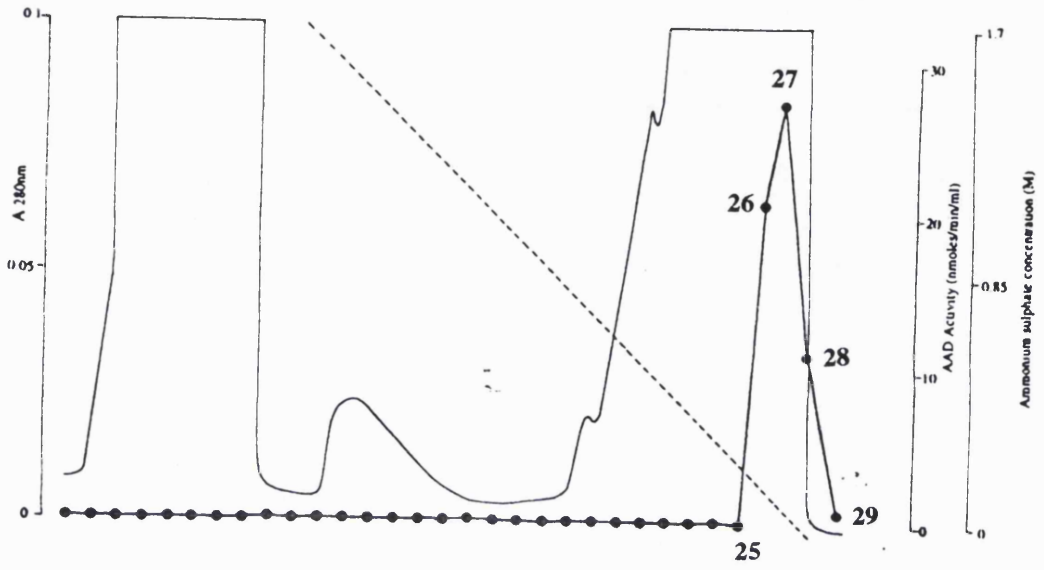


Fig. 4.3-Elution profile of ANT(3")-I from PhenylSuperose column. A linear gradient of ammonium sulphate was applied. The collected fractions are indicated.

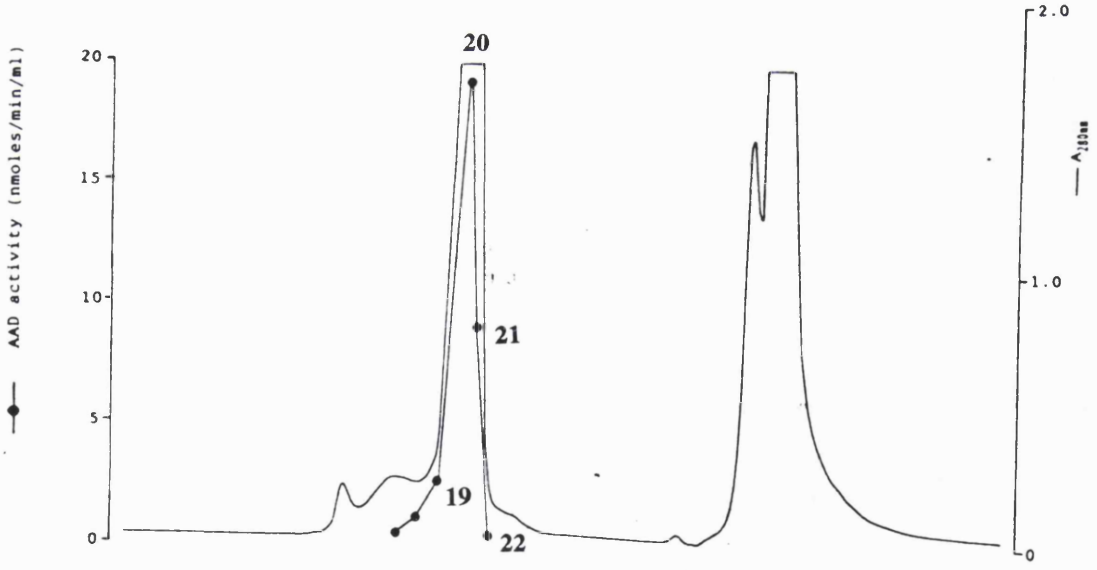


Fig. 4.4-Elution of ANT(3")-I from Superdex 75 gel filtration column. The collected fractions are numbered.

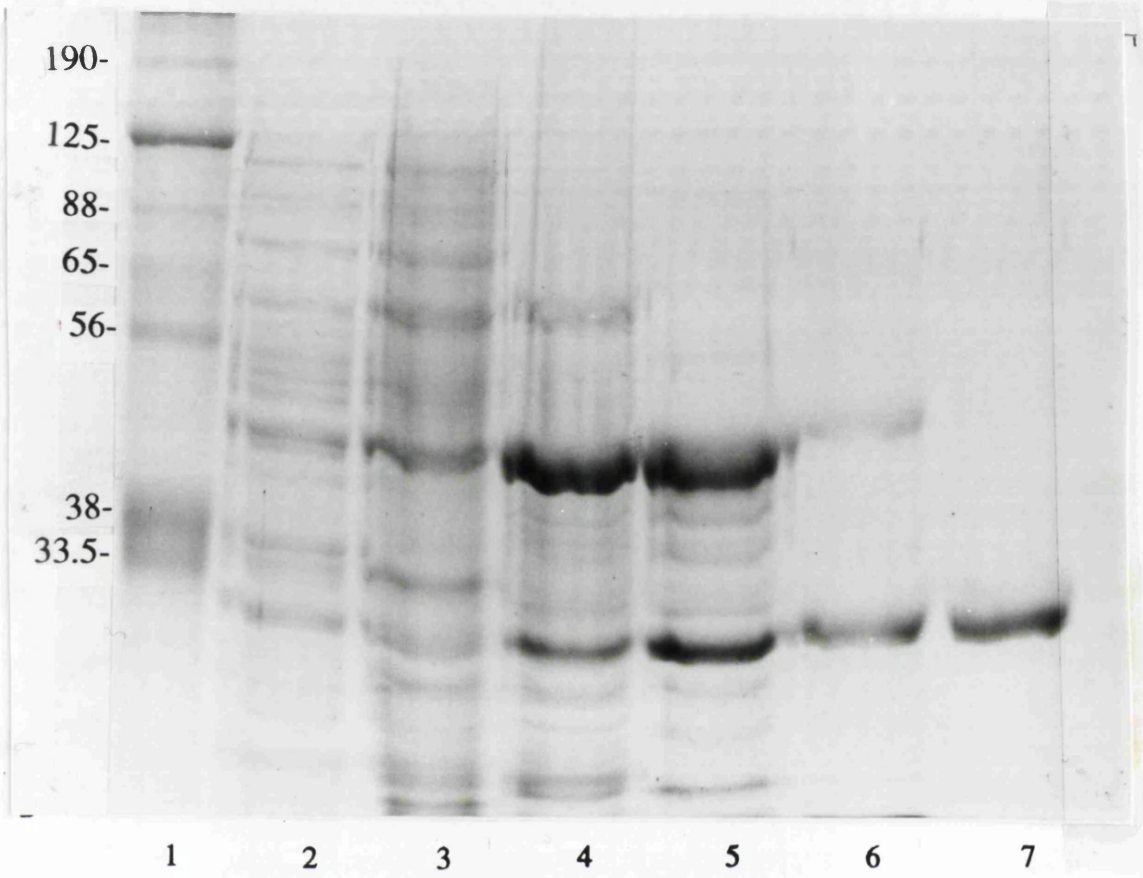


Fig. 4.5-SDS-Polyacrylamide gel showing the different stages of purification. 1-molecular weight marker; 2-Total cell lysate; 3-Ammonium sulphate precipitate; 4-Pooled fractions following Superdex 200 column; 5-Pooled fractions following Mono Q; 6-Pooled fractions following PhenylSuperose; 7-Pooled fractions following Superdex 75

Purification step	Total Activity (nmoles/min)	Total Protein (mg)	Sp Activity (nmoles/min/ mg)	% Yield	Purification Factor
Lysate	716.0	370	1.94	100	1.0
$(NH_4)_2SO_4$	622.92	121.66	5.12	87	2.64
S-200	379.9	26.6	14.29	53.1	7.4
Mono Q	166.10	5.8	28.60	23.2	14.7
Phenyl S	79.5	0.74	107.00	11.1	55.2
S-75	64.3	0.23	279.57	9	144.3

Table 4.3-Purification of ANT(3")-I enzyme. Sp Activity, specific activity

4.2 CHARACTERISATION

4.2.1 Minimum Inhibitory Concentration (MIC) studies

Tests have been carried out to determine the Minimum Inhibitory Concentration (MICs) of the two substrates (table 4.4) (see section 2.2.2.3). These tests were performed on *E. coli* cells carrying the pQR606 construct. The enzyme does not confer resistance to any other aminoglycoside (kanamycin, neomycin, gentamicin, butirosin, kasugamycin, amikacin were tested, data not shown).

Specificity of the enzyme for other nucleotides was not tested as ANT(3")-I enzymes are only able to utilise ATP as co-substrate (Yamada *et al.,* 1968).

4.2.2 Determination of the pI of ANT(3")-I

Isoelectric focusing of the purified extract was carried out to determine the pI of the enzyme. This was carried out using broad range (pH 3-9) pre-cast isoelectric focusing gels on a Pharmacia phastgel system (see section 2.13.3).

The pI of ANT(3")-I was shown to be 5.91, running as a single band (a further confirmation of the purity of the enzyme). This was not consistent with the value of 4.68 obtained by Shaw *et al.* (1993). The latter was a predicted value calculated from the amino acid sequence of the protein.

4.2.3 Estimation of the relative molecular weight of the enzyme

The relative molecular weight of ANT(3")-I as determined on 12.5%(w/v) SDS-polyacrylamide gels is approximately 29.5-31 kDa.

By comparing the relative mobility of pure enzyme to proteins of standard molecular weight, the subunit size was estimated to be 29.5 kDa. This compared well with the predicted molecular weight of 31.6 kDa (Shaw *et al.*, 1993) (see table 1.2). Furthermore, pure enzyme eluted from the Superdex 75 column with a relative retention time consistent with a molecular weight of 29-30 kDa, indicating a monomeric enzyme in the native state. Most nucleotidyltransferase enzymes are believed to exist as monomers (Davies & Smith, 1978). However, Sakon *et al.* (1993) discovered upon crystallographic analysis that kanamycin nucleotidyltransferase (originally isolated from *S. aureus*) exists as a dimer.

The summary of the physical characterisation of the enzyme is shown in table 4.4

Table 4.4- Physical properties and resistance profile of ANT(3")-I. The results were obtained from the pQR601 construct in *E.coli* JM107.

Antibiotic Substrate	MIC μg/ml
Strepomycin	120
Spectinomycin	450

Size 29.5 kDa (monomeric)

pI 5.91

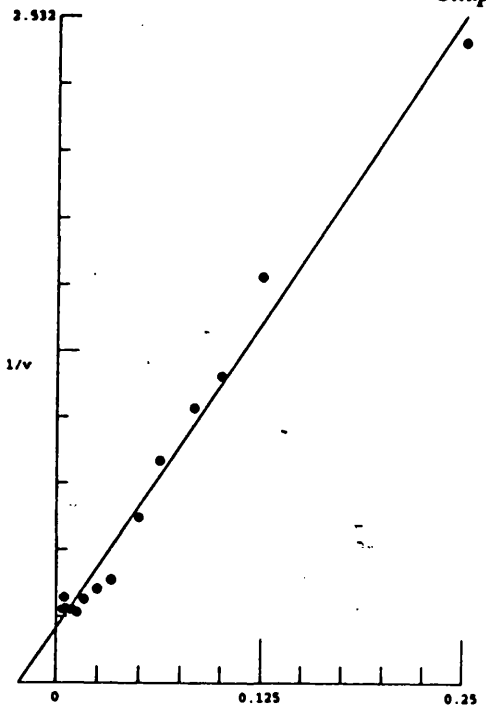
4.2.4 BIOCHEMICAL CHARACTERISATION

Kinetic studies of the enzyme were carried out to determine K_m and V_{max} for each substrate. The K_m and V_{max} values obtained for all three substrates are shown in table 4.5. The Lineweaver-Burk plots for each substrate are represented in fig.4.6.

Table 4.5-Table of kinetic characterisation of ANT(3")-I. The enzyme's K_m and V_{max} values were determined using the hyperbolic regression analysis with respect to all three substrates. The values quoted are the mean values of two individual experiments.

Substrate	K_m (μM)	V_{max} (nMol/min/mg)
ATP	81.53	361.45
Streptomycin	0.64	396.3
Spectinomycin	36.0	384.4

A:



B:

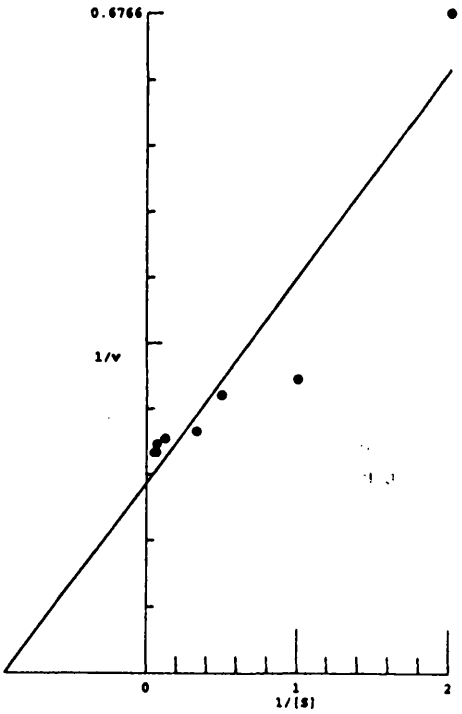
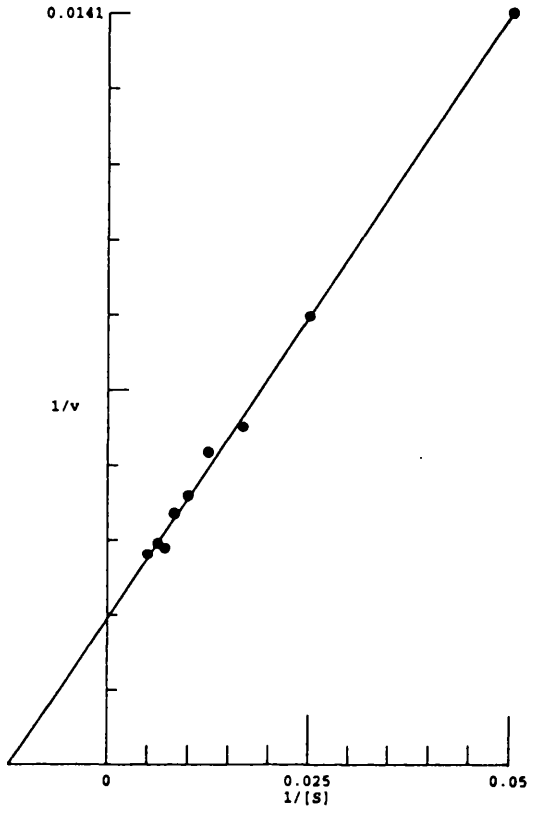


Fig. 4.6-Lineweaver-Burk plots for the three substrates. A, plot for ATP; B, plot for spectinomycin; C, plot for streptomycin. To obtain K_m and V_{max} values for streptomycin and spectinomycin, the assays were performed at 500mM ATP. The assay buffer used to calculate the K_m for ATP contained 500mM spectinomycin.

C:



4.3 DISCUSSION

The purification strategy employed, avoided the use of affinity separation techniques as the protocol was to be ultimately used to purify mutant enzymes. The presence of mutations within the coding sequence of the gene may alter the affinity of the enzyme for the aminoglycoside substrate or co-substrate (ATP). Consequently, techniques involving separation on the basis of affinity of the enzyme for either substrate was not considered.

A number of different columns were tested in many different combinations (both high and low pressure) until the final combination of columns described in this chapter, was used for the purification of ANT(3")-I.

During the purification process, problems were encountered in the stability of the partially purified enzyme, resulting in low yields of the pure enzyme (see table 4.3). This instability was thought to be as a result of proteolytic degradation by intrinsic catabolic enzymes and the natural degradation of proteins upon cell lysis. To minimise degradation by proteases, a cocktail of protease inhibitors was added to the extracts following each step of purification. This was thought to be necessary as some of the protease inhibitors used were revesible inhibitors and therefore could be diluted out or separated from the enzyme by the various separation techniques.

As the partially purified enzyme was found to be rather unstable during freeze/thawing procedures the purification protocol had to be completed consecutively, without storage of the partially purified protein. As a result, the purification process was performed in approximatly 15-20 hours. Once the protein had reached homogeneity it was found to be very stable and was stored at -70°C until required.

To improve the yield of the pure enzyme, other methodologies were tested. The first of these was to replace the ammonium sulphate precipitation of protein with PEG precipitation. Precipitation of proteins by organic polymers such as PEG, is often prefered as a first step in purification as PEG does not normally interfere with the following steps in the purification such as chromotography. However, as can be seen from table 4.2, the enzyme could not be precipitated using this organic polymer. Other methods were also tested. Another procedure that was examined was to omit the precipitation step altogether. For this, the cleared cell lysate was loaded directly onto a high load ion exchange column (Mono-Q) as the first step of purification. This

resulted in a 95% recovery of the enzyme. However, using this procedure, the enzyme could not be purified to homogeneity even following a chromatofocusing column (results not shown). It was decided therefore, that precipitation of the enzyme by ammonium sulphate was an essential first step in the purification as it resulted in the removal of other proteins that could not be fractionated successfully by other methods. Another strategy that was employed was to transform another *E.coli* strain with the plasmid carrying the *ant(3")-Ia* gene. *E.coli* strain BL21(DE3) carrying the pQR606 construct was tested for stability and expression of the enzyme. This strain lacks some of the major proteases in the cell and was thought to be suitable for purification purposes. However, the level of expression from this construct was found to be insufficient for bulk purification (results not shown).

K_m and V_{max} values of pure ANT(3")-I have been determined for the three enzyme substrates; streptomycin, spectinomycin, and ATP (table 4.5).

On comparing the K_m values obtained for the two aminoglycoside substrates, it can be seen that the enzyme has an approximate 50 fold higher affinity for streptomycin as compared to spectinomycin whilst the V_{max} of the enzyme for either substrate remains essentially the same. This indicates that although ANT(3")-I binds streptomycin with a higher affinity, the enzyme's maximum catalytic rate is the same when modifying either aminoglycoside. However, when challenged with both substrates simultaneously, one would expect ANT(3")-I to saturate with streptomycin first and therefore, this would be preferentially modified..

When comparing the minimum inhibitory concentrations obtained for the two antibiotics and their K_m values, there is obviously a clear correlation between K_m

values and MICs. In the case of streptomycin, the lower K_m value corresponds to the lower MIC obtained for this antibiotic, as expected. As this antibiotic is more lethal to the bacterial cell, there is a need for this drug to be inactivated as soon as it enters the cell. This supports pervious findings by Vatstola *et al.*(1980) for an *E.coli* strain with an ANT(2")-I enzyme where a higher affinity for a substrate usually indicates a lower tolerance for the drug.

These findings further support the rate competition concept where rate of modification versus accumulation of antibiotic (Dickie *et al.*, 1978, Holtje, 1979). If the affinity of the enzyme for the antibiotic is sufficiently high, modification of the drug commences at the lowest concentration at which the aminoglycoside is accumulated. Therefore, in order for the enzyme to be effective and produce resistance, it is vital that the enzyme shows a high affinity for the antibiotic to prevent accumulation of the unmodified form of the drug.

There is little understanding of events which occur in the cell following modification by the enzyme. It is unclear what happens to the aminoglycoside antibiotic following modification. Studies carried out in a strain that acetylates gentamicin (Davies, J., personal communications), showed the presence of the acetylated drug in the resistant bacterial cells which remained in the cell cytoplasm. No modified antibiotic was released into the medium. Further studies indicated that the concentration of modified antibiotic remained constant within the cell. As aminoglycosides enter the cell by a proton gradient mechanism, it has been assumed that once the drug reaches a saturating concentration in the cytoplasm, which may be low in the absence of

ribosome binding, no more antibiotic is able to enter the cell. In this instance, the amount of modified antibiotic within the cell remains stable until it is simply diluted out with growth (Davies, J., personal communications). To date, no studies have been carried out to determine the fate of the inactivated drug after several generations of growth in the absence of antibiotic.

The determined value for pI is some what different to the predicted value for the enzyme. As previously mentioned, this predicted value was based upon the amino acid sequence of the protein which does not take into account the tertiary structure of the proteins and the subsequent interactions of the side groups with one another. It is therefore feasible to assume that the pI value (5.91) obtained through this study is the more accurate value.

The molecular weight of the enzyme was also determined, again, there are discrepencies between the previously published molecular weight (Hollingshead and Vapnek, 1985: Shaw *et al.,* 1993) and the molecular weight obtained during the course of this study. However, this difference has little significance as conditions during electrophoresis of proteins may effect the migration patterns observed. A more accurate estimation of the size of the enzyme was obtained through the elution profile obtained from gel filtration chromatography. The molecular weight was found to be 29.5kDa which still agrees well with the previously published data, and shows the enzyme to be monomeric.

CHAPTER FIVE

SEQUENCE ANALYSIS AND HOMOLOGY STUDIES

SUMMARY

Amino acid sequence homology studies carried out on ANT(3")-I proteins isolated from various sources has revealed extensive homology throughout this class of enzymes (see section 1.6), however, certain residues within the protein seem to be more conserved than others. Comparative amino acid sequence analysis between nucleotidyltransferases has implicated two acidic residues in the N-terminal region of the protein in binding of the nucleotide.

Secondary structure prediction of the encoded protein has been carried out together with a number of other protein and amino acid analysis studies.

5.1 *ant(3")-Ia* Gene

The *ant(3")-Ia* gene from R-plasmid R538-1 used in this study was originally isolated and sequenced by Hollingshead and Vapnek (1985). The derived amino acid sequence was predicated by performing the Fickett test (Fickett, 1982) on the coding region of *ant(3")-Ia* gene. This test gave a 92% probability that the deduced amino acid sequence from *ant(3")-Ia* gene is as reported.

Previous studies had indicated that the enzyme was periplasmic (Benveniste *et al.*, 1970) as it could be released from the cells by osmotic shock treatment. The protein was therefore thought to possess a signal sequence at the 5' end of the amino acid sequence to facilitate the passage of proteins, made in the cytoplasm, through the cellular membrane. However, further studies on this enzyme and other aminoglycoside modifying enzymes indicated that these enzymes were membrane associated and not periplasmic and hence did not require a signal sequence. Moreover, sequence analysis carried out by Fling *et al.* (1985) on Tn7 encoded *ant(3")-Ia* gene indicated a possible initiation codon at nucleotide position 402 of the originally published sequence. This was further confirmed by

sequence homology studies carried out between the two sequences for positions 402 to the end of the gene, where a 99% homology was shown.

5.2 Computer Analysis of Protein and DNA Sequences

The main DNA and protein sequence databanks (GenBank, the EMBL Nucleotide Sequence Data Library, the NBRF DNA and protein sequence databanks, and Swiss-Prot) are the central repositories for sequence data produced around the globe and now contain more than 40 million bases of DNA sequence and some 5 million residues of protein sequences. These data bases were used during the course of this study to retrieve protein and DNA sequences and to carry out theoretical sequence analysis.

5.2.1 Data-base searching and multiple sequence alignments for protein modelling

For the purposes of molecular modelling, the ultimate goal of a sequence data-base search is to find a matching sequence that has two essential characteristics:

A) that it is similar enough to the query sequence that one can assume both proteins have the same general structure or fold (there are cases where the structure of two proteins are similar but share no significant homology in primary sequence) and

B) that the matching sequence corresponds to a known 3D structure whose co-ordinates are obtainable.

A number of searches were carried out to identify other matching sequences to the aminoglycoside nucleotidyltransferase enzymes using programs BLAST (Altshul *et al.,* 1990) and FASTA (Pearson and Lipman, 1987). Each of these methods uses a different scoring system and evaluation of statistical significance.

However, no matches other than other aminoglycoside nucleotidyltransferase enzymes were identified.

The most interesting recent development in the database field is the construction of more 'intelligent' object-oriented databases which allow very sophisticated searches to be made with a minimum effort. Such databases on protein sequence and structure were used to obtain the secondary structure prediction of aminoglycoside (3") nucleotidyltransferase using the PHD program at Hiedelberg.

5.3 Amino acid homology studies

Predicted amino acid sequences from a number of aminoglycoside nucleotidyltransferase enzymes were compared by using various multiple sequence analysis program software packages of the University of Wisconsin Genetics Computer Group. The programs most often used to compare sequences were ClustalV (Higgins *et al.*, 1992), Pileup (Feng and Doolittle, 1987) and Bestfit (Rechid *et al.*, 1989) (the latter being used for pairwise alignments only). ClustalV and Pileup create multiple sequence alignments using progressive, pairwise alignments. All programs use a scoring system for alignments of the protein sequences.

Once the nucleotide or amino acid sequence of resistance genes have been compared, it is possible to demonstrate the evolutionary relatedness of genes. Being assigned to different homology groups does not necessarily imply that the determinants have evolved independently. However, if a small degree of homology confined to the active site of the enzyme is found, it could be argued that this is due to convergent evolution. Convergence would certainly be suspected where no homology was detectable (Ambler, 1980, Jaurin and Grundstrom, 1981).

5.4 Evolutionary trees

Amino acid sequence alignments carried out on ANT family of proteins were used to construct phylogenetic trees of this group of proteins. The trees give a "branching order" , or "topology", which is a reflection of the gene duplications giving rise to new gene products or the point of divergence for organisms that share a common ancestry. The branch lengths are indicative of the evolutionary distance separating the sequences that existed when these events occurred and the present sequences.

Family trees were constructed to determine the evolutionary relationship and the relatedness of the nucleotidyltransferase enzymes. These trees were constructed using the progressive alignment program Pileup, where two closely related sequences are first paired and gaps created, the next most similar sequence is then added to what is essentially the average sequence of the first pair. The process is then repeated until all the sequences have been aligned.

5.5 RESULTS

The derived amino acid sequence of ANT(3")-I from plasmid R538-1 was subjected to a number of data-base searches and profile network analysis prior to multiple sequence alignment studies with other members of aminoglycoside nucleotidyltransferase enzymes. The result of these searches were as follows:

5.5.1 Secondary structure analysis

The secondary structural analysis of ANT(3")-I was predicted by the profile network method (Rost and Sander, 1994) with an accuracy of 65-71.4%. This indicated an α-helix content of 49.0%, a β-structure content of 14.1%, and 36.9%

loop content indicating that the protein belongs to the mixed class of proteins. The full prediction of the secondary structure can be found in appendix 3.

5.5.2 Tertiary structure prediction

Various predictive methods of homology search have failed to reveal any sequence homology between the family of ANT and any other protein of a known structure. Although a kanamycin nucleotidyltransferase (KNT) enzyme has recently been crystallised (Sakon *et al.,* 1993), there appears to be vast structural differences between the primary sequence of this enzyme and aminoglycoside (3") nucleotidyltransferases. One of the main differences between the two enzymes is the fact that the kanamycin nucleotidyltransferase enzyme is present as a dimer, whereas, the ANT(3")-I enzyme is found to be a monomer. Due to these differences, the tertiary structure of KNT was not recognised through the homology searches and could not be used as a model for the prediction of the tertiary structure of ANT(3")-I group of enzymes.

Using the predicted secondary structure, one may be able to predict the tertiary structure of the protein. However, the secondary structure of the ANT family has not been predicted with a high degree of confidence due to the lack of homology of these proteins with others present in the data base.

5.5.3 Nucleotide-Binding Site

Although this family of enzymes bind ATP, no nucleotide binding motif was found within the sequence. There is no definite indication of a P-loop (Saraste *et al.*, 1990) or the Walker motif found in a number of nucleotide binding proteins (NTP) (Walker *et al.*, 1982). However, there seems to exist limited similarities between parts of the gene and the pyrophosphate motif (PP-motif) (Bork and Koonin, 1994). This is a highly conserved motif found in several groups of

enzymes cleaving the α–β phosphodiester bond in ATP and appears to be a modified version of the P-loop found in β–α NTPases.

Secondary structural predication of the PP-motif suggest a β–α–β organisation that resembles several nucleotide triphosphate binding (NTP) motifs including the classical NAD/FAD-binding motif, the Walker type NTP-binding motif A as well as phosphate binding motifs in phosphorylases. Similarly to the PP-motif, these binding sites consist of a glycine-rich loop preceded by an interior hydrophobic β–strand and succeeded by an α–helix. Furthermore, the PP-motif seems to reside in a distinct domain. The motif is either located near the N-terminus or is immediately downstream from a functionally well-characterised domain.

The similarities found between regions of the ANT(3")-I protein and the PP-motif lie close to the N-terminal region of the protein. This region (between amino acids 32-53) consists of a glycine rich loop followed by a hydrophobic region. The essential difference in ANT(3") is that this region is succeeded by another β-strand (as predicted by the secondary structure prediction, see appendix 3) rather than an α-helix. The consensus of the PP-motif can be seen in fig 5.1.

5.5.4 Sequence alignment studies

Sequence alignments were first carried out on members of ANT(3")-I family of proteins (fig. 5.1). These alignments revealed that the members of this group share large regions of homology throughout the predicted amino acid sequence.

There seems to be considerable homology between the eight ANT(3")-I enzymes shown in fig. 5.1 with certain blocks having long stretches of complete homology (boxed regions). whilst the N- and the C-termini show the least degree of homology. When the ANT(9)-I protein is included in the alignment (fig. 5.2), the number of completely conserved residues drops but certain regions still stand out as being highly conserved. These are within the boxed regions and the first of

these regions maintains 11 out of 16 residues completely conserved, with 3 conservative changes. There are other shorter regions of complete conservation scattered throughout the centre of the protein alignment. This indicates that an overall frame work in the tertiary structure is shared by this group of proteins.

A phylogenetic tree was also constructed which shows the evolutionary relationship of these genes with respect to each other (fig. 5.3). In the construction of the tree diagram, the enzyme ANT(9)-I was also included to determine the relationship between this protein and the other ANT(3")-I enzymes.

The *ant(3")-Ia* gene product shows close to 100% identity at the amino acid level among bacteria such as *E.coli* (Fling *et al.*, 1985), and *K.pneumoniae* (Tolmasky, 1990). There are two exceptions where the protein shows a lower degree of conservation at the amino acid level. The ANT(3")-I isolated from *Agrobacterium tumefaciens*, which shows 82% homology to other ANT(3") proteins. The second being the protein encoded by the *ant(3")-Ia* gene isolated from *Salmonella choleraesuis* (Leung *et al.*, 1992) where only 44% homology is observed when compared with amino acid sequences of other highly conserved ANT(3") proteins (fig. 5.3). This suggests that the *ant(3")-Ia* genes of *S.choleraesuis* and *Agrobacterium tumefaciens* diverged from the other members of this family earlier in evolution.

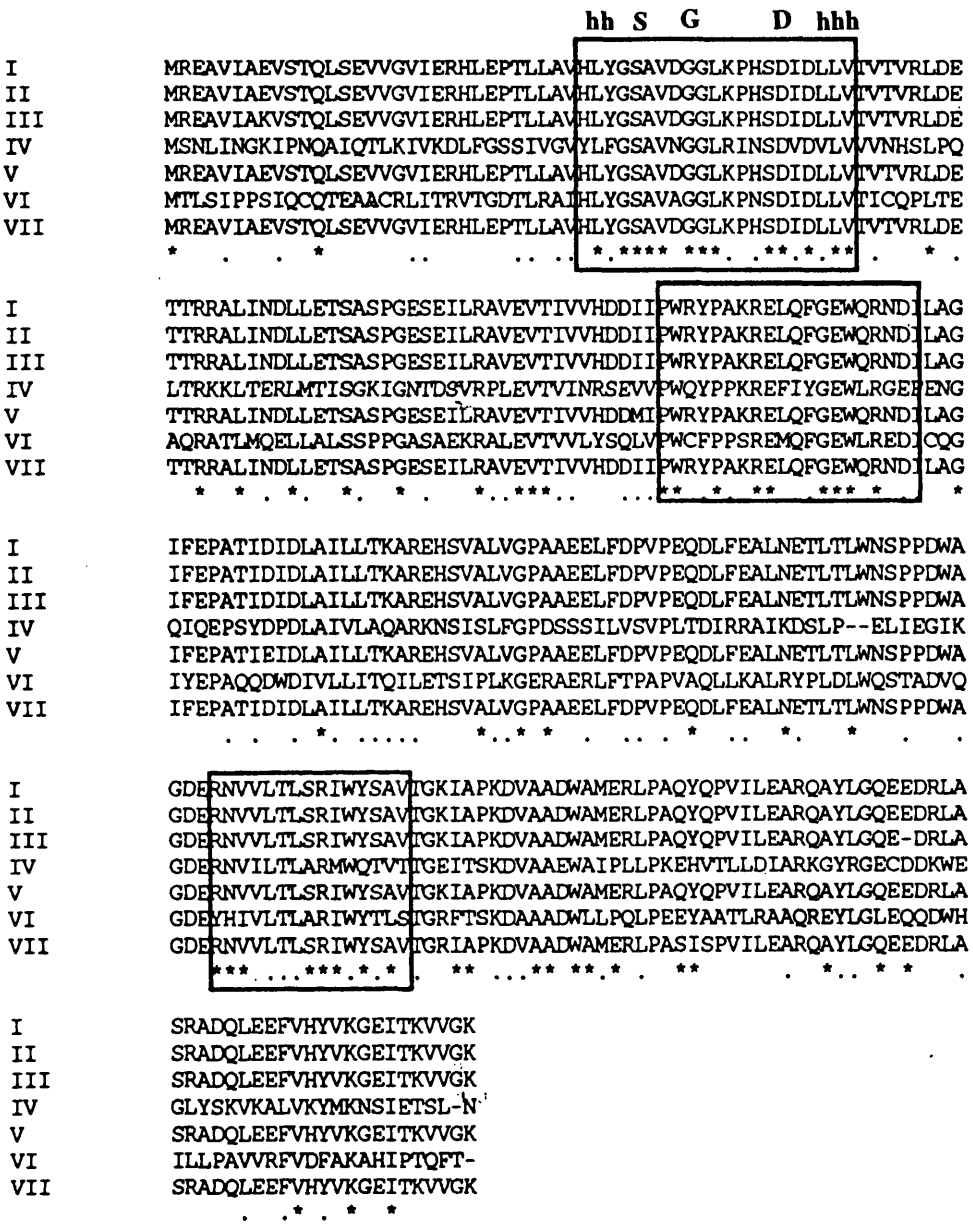


Fig. 5.1- Amino acid sequence alignment of the ANT(3")-I enzymes isolated from various sorces: I, R538-!; II, *E.coli*; III, Tn1331; IV, pSa; V, Tn21; VI, *S.choleraesuis*; VII, R46. The boxed regions are areas of high conservation of amino acids seen in all sequences. * indicates identical residues, . conserved residues. Top line: PP-motif (see 5.5.3), h, hydrophobic positions, bold capitals, invarient amino acids.

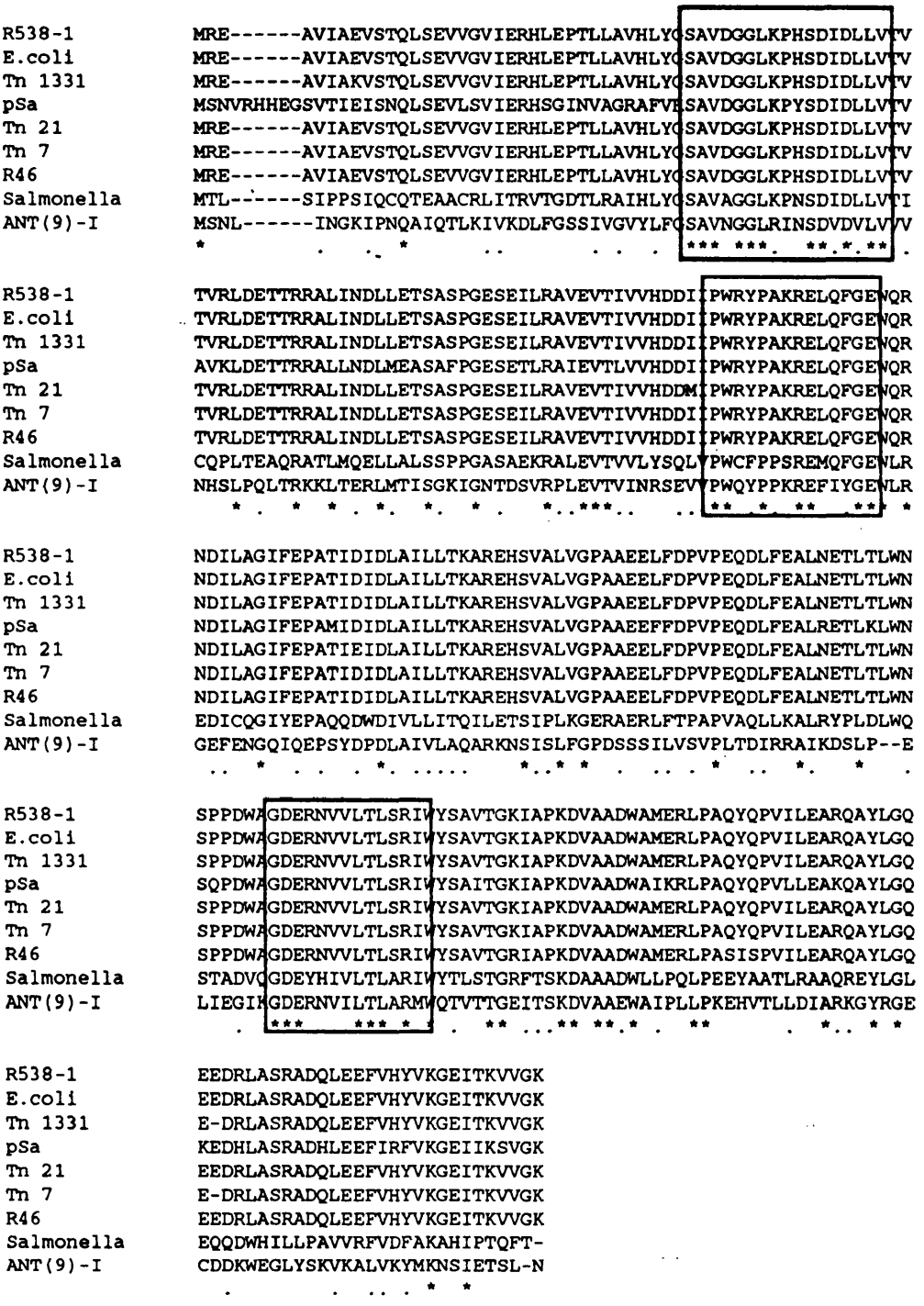


Fig. 5.2-Amino acid sequence alignment of ANT(3")-I enzymes and ANT(9)-I enzyme. The alignment was performed by ClustalV multiple sequence alignment program. *denotes identical residues, . conserved residues. The first 8 sequences all blong to ANT(3")-I family of proteins isolated from various sources. The boxed regions are areas of high conservation of amino acids seen in all sequences.

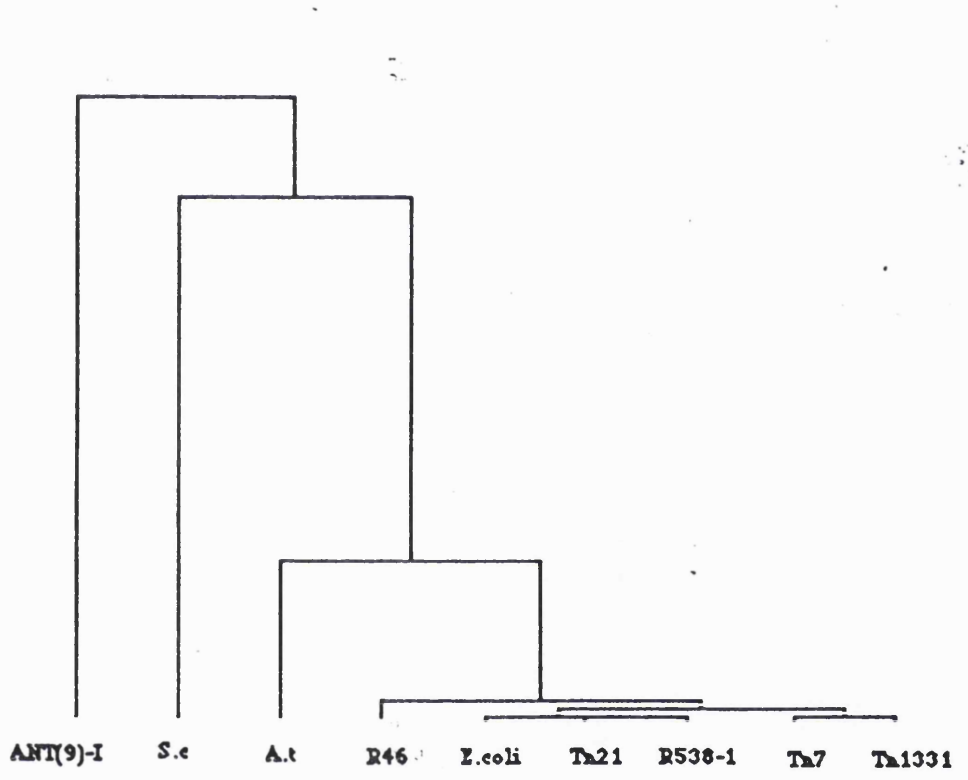


Fig. 5.3-Phylogenetic tree of ANT(3")-I family of enzymes showing their relationship to one another and the ANT(9)-I enzyme. S.c., *Salmonella choleraesuis*; A.t., *Agrobacterium tumefaciens*

Other members of the aminoglycoside nucleotidyltransferase family were then included in the amino acid sequence alignment together with the derived protein sequence from two other nucleotidyltransferase genes which result in antibiotic resistance in host strains. The first was the protein derived from the lincosaminide nucleotidyltransferase gene (linA) isolated from *S.aureus* which confers resistance to the lincosaminide antibiotic lincomycin (Brisson-Noel *et al.*, 1988). Although this enzyme does not display any overall sequence homology with any aminoglycoside nucleotidyltransferase (fig. 5.4), there seems to exist some conservation of acidic charged residues between this enzyme and the ANT family of enzymes (fig. 5.5).

Another enzyme included in the amino acid sequence analysis was the kanamycin nucleotidyltransferase (KNT) enzyme. This enzyme was also originally isolated from *Staphylococcus aureus* and catalyses the transfer of a nucleoside monophosphate group from a nucleotide triphosphate to the 4'-hydroxyl group of kanamycin (LeGoffic *et al.,* 1976). KNT can utilise ATP, GTP, or UTP as the nucleoside monophosphate donor and can inactivate a wide range of aminoglycosides including kanamycins A, B, and C, gentamicin A, amikacin, tobramycin, and neomycins B and C (Davies and Smith, 1978; Le Goffic *et al.,* 1976b). Although the enzymatic mechanism of KNTase has not yet been fully established, it is possible that this enzyme operates in a similar manner to that of aminoglycoside nucleotidyltransferase 2"-I (Gates & Northrop, 1988) and other aminoglycoside nucleotidyltransferases.

Fig. 5.4-Amino acid sequence alignment of ANT(3")-I (R538-1), kanamycin nucleotidyltransferase (KNT) and lincosaminide nucleotidyltransferase (LNT). Boxed region indicates the conserved amino acid residues identified in the nucleotidyltransferase enzymes. Amino acid sequence aligenment was performed by ClustalV multiple sequence alignment program. * denotes identical residues; . conserved residues.

```
AADS        EKNEDVRVLL LTSSLVNPLA LVDEFSDLDI EFVFEDNTNY ISDKSWTLKF
AADK        LNDERIRLVT LEGSRTNRNI PPDNFQDYDI SYFVTDVESF KENDQWLEIF
LNT         .......... .......MKI DNVT..EKDL FYILDLF.EK MEVTHWLDGG
R538-1      LE...PTLLA VHLYGSAVDG GLKPHSDIDL LVTVTVRLDE TTRRALINDL
Shigella    LE...PTLLA VHLYGSAVDG GLKPHSDIDL LVTVTVRLDE TTRRALINDL
psa         SG...INVAG RAFVRSAVDG GLKPYSDIDL LVTVAVKLDE TTRRALLNDL
Salmonella TG...DTLRA IHLYGSAVAG GLKPNSDIDL LVTICQPLTE AQRATLMQEL
AADE        LK...NNLIG TYMFGSGVES GLKPNSDLDF LVVVSEPLTD QSKEILIQKI
ANT(4)-II   REAYPHAV.A ILLKGSYARG EASAWSDIDF DVLVSDEEVE EYR.......
KNT         LDKYGDDVKA IGVYGSLGRQ TDGPYSDIEM MCVMSTEEAE FSH.......
```

Fig.5.5- Multiple sequence alignment of nucleotidyltransferase enzymes. The region shown is towards the N-terminus. The boxed region shows the conserved aspartic acid/glutamic acid residues identified in nucleotidylating enzymes. The sequence alignment was performed by Pileup multiple sequence alignment program.

The evolutionary tree constructed for nucleotidyltransferases (fig. 5.6) shows four clusters. The genes in each cluster probably diverged early on in the evolution from the other three. One branch is made up of *aadS*, *ant(6)*, and *ant(4)-I* genes. The second branch contains genes for *ant(2)* and *linA* (encoding the LNT protein). The branch that shows the highest degree of relatedness is the one encoding the *ant(3")* genes. The gene coding for *ant(9)* is also included in this group. The fourth cluster includes the genes coding for KNT and ANT(4)-II enzymes.

The clustering of these genes indicates that although these genes may have had a common ancestor, they diverged very early on and have probably evolved separately, as their sequences do not show any significant homology to sequences in the other clusters.

Previous sequence analysis studies between members of the aminoglyoside nucleotidyltransferase family has failed to show any significant homology within this group of enzymes, at the amino acid level (Shaw *et al.*, 1993). However, during the course of this study, certain regions within the proteins were identified which show a large degree of homology and conservation throughout all classes of nucleotidyltransferase enzymes (figs. 5.2 & 5.5). The largest percentage homology was observed towards the N-terminal region of the protein.

It is interesting to note that although the percent identity between these enzymes is very low, the acidic residues seem to have been conserved (fig.5.5), perhaps indicating a common structure/function relationship.

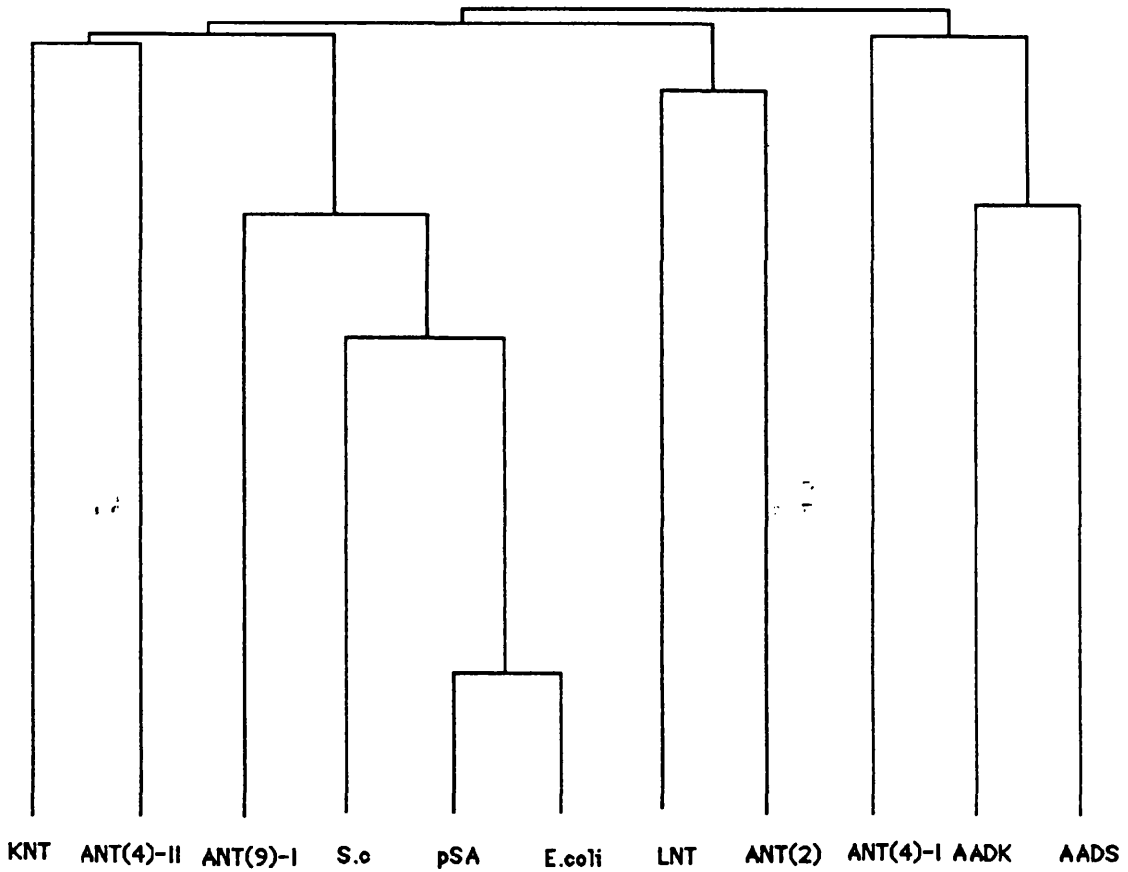


Fig. 5.6-Phylogenetic tree of aminoglycoside nucleotidyltransferase enzymes and lincosaminide nucleotidyltransferase. The tree was constructed from amino acid sequence alignment studies. Alignment of protein sequences was performed by multiple sequence alignment analysis program software. Abbreviations: LNT, lincosaminide nucleotidyltransferase; KNT, kanamycin nucleotidyltransferase; S.c. ANT(3")-I isolated from *Salmonella choleraesuis*; E.coli, ANT(3")-I isolated from *E.coli*.

5.6 DISCUSSION

Amino acid sequence analysis studies of the ANT family of enzymes has not shown significant homology between members of this family and other nucleotidyltransferase enzymes. The ANT(4')-Ia and ANT(4')-IIa proteins, although members of the same class of proteins, do not show significant overall homology to one another and actually fall into different clusters (fig. 5.6). However, the predicted amino-terminal sequences of these proteins show significant sequence similarity between residues 38-63 of the ANT(4')-Ia protein and residues 26 and 51 of the ANT(4')-IIa protein.

Previously, the AADS protein had been shown to possess a strong relationship with a number of streptomycin nucleotidyltransferase enzymes, in particular ANT(6) from *Bacillus subtilis* (Ohmiya *et al.*, 1989) and AADE from *Entrococcus faecalis* plasmid, pJH1 (Ounissi and Courvalin, 1987). Comparisons with other Gram-positive aminoglycoside modifying enzymes (AAC or APH) or other streptomycin resistance genes of Gram-negative origin (ANT(3")-Ia or ANT(2')) has shown no significant homology at either the protein or nucleic acid level (Smith *et al.*, 1992).

Amino acid sequence homology studies between KNT and LNT enzymes and aminoglycoside nucleotidyltransferases have revealed highly conserved regions within the ANT(3")-I sequences and a suggestion of a shared motif between this group and the KNT and LNT enzymes. Since conserved sequence motifs usually correspond to a functional site within a larger domain with a conserved topology, these regions were thought to be important.

Recently, mutants of the enzyme kanamycin nucleotidyltransferase (KNT) have been crystallised and the tertiary structure of this enzyme determined (Sakon *et*

al., 1993). Comparison of the secondary structure prediction of ANT(3")-I with the determined tertiary structure of KNT has indicated some degree of conservation of these regions at the structural level, however, as can be seen in fig 5.4, the primary amino acid sequences of the enzymes do not show significant homology other than that shown in fig. 5.5. Furthermore, the conserved region of the protein at the N-terminal part, and in particular the aspartic acid residue found at positions 50 and glutamic acid residue at position 52 of the KNT enzyme (corresponding to aspartic acid residues 47 and 49 of ANT(3")-I) have been implicated in binding of metal ions (Sakon *et al.*, 1993). Amino acid sequence alignment studies carried out on other members of the ANT family have revealed the complete conservation of these charged residues (fig. 5.5). Although the aminoglycoside nucleotidyltransferase group of enzymes possess very limited sequence homology to one another, these acidic residues seem to be conserved throughout the family. Since substrate specificity of these enzymes are varied, it is reasonable to assume that this region may in some way be involved in nucleotide binding

Charged residues, in particular aspartic acid residues in other nucleotide binding proteins have been associated in binding of Mg^{2+} ions and other metal ions such as zinc and manganese (Sakon *et al.*, 1993, Sawaya *et al.*, 1994). Furthermore, they have also been associated with binding of Ca^{2+} in amylases and proteases. (Rogers, 1985).

Structural studies in other nucleotidyltransferase enzymes have indicated that these conserved aspartic acid residues are spatially clustered together so that they may co-operatively co-ordinate the binding of the metal ions (Sawaya *et al.*, 1994) which in turn probably participate to some extent in the positioning of the incoming nucleotide by neutralising the charges on the phosphate groups.

Furthermore, mutation studies carried out by Sawaya *et al.* (1994) on these residues in the β-subunit of the DNA polymerase enzyme resulted in a decreased K_{cat} of nucleotidyl transfer by 100 to 50,000 times.

To further investigate the role of these invariant acidic residues , it was decided to carry out site-directed mutagenesis on these conserved residues and study the mutants for their substrate specificity and enzymatic activity.

CHAPTER SIX

SITE-SPECIFIC MUTATION STUDIES

SUMMARY

Amino acid sequence analysis of the proteins derived from *ant(3")-Ia* genes from various sources has revealed extensive homology throughout, although certain regions of the protein appeared to be more highly conserved than others. In order to test the biological significance of these regions, site-directed mutagenesis was carried out to alter the highly conserved residues present.

6.1 Amino Acid Sequence Analysis

Predicted amino acid sequences from a number of aminoglycoside nucleotidyltransferase enzymes were compared by using various multiple sequence analysis program software packages of the University of Wisconsin Genetics Computer Group (see chapter 5). Highly conserved regions within the gene have been identified and postulated to be involved in catalysis (see figs 5.1&5.2). To test the significance of these areas, site-specific mutagenesis studies were carried out using a number of synthetic oligonucleotides designed to alter some of the most highly conserved residues within this conserved region of the protein.

The surprising sequence similarity in these enzymes, which bind widely differing antibiotic structures such as streptomycin and spectinomycin, suggests that the conserved residues might be involved in a common function, such as ATP binding and adenylation of the substrate. Most of the highly conserved amino acids seem to be located in the N-terminal portion of the aminoglycoside nucleotidyltransferases, although the rest of the gene also shows a high degree of conservation with other aminoglycoside (3") nucleotidyltransferases.

6.2 Site-Directed Mutagenesis

Plasmid pQR601 was used for mutagenesis studies.This plasmid was constructed by ligation of the *ant(3")-Ia* gene on a 1.95Kb *Hin*dIII fragment into the pBluescriptKS$^-$ vector (section 3.1). Site-specific mutations were carried out using the Transformer mutagenesis kit obtained from Clontech Laboratories, Inc. The mutagenesis was performed as manufacturer's instructions (see section 2.9) using the Trans Oligo *ScaI/Stu*I (Fig. 6.1) as the selection primer. This primer was recommended by the manufacturer for use with the pBluescriptKS$^-$ plasmid.

Trans Oligo *Sca I/Stu I* GTGACTGGTGAG**G C**CTCAACAAGTC

StuI

Fig. 6.1- The coding sequence for the Trans Oligo used during the mutagenesis. The underlined portions of the sequences represent the second restriction enzyme site, the bold sequences represent base pairs changed during mutagenesis. The DNA sequences flanking both sides of the restriction site match those found on the plasmid. The incorporation of this selection primer changes the *Sca*I site of the vector to a *Stu*I site allowing selection against the non-mutated parent strain by cleavage with *Sca*I

Synthetic oligonucleotides were used to alter specific sites within a highly conserved region of the gene close to the N-terminal part of the protein. The mutagenic oliogonucleotides were designed to alter the aspartic acid residues at positions 47 and 49 to alanine (fig. 6.2). Figure 6.2 shows the region of the gene chosen and the synthetic oligonucleotides used to change the aspartic acid residues.

Wild type gene sequence:

```
                    47      49
5' G CCA CAC AGT GAT ATT GAT TTG CTG GTT 3'
     P   H   S   D   I   D   L   L   V
```

Mutagenic oligonucleotide 1:

```
5' G CCA CAC AGT G C T ATT GAT TTG C 3'
     P   H   S   A   I   D   L
```

Mutagenic oligonucleotide 2:

```
5' C AGT GAT ATT G C T TTG CTG GTT A 3'
     S   D   I   A   L   L   V
```

Mutagenic oligonucleotide 3:

```
5' G CCA CAC AGT G C T AAT G C T TTG CTG GTT A 3'
     P   H   S   A   I   A   L   L   V
```

Fig. 6.2-The DNA sequence and the derived amino acid sequence of the highly conserved region of the wild type gene. The three mutagenic oligonucleotides used to alter the aspartic acid residues at positions 47 and 49 are also shown. The bold under lined sequences indicate the base pair changes during mutagenesis. The bold capitals underneath the DNA sequence represents the derived amino acid sequences (shown as single letter codes).

Mutagenesis was carried out as described in materials and methods section 2.9. Following mutagenesis, the ligation mixtures were used to transform competent *E.coli* JM107 cells. The transformation mixture was then plated out on nutrient agar plates containing ampicillin as the selection marker. Streptomycin or spectinomycin were not used at this stage as the effect of the mutation on the gene was not known.

6.3 RESULTS

Two mutants were obtained by this method. Attempts at isolation of the third mutant were however, unsuccessful. The mutants isolated carried the mutagenic oligonucleotides 2 and 3 where the changes were; aspartic acid residue at position 49 to alanine and changes of both aspartic acid residues at positions 47 and 49 to alanine, respectively. The plasmid carrying the mutation derived from oligonucleotide 2 was called pQR610 and the plasmid carrying the mutation derived from oligonucleotide 3 was named pQR611. The third mutation, which was unsuccessful, would have changed the Asp 47 to Ala while keeping the Asp 49.

Mutagenic oligonucleotide	Amino Acid Position			Plasmid
	47	48	49	
Parent	**D**	**I**	**D**	pQR601
oligo 2 mutation	**D**	**I**	**A**	pQR610
oligo 3 mutation	**A**	**I**	**A**	pQR611

Table 6.1-The amino acid sequence of the gene carried on the parent plasmid, pQR601, and the mutations carried on the derived plasmids following mutagenesis.

To ascertain the presence of the specific mutation within the desired region, sequencing reactions were carried out using a specially designed synthetic oligonucleotide as the sequencing primer. This oligonucleotide was designed to hybridise to the gene approximately 50 base pairs upstream (on the 5' side) of the site of the mutations. The sequencing autoradiographs confirmed the desired alterations within each strain.

The resistance phenotype of each mutant was tested by using various antibiotic concentrations to measure the minimum inhibitory concentrations (MICs) of antibiotics above which there was no growth. The MICs of these mutants were then to be compared to those of the wildtype strain. No resistance was detected to streptomycin or spectinomycin in the two mutant strains. Resistance to other aminoglycosides was also tested (table 6.2) to investigate the substrate specificity of the mutants. Again, the mutants did not confer resistance to any of the aminoglycoside antibiotics tested.

plasmid	Sm	Spcm	Amk	Gm	Km	Nm	Kgm	Bt
pQR601	+	+	-	-	-	-	-	-
pQR610	-	-	-	-	-	-	-	-
pQR611	-	-	-	-	-	-	-	-

Table 6.2-Resistance confered to aminoglycoside antibiotics by the wild type enzyme (pQR601) and the mutant enzymes.Sm, streptomycin; Spcm, spectinomycin; Amk, amikacin; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Kgm, kasugamycin; Bt, butirosin.

Polyclonal antibodies were raised against the ANT(3")-I enzyme to be used in Western Blot analysis. The specificity of the polyclonal antibodies were tested by performing Western Blot analysis on *E.coli* JM107 cell lysate carrying pBluescript plasmid with no *ant(3")-Ia* gene insert. As no bands were visulised following immunological staining (results not shown) of the Western Blot, it was assumed that the antibodies were

specific for ANT(3")-I enzyme.Cleared lysates of the wild type and the mutant strains were analysed by Western Blotting to verify the presence of ANT(3")-I and the integrity of the protein. The results of the western blots show that in the mutant strains the enzyme is still being produced and is present although inactive. This can be seen in fig. 6.3 where the signals obtained for the mutant enzymes are of the same intensity as that of the wild type enzyme. As the bands obtained from the mutant enzyme are of the same intensity as that of the parent, it is assumed that the expression of the gene has not been affected by the mutations. Therefore, the lack of activity from the mutant enzymes can not be attributed to lower expression levels from these mutated plasmids. It is interesting to note that 2 bands were detected in all crude cell lysates and only one band was present on the purified enzyme sample. This may have been as a result of protease activity and the consequent degredation of the protein in the crude cell extracts.

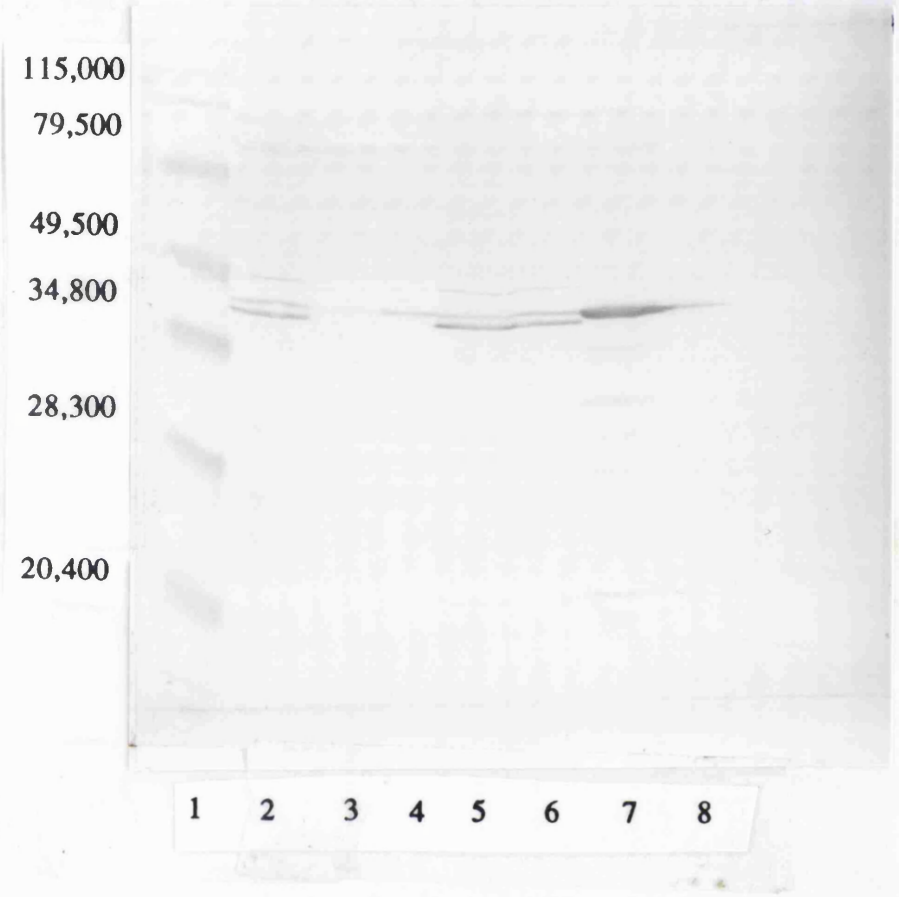


Fig. 6.3-Western blot analysis of mutant constructs to confirm the presence of the enzyme. 1, molecular weight marker; 2, pQR601; 3, pQR602; 4, pQR606; 5 pQR610; 6, pQR611; 7, pQR609; 8, pure enzyme.

To characterise the effect of each mutation at the enzymatic level, ANT activities of crude enzyme preparations from wild type and the mutant strains, were assayed by the phosphocellulose binding assay (see chapter 2, section 2.11.1). These indicated that there was no ANT activity detectable in the mutant strains. Activity of aminoglycoside modifying enzymes, in particular the ANTs, has been reported to increase with partial purification of these enzymes (Coombe and George, 1981). This is probably due to the

removal of contaminating ATP utilising enzymes. However, attempts at partial purification of the mutant enzymes by gel filtration chromatography (Superdex 75) were unsuccessful and no enzyme activity was detected in assayed fractions following gel filtration. To confirm the presence of the mutant proteins in the eluted fractions, Western Blot analysis was carried out. The elution profile of the mutant enzymes was found to be the same as the wild type enzyme although a much fainter band was visualised. The reduction in the intensity of the band is as a result of the dilution of the samples as it is put through the gel filtration column. The mutant protein is likely to be folded in the same way as the wildtype enzyme as the mutant protein elutes from the gel filtration column at the same position as the wildtype.

6.4 DISCUSSION

Results indicate that the aspartic acid residue at position 49 is important in the catalysis reactions by the enzyme. Change of this residue to alanine has resulted in a complete loss of resistance in the mutant strain indicating the importance of this residue. The second mutant also showed loss of resistance to either antibiotic, however, in this mutant no further conclusions may be drawn as the same residue is affected together with Asp^{47}. the importance of the conserved aspartic acid at this position could not be tested as the mutation precedures for this were unsuccessful.

As already discussed in the previous chapter, these conserved charged residues may play a role in the action of the enzyme; possibly involved in the binding of the Mg^{2+} ions that bind to the phosphate groups of ATP. This seems feasible due to the conservation of these residues in different classes of nucleotidyltransferases with different substrate specificities. As already mentioned in the previous chapter, even

derived protein sequences that only show limited homology to the ANT(3")-Ia protein, seem to show conservation of these residues.

Furthermore, these conserved aspartic acid residues are close to sequences, in particular a glycine rich region, that loosly resembles an ATP binding motif although does not exactly match the recognised motifs for nucleotide binding. Also conserved aspartic acid and glutamic acid residues have been implicated in ATP binding in a number of different enzymes including the kanamycin nucleotidyltransferase enzyme (Sawaya *et al.*, 1994).

CHAPTER SEVEN

SUMMARY & DISCUSSION

Summary and Discussion

The gene conferring resistance to aminoglycoside antibiotics streptomycin and spectinomycin, *ant(3")-Ia* originally isolated from R-plasmid R538-1 has been cloned into a number of expression vectors on a 1.95Kb *Hin*dIII fragment.
Subsequent expression studies indicated low levels of expression of the gene from these constructs. To further enhance expression, two synthetic oligonucleotides were constructed to remove the non-coding sequences flanking the gene and to insert two unique restriction sites at either end of the gene using PCR. The restriction sites inserted were *Eco*RI and *Nde*I upstream of the start codon and *Hin*dIII and *Bam*HI at the end of the gene.

The *ant(3)-Ia* gene, isolated as a 781 bp *NdeI/Bam*HI PCR fragment was further cloned into two expression vectors, pMTL2023 and pMTL1005 carrying the *trp* promoter (resulting in pQR606) and the *E.coli* malate dehydrogenase (*mdh*) promoter (giving rise to recombinant plasmid pQR609), respectively.
Comparison of expression from each constructs revealed a 2 fold increase in expression from construct pQR606 and a 22 fold increase from cells carrying the recombinant plasmid pQR609, as compared to the first isolate carrying the 1.95Kb *Hin*dIII fragment. Two conclusions may be drawn from this, the first, that non-coding sequences flanking the gene probably affected the expression levels as their removal resulted in an increase in the expression of the gene. The second is that the malate dehydrogenase promoter seems to be a much stronger promoter than the *trp* promoter (itself already considered a strong promoter and used in many expression systems).

The malate dehydrogenase expression system has been shown to be very efficient for the high level expression of heterologous genes as well as of other *E.coli* genes (Alldread *et al.*, 1995). It has successfully been used in the expression of immunoglobulin binding proteins G' and L'(Murphy, J.P., personal communications) and several interleukins (Alldread *et al.*, 1995). This high efficiency is not merely due to the large copy number of the plasmid vector that carries the *mdh* promoter as the recombinant plasmid pQR606 carrying the trp promoter is a vector with similar copy number. The high expression levels observed must therefore be the consequence of the transcription and translation signals present in the *mdh* system.

Several, but not all, of the features seen in many strong *E.coli* promoters and most of the signals considered to facilitate translation at high rate are contained in the malate dehydrogenase system. The sequences TAAGGT and TTGTAA (see appendix 3) in positions 68-73 and 45-50 respectively are presumed to be the -10 and -35 regions of homology seen in a number of *E.coli* promoters (Harley and Reynolds, 1987). In the -10 consensus sequence, TATAAT, the T at the end is invariant and the TA at the beginning is highly conserved in many promoters. This is seen to apply to the *mdh* promoter. In the -35 consensus sequence, TTGACA, the TTG is highly conserved in many promoters, and this holds true for the *mdh* promoter. Furthermore, the spacing between the -35 and -10 sequences in the *mdh* promoter is 17 bp, which is considered to be the optimum for gene expression. The transcription start point (+1 position) in the *mdh* system has been shown to be the G in position 81 (Vogel *et al.*, 1987). On this basis the distance between the 3' end of the -10 sequence and the transcription start point is 7 bp, which is the most common separation seen in promoters (Harley and Reynolds, 1987). Translation initiation codon used for the mdh system was AUG which is the generally preferred codon for initiation in *E.coli* (Van Knippenberg, 1990).

The ribosome binding site which also has the Shine-Dalgarno sequence is also present upstream of the start codon.

TCA cycle enzymes function in two ways under aerobic conditions: firstly, to generate power for the production of ATP by oxidative phosphorylation, and secondly, to provide precursor compounds for biosynthetic processes. As malate dehydrogenase is an enzyme involved in the TCA cycle, glucose represses MDH synthesis therefore, strong (90%) repression of expression from this promoter can be achieved by addition of 1% glucose (w/v). This repression may be due to the fact that under these conditions the need for the organism to produce precursors for the biosynthesis will be greatly reduced and glycolytic catabolism of glucose will yield ATP and alleviate the need for ATP production by oxidative phosphorylation.

Strong repression of expression was also observed when the bacterium carrying the *mdh* promoter was grown under anaerobic conditions (Alldread *et al.*, 1995). The repression observed under these conditions can be understood, as the functioning of the TCA cycle will be much less essential owing to the lack of oxidative phosphorylation and to the provision of biosynthetic intermediates by the complex medium.

This system is therefore obviously able to provide certain advantages as comparable to and in some cases better than other expression systems already established and in use. One of the main characteristics of the *mdh* system is that heterologous genes may be expressed in *E.coli* hosts at a very high rate. It has been shown that the cell may contain up to 40-50% of the total soluble cell protein as the encoded protein under the control of *mdh* promoter (Alldread *et al.*, 1995). The second important feature of this system is the ease with which the promoter is repressed in the presence of more than 1% glucose. This provides a cost effective, efficient and easy method of production of proteins and in particular proteins that

may be toxic to the host cells. Transcription of proteins that are toxic at high concentrations may be repressed by addition of glucose to the media. Once the glucose concentration falls below 1% (w/v), the promoter is able to function and express the protein.

The mechanism for the activation of transcription from this promoter is however still under study and as yet not fully understood.

Relatively few of the aminoglycoside modifying enzymes, in particular members of the phosphotransferases (Mitsuhashi *et al.*, 1976) and acetyltransferases (Williams and Northrop, 1976) have, in the past, been purified and characterised. However, previous attempts by other groups to purify members of the nucleotidyltransferase family of enzymes to homogeneity in an active form have, by and large, met with little success. Only two nucleotidyltransferase enzymes have been partially purified and characterised as to size and various properties. One of these is the ANT(2")-I enzyme (Smith and Smith, 1974) where the enzyme has been partially purified, characterised and inhibitors of its action identified. The second one and the only member of ANT(3")-I family of enzymes was the enzyme isolated from transposon Tn7 which has been purified by preparative gel electrophoresis methods which have generally resulted in an inactive enzyme (Chinault *et al.*, 1986). As a result physical characterisation was carried out on this enzyme but no kinetic characterisation could be performed.

The main difficulty in purification of these enzymes has been the low levels of enzyme present in cells carrying the gene and the instability of these enzymes upon cell lysis.

During the course of this study, the protein expressed in *E.coli* JM107 carrying the recombinant plasmid pQR606 was purified to homogeneity in an active form and kinetic characterisation studies carried out on the pure enzyme. Although the

expression of the gene from plasmid pQR609 was found to be much higher, this plasmid had not been constructed at the onset of purification. Consequently, purification was carried out from pQR606 (*trp* promoter) which was later shown to have lower expression than that of pQR609 (*mdh* promoter) but higher expression with respect to pQR601 (*lac* promoter).

The purification protocol was devised to avoid the use of affinity methods for separation of proteins as the same protocol was to be used for the purification of the mutant enzymes. To this end, the enzyme was purified by a combination of ammonium sulphate precipitation, gel filtration chromatography, ion exchange chromatography and hydrophobic interaction chromatography. The main problem encountered was that of protein stability during the purification processes. Addition of protease inhibitors to the fractionated protein did not significantly reduce the degradation and loss of the protein.

Attempts were made to increase the yield by alteration of purification steps. To try and optimise the conditions, ammonium sulphate precipitation of protein which resulted in a 13 % loss of protein (see table 4.1) was omitted from the protocol, instead a high load ion exchange column was used to fractionate the cleared cell lysate as the first step in the purification. This step resulted in a 95% recovery of protein (results not shown). The following procedure was then performed on the pooled fractions: Gel filtration chromatography (S-200), PhenylSuperose column and a final Gel filtration column (S-75). However, SDS-PAGE analysis carried out on the final fractionated protein showed that the protein was not pure. As the pI had already been established at this stage, chromato focusing was carried out to remove the contaminating proteins. The active fractions from this column were analysed by SDS-PAGE analysis. Again, all fractions collected showed more than

one protein band present. The results obtained indicated that the ammonium sulphate treatment of the cell lysate resulted in the removal of proteins that could not be separated by other steps in the purification protocol devised.

Precipitation of proteins by PEG as a first step was also tested as PEG does not interfere with the subsequent purification steps. However, no activity was detected in the precipitate at 30% concentration (table 4.2). The concentration of PEG could not be increased as this would result in a viscous solution, making the recovery of the precipitate difficult.

It was decided that the ammonium sulphate precipitation was therefore an essential first step in the purification of ANT(3")-I protein as it seemed to remove proteins which could not be separated by other methods in the purification protocol.

Two other steps in the purification that resulted in a major loss of protein were that of gel filtration (S-200 column) and ion exchange chromatography using Mono Q column (see table 4.3). Approximately half of the activity was lost from the fractions following each of these steps. This loss in activity could be attributed to the natural degradation of the proteins in the cell lysate, this may be especially true of the fractions collected following the S-200 column as this step was one of the first and longest steps in the purification taking on average 2 hours for the protein to elute from the column. A small amount of protein loss may be attributed to the use of reversible protease inhibitors (pepstatin, leupeptin) which may have been diluted out during the chromatography steps. However, addition of extra protease inhibitor cocktail following each step of the purification protocol did not have a significant effect on the final yield of the enzyme.

Although the total percentage yield of the enzyme was rather low (9%), the protein was purified 144 fold with a specific activity of 279.57nmoles/min/mg and the enzyme was found to be very stable at -70°C.

Characterisation studies (physical & chemical) of the enzyme were carried out to determine the size, pI, K_m and V_{max} values for all substrates.

Indirectly, substrate specificity of the enzyme was studied by minimum inhibitory concentration (MIC) tests (section 4.2.1) for both streptomycin and spectinomycin as well as other aminoglycoside antibiotics. The enzyme does not confer resistance to any other aminoglycoside antibiotics (kanamycin, gentamicin, kasugamycin, butirosin, neomycin and amikacin). The enzyme was found to confer a higher MIC for spectinomycin as compared to the value obtained for streptomycin.

E.coli cells carrying the recombinant plasmids pQR601 (*lac* promoter), pQR606 (*trp* promoter) and pQR609 (*mdh* promoter) constructed during the course of this study were used to determine the substrate range of the enzyme and to investigate the effect of high expression of the gene on MIC values. The results indicated that the higher expression of the gene results in a higher MIC value for both aminoglycoside substrates. Previous studies by Prince and Jacoby (1982) have shown that cloning of the *aac(6')* gene into a high expression vector significantly increased enzyme activity resulting in higher resistance.

To ascertain if higher enzyme concentration within the cell results in altered specificity, MIC tests were carried out for a number of other aminoglycoside antibiotics. No resistance to other aminoglycoside antibiotic was observed from any of the constructs. This indicates that the higher enzyme concentration present in the cell does not bring about resistance to other aminoglycoside antibiotics, confirming that the enzyme is very specific in its action and recognition sites.

The specificity seen in the ANT(3")-I enzyme arises from the ability of the enzyme to recognise a specific site on the antibiotic. The enzyme adds an adenylyl

(AMP) group to the 3'-hydroxyl group of the N-methyl-L-glucosamine moiety of streptomycin (see fig. 1.4) only when the stereochemistry is that of the *D*-threo configuration. If one examines the structure of spectinomycin as shown in fig. 1.1, it is clear that there is only one *D*-threo-methylamino alcohol moiety in actinamine with the same stereochemistry as in the N-acetyl-L-glucosamine residue of streptomycin. It is this hydroxyl that is adenylated. This stereochemistry is not present in other aminoglycoside antibiotics. Consequently, the enzyme is not able to adenylate other aminoglycoside antibiotics.
Furthermore, it has been shown that actinamine which is not an antibiotic but does contain the *D*-threo-methyl-amino moiety, is as effective a substrate as spectinomycin for ANT(3")-I enzyme (Benveniste *et al.,* 1970). This conformation therefore, accounts for the cross resistance pattern caused by this enzyme, even though the overall structure of these two antibiotics is extremely different.

The purified enzyme was characterised and kinetic studies were performed to determine the K_m and V_{max} of the enzyme for both antibiotic substrates as well as for the co-substrate, ATP. The K_m of the enzyme for streptomycin (0.643μM) was found to be 56 fold lower than its K_m for spectinomycin (36.04μM). This indicates that the enzyme has a higher affinity for streptomycin as compared with the affinity that is shown for spectinomycin. This result is consistent with previous findings where a lower MIC for a substrate usually indicates a higher affinity and consequently a lower K_m (Vestola *et al,* 1980). Similar findings were found with AAC(3')-I and AAC(2') enzymes for sisomicin and 5-episisomicin as well as with AAC(3')-I for gentamicin and 5-epigentamicin B (Bryan, 1984). The high K_m values for the aminoglycoside substrates allows low amounts of enzyme to produce resistance to an effective substrate.

There is therefore a correlation between MIC for a specific substrate and the K_m value obtained for that substrate. The MICs for streptomycin and spectinomycin were found to be 120μg/ml and 450μg/ml respectively. The lethality of streptomycin, therefore, dictates a need for this antibiotic to be inactivated much more quickly as there is a lower tolerance for this antibiotic. This high affinity is important as the aminoglycoside is modified at the lowest concentration at which it is accumulated.

K_m values for many aminoglycosides to which resistance is effectively specified by aminoglycoside modifying enzymes have often been reported to be in the range of 1μM (Davies,1983), although some values are noticeably higher (Williams & Northrup, 1976; Vestola *et al.*, 1980)as with the K_m of spectinomycin.

The difference in lethality of streptomycin and spectinomycin may arise from the mechanism and rate of uptake into cells. Spectinomycin is not a "true" aminoglycoside, as a result, the uptake of this antibiotic into cells is somewhat different to streptomycin. Moreover, spectinomycin is not a particularly active compound and is considered to be more bacteristatic rather than bactericidal (Garrod *et al.*, 1981). Compared to streptomycin, spectinomycin binds slowly and reversibly to the 30S subunit of ribosomes.

The molecular weight of the enzyme has previously been reported to be in the range of 27.5-31.6 kDa, as estimated by SDS-gel electrophoresis (Alton and Vapnek, 1979, Hollingshead and Vapnek, 1985, Shaw *et al.*, 1993). Analysis carried out during the course of this study, on SDS-PAGE and the elution profile from gel filtration column S-200 indicated the presence of a monomeric enzyme with a molecular weight of 29.5 kDa. This agrees well with the previously published data for this enzyme (Hollingshead and Vapnek, 1985). The

inconsistency in the size of the enzyme does not have a major significance as conditions of electrophoresis greatly affects the migration of proteins. Another contributing factor is the state of the protein during electrophoresis. The discrepancy between apparent relative masses and real molecular weights underlines the uncertainty in deducing molecular masses of proteins from their mobility in electrophoretic gels.

Some aminoglycoside modifying enzymes such as APH(3")-III exist predominantly as monomers but can also exist as dimer molecules (Davies and Smith, 1978). AAC(3)-I is a tetramer composed of four identical subunits (Williams and Northrop, 1976) and KNT is a dimer composed of two identical subunits (Sakon *et al.*, 1993).

The pI value (5.91) obtained through isoelectric focusing also varies with the predicted value (4.68) obtained by Shaw *et al.* (1993) where the pI was predicted from the derived amino acid sequence of the protein. This does not take into account the tertiary structure of the protein. As isoelectric focusing is carried out under non-denaturing conditions, the protein retains its tertiary structure. The difference between the predicted pI and the determined pI may arise from the interaction of the side chains which may result in shielding of certain residues. Some residues may also be buried and not able to interact with the solvent or may have metal ions bound.

Comparative sequence analysis was carried out on the derived protein sequence of ANT(3")-I using data-base searching. Sequence similarity searches were carried out to first identify closely related sequences and carry out multiple sequence alignments using these sequences. A number of network-based searches were also carried out to identify important patterns and motifs that may be present in the

protein sequence such as well characterised motifs for nucleotide binding sites. These searches however, did not identify any region within the protein that may be implicated in the binding of the nucleotide or the substrate.

Secondary structural analysis was carried out using the profile network method (see section 5.5.1). However, only about 70% confidence could be placed on the secondary structure prediction as the number of similar sequences to that of the query sequence (ANT(3")-I) in the data-bases searched was limited and not enough information could be gathered for a more accurate prediction. The aim of obtaining the secondary structure of the protein was to try and identify residues that may be involved in catalysis by the prediction of positions of the side chains and reactive groups. Furthermore, this secondary structure prediction was used in comparative analysis with the published tertiary structure of the kanamycin nucleotidyltransferase enzyme (Sakon *et al.,* 1993) to see if any of the residues implicated in binding of either substrate or nucleotide was in the same approximate position in ANT(3")-I family of enzymes.

Following extensive amino acid sequence homology studies between members of aminoglycoside nucleotidyltransferase family, a conserved region within these proteins was identified. Previous sequence analysis performed on aminoglycoside modifying enzymes (Shaw *et al.,* 1993) had failed to recognise any sequence homology between members of nucleotidyltransferase family of enzymes, although presence of conserved motifs were reported in phosphotransferases and acetyltransferases. Consequently, it has been assumed that the ANT family of enzymes show no homology at amino acid level. During the course of this study, not only have conserved regions been identified but two aspartic acid residues close to the N-terminal region of the protein have been implicated in binding of the nucleotide.

To carry out amino acid sequence analysis on members of nucleotidyltransferase enzymes for the purpose of identifying conserved regions within the proteins, progressive alignment programs were used. This type of program was chosen as it has the ability to align sequences with various degrees of similarity. To do this, highly homologous sequences were first aligned and gaps created. From this point on, the operating rule is: once a gap, always a gap. Once closely related sequences were aligned, more distantly related sequences were added to the alignment. This alignment was then used to construct a refined matrix to derive the evolutionary tree (see chapter 5). The difference between this progressive alignment system and multiple-alignment schemes that depend on global optimisation is that more trust is put in the alignments of the most recently diverged sequences. A number of different alignment programs (ClustalV& Pileup) were first used to align the same set of sequences. Each program uses a different matrix and therefore results in a different alignment. Two multiple alignment programs were chosen to perform the comparison studies. ClustalV was used where the sequences were members of the same family and hence of a similar length. This program was not very useful when aligning sequences of vastly varied lengths.

A more useful alignment program for aligning distantly related sequences was the Pileup multiple sequence alignment program which was used as the main alignment program during this study. The reason for this being that early on in the study, analysis carried out by "eye" had indicated certain regions within the ANT family of proteins to show a high degree of conservation. However, once the alignment was performed by the various alignment programs, these regions were not recognised and had failed to be aligned in the ClustalV program. The program Pileup showed a better type of alignment and was chosen for further analysis. The differences between the these programs arise from the various matrices used by

each to create some type of mathematical optimisation according to a certain algorithm. Furthermore, ClustalV and Alien seem to ignore the different lengths of the various sequences in the multiple alignment and try to align each sequence to an average length by creating gaps in the alignment. This posed as a particular problem when the LNT sequence was included in the multiple sequence alignment studies. This is a very small protein consisting of only 162 residues, consequently when it was included in the multiple alignment studies too many gaps were created resulting in an alignment with little significance.

On performing these sequence alignment studies, it became clear that the central region of the nucleotidyltransferase enzymes is highly conserved as most amino acid sequence homology was observed in this region (see section 5.5.4, fig. 5.1). The N- and C-termini however, showed little homology throughout this class of enzymes. Homologous proteins normally contain a core region where the general folds of the polypeptide chains are very similar. This core region contains mainly the secondary structure elements that build up the interior of the protein. One can therefore assume that this conservation of sequences is indicative of a common structure in the core region of these proteins. Since conservation of structure normally relates to function in homologous proteins, it is safe to assume that the catalytic site of the enzyme is in the core region of the protein and does not involve the N- or the C-termini.

Within this core region, regions of complete conservation of amino acids have been identified (fig.5.1). Three regions of the gene show complete conservation of more than 8 amino acids with 3-5 conservative changes. In particular, one region stands out as having the most amount of homology. In this region, 11 out of 18 amino acids seem to be totally conserved. Furthermore, on performing multiple sequence alignment of more distantly related nucleotidyltransferases, only this highly conserved region appeared to retain some conservation with respect to the

charged residues present (fig. 5.5). The charged residues were either totally conserved or conservative changes had taken place.

Site-directed mutagenesis was carried out to study the structure/function relation in this region of the protein. This conserved region was thought to be of importance due to the conserved charged residues (aspartic acid and glutamic acid) present through-out vastly differing aminoglycoside nucleotidyltransferase enzymes. Previous analysis on some nucleotide binding proteins (in particular KNT, and the rat DNA polymerase, β-subunit) have implicated that charged residues may be important in either the binding of the substrate or the metal ions (Sawaya *et al.,* 1994; Sakon *et al.,* 1993). This is particularly true in the case of the enzyme kanamycin nucleotidyltransferase In the case of KNT, a ring of seven negatively charged amino acid residues surround a metal binding site suggesting that this region may bind either the positively charged aminoglycoside or be involved in binding of the nucleotide (Sakon *et al.,* 1993). In the β-subunit of the rat DNA polymerase enzyme, two aspartic acid residues are implicated in the binding of nucleoside triphosphate molecule (Sawaya *et al.,* 1994).

In addition, these conserved residues were preceeded, in a number of ANT sequences analysed, by a glycine rich region within the protein and succeeded by a hydrophobic region. A glycine rich flexible loop is the single conserved element in all of the nucleotide binding regions, whose apparent function is to serve as a phosphate anchor (Fry *et al.,* 1986, Knighton *et al.,* 1991). The hydrophobic amino acids, such as leucine, isoleucine and valine may help to accommodate the nucleotide and position the phosphate groups as donors for catalysis.

The predicted secondary structure of the ANT(3")-I enzyme indicates the position of these conserved amino acid residues in a loop region. Generally, loops function

as connections between the secondary structural elements (α-helices and β-strands) within the protein, however, they also frequently participate in forming binding sites and enzyme active sites (Branden and Tooze, 1991).

The alteration of the Asp^{49} to alanine resulted in an inactive enzyme as no activity could be detected following assays performed on cleared cell lysates. Western Blot analysis was carried out to verify the presence of ANT(3")-I in both wild type and mutant enzyme. On comparative analysis, the immunoblot signals of the mutant were quite similar to that of the positive control (wild type), which indicated that the single amino acid change in this mutant did not affect the synthesis or processing of the ANT(3")-I enzyme. A second mutant was also isolated, in this mutant the aspartic acid residues at positions 47 and 49 were both changed to alanine. As expected, no nucleotidyltransferase activity was detected and no further conclusions could be drawn as the same residue (Asp^{49}) was affected in both mutants. The significance of the conserved aspartic acid residue at amino acid position 47 could not be investigated as problems were encountered with the isolation of this mutant. It would be very interesting to investigate the effect of other residues such as glutamate which may have similar ion binding properties in these positions. Indeed, one of the enzymes included in this study, KNT has a glutamic acid residue in the second aspartate position.

Attempts were made at partial purification of the mutant enzyme by gel filtration chromatography (S-75 column). Previous studies have suggested that activity of aminoglycoside modifying enzymes, in particular nucleotidyltransferases increases with partial purification as other ATP utilising enzymes may be removed (Coombe & George, 1981). It was hoped that by partially purifying the enzyme, some activity would be detected in the mutant. However, no activity was

observed in the fractions following gel filtration chromatography. Due to this lack of activity, Western Blot analysis was performed on the fractions following gel filtration which verified the presence of the enzyme.

In conclusion, the importance of conserved acidic charged residues (aspartic acid and glutamic acid) towards the N-terminal region of the protein in the action of the ANT(3")-I enzyme is strong. Site-specific mutagenesis of Asp-49 has shown that this residue is essential for the function of the enzyme. Although the exact effect of this change has not been fully investigated, the evidence gathered throughout the course of this study suggests that the effect may have been on the ability of the enzyme to bind ATP. This hypothesis may be further investigated by photo-affinity labelling of the mutant and the wildtype enzymes to determine if the ATP binding ability of the enzyme has been altered. Photo-affinity labelling studies were carried out on the pure enzyme during the course of this study, however, due to time constraints the experiments did not reach completion.
More information as to the mechanism of the action of the enzyme may be gathered through studies such as X-ray crystallography to fully investigate the involvement of these conserved residues in catalysis. By elucidating the tertiary structures of these enzymes, one may be able to seek specific inhibitors for aminoglycoside nucleotidyltransferase enzymes which in the long run, may offer therapeutic potential to extend the effective range of this class of antibiotics.

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APPENDICES

```
  1  ATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGT   50
     M  R  E  A  V  I  A  E  V  S  T  Q  L  S  E  V  V

 51  TGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGT  100
      G  V  I  E  R  H  L  E  P  T  L  L  A  V  H  L  Y

101  ACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTG  150
       G  S  A  V  D  G  G  L  K  P  H  S  D  I  D  L

151  CTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGAT  200
     L  V  T  V  T  V  R  L  D  E  T  T  R  R  A  L  I

201  CAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCC  250
      N  D  L  L  E  T  S  A  S  P  G  E  S  E  I  L  R

251  GCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGT  300
       A  V  E  V  T  I  V  V  H  D  D  I  I  P  W  R

301  TATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACAT  350
     Y  P  A  K  R  E  L  Q  F  G  E  W  Q  R  N  D  I

351  TCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCT  400
      L  A  G  I  F  E  P  A  T  I  D  I  D  L  A  I  L

401  TGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCG  450
       L  T  K  A  R  E  H  S  V  A  L  V  G  P  A  A

451  GAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAA  500
     E  E  L  F  D  P  V  P  E  Q  D  L  F  E  A  L  N

501  TGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGC  550
      E  T  L  T  L  W  N  S  P  P  D  W  A  G  D  E  R

551  GAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGC  600
       N  V  V  L  T  L  S  R  I  W  Y  S  A  V  T  G

601  AAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCC  650
     K  I  A  P  K  D  V  A  A  D  W  A  M  E  R  L  P

651  GGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGAC  700
      A  Q  Y  Q  P  V  I  L  E  A  R  Q  A  Y  L  G  Q

701  AAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTC  750
       E  E  D  R  L  A  S  R  A  D  Q  L  E  E  F  V

751  CACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAA  792
     H  Y  V  K  G  E  I  T  K  V  V  G  K  *
```

Amino acid and nucleotide sequence of ANT(3")-I isolated from R538-1.

```
40                                                          80
TGGTCATTGTAAACGGCAATTTTGTGGATTAAGGTCGCGGCAGCGGAGCA
          -35                                 -10          TS

ACATATCTTAGTTTATCAATATAATAAGGAGTTTAGGATGAGGGAAGCGG
                                    SD          M  R  E  A

TGATC
V  I
```

Nucleotide sequence of the MDH gene promoter region from *E.coli* K12 W3899. The N-terminal amino acid sequence of ANT(3")-I deduced from the gene sequence is also shown using bold single letter abbreviations for the amino acids.
The inferred -10 and -35 promoter consensus sequences, Shine-Dalgarno sequence (SD) are shown and underlined. The transcription start site (TS), determined by Vogel *et al.* (1987), is also shown.

```
          ....,....1....,....2....,....3....,....4....,....5....,....6
AA       |MREAVIAEVSTQLSEVVGVIERHLEPTLLAVHLYGSAVDGGLKPHSDIDLLVTVTVRLDE|
PHD sec  |  HHHHHHHHHHHHHHHHHHHHH    EEEEEEEEEE            EEEEEEE   H|


          ....,....7....,....8....,....9....,....10...,....11...,....1
AA       |TTRRALINDLLETSASPGESEILRAVEVTIVVHDDIIPWRYPAKRELQFGEWQRNDILAG|
PHD sec  |HHHHHHHHHHHHHH       EEEEEEEEEEE                      HHHHH |


          2...,....13...,....14...,....15...,....16...,....17...,....1
AA       |IFEPATIDIDLAILLTKAREHSVALVGPAAEELFDPVPEQDLFEALNETLTLWNSPPDWA|
PHD sec  |       HHHHHHHHHHHHH   EEE   HHHH     HHHHHHHHHHHHH          |


          8...,....19...,....20...,....21...,....22...,....23...,....2
AA       |GDERNVVLTLSRIWYSAVTGKIAPKDVAADWAMERLPAQYQPVILEARQAYLGQEEDRLA|
PHD sec  |    EEEEHHHHHHHE    E   HHHHHHHHHHHHHHHHHHHHHHHHHHHHH     HHH|


          4...,....25...,....26...,....27...,....28...,....29...,....3
AA       |SRADQLEEFVHYVKGEITKVVGK|
PHD sec  |HHHHHHHHHHHHHHHHHHHHH   |
```

Secondary structure prediction of ANT(3")-I enzyme as predicted by Rost and Sander (1994) method. H, α-helix; E, β-strand; blank spaces represent loops.