CLONING OF AMINOGLYCOSIDE-RESISTANCE DETERMINANTS IN STREPTOMYCES

by

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P.A. SKEEGGS

3rd March 1986.
CHAPTER 3 GENERAL METHODS

1. Origin and Maintenance of Bacterial Strains 41
2. Bulk Growth of Organisms 41
3. Determination of Minimum Inhibitory Drug Concentrations (MIC Values) 42
4. Preparation of Total DNA 42
5. Large Scale Preparation of Plasmid DNA 43
6. Religation of Vector and Genomic DNA 45
7. Analysis of DNA by Agarose Gel Electrophoresis 45
8. Preparation of Streptomyces lividans TK21 Protoplasts 46
9. Transformation and Regeneration of Streptomyces lividans TK21 Protoplasts 48
10. Small Scale (Mini-lysate) Preparation of Plasmid 49
11. Preparation of Ribosomes and Post-ribosomal Supernatant 51
12. Assay for Cell-Free Protein Synthesis
   12.1 Choice of System 52
   12.2 Polyphenylalanine Synthesis 53
13. Southern Transfer and Hybridisation of DNA 54
   13.1 DNA Transfer 55
   13.2 Preparation of Radioactive Probe 57
   13.3 Hybridisation of DNA 59
14. MATERIALS
   14.1 Enzymes 61
   14.2 Biochemicals 61
   14.3 Radiochemicals 61
   14.4 Antibiotics 62
   14.5 Other Chemicals 62
CHAPTER 4 CLONING OF THE VIOMYCIN-RESISTANCE DETERMINANT FROM

STREPTOMYCES VINACEUS

1. INTRODUCTION 63

2. METHODS

2.1 Preparation of Cell Extracts from Streptomyces

lividans TK21 for Coupled Transcription-Translation 65

2.2 Assay of Coupled Transcription-Translation Activity 66

2.3 Analysis of Protein Products on Polyacrylamide Gels 67

3. RESULTS AND DISCUSSION

3.1 Construction of Viomycin-Resistant Clones 69

3.2 Biochemical Analysis of a Viomycin-Resistant Clone 71

3.3 Analysis of the Product(s) of the Viomycin-Resistance Determinant 72

CHAPTER 5 CONSTRUCTION OF AMINOGLYCOSIDE-RESISTANT CLONES OF STREPTOMYCES LIVIDANS

1. INTRODUCTION 75

2. METHODS

2.1 Size-Fractionation of Genomic DNA from

S.tenjimariensis and S.tenebrarius 76

2.2 Phosphatase Treatment of Vector DNA 77

3. RESULTS

3.1 Cloning of Aminoglycoside-Resistance Determinant(s) from S.tenjimariensis 78

3.2 Cloning of Aminoglycoside-Resistance Determinant(s) from S.tenebrarius 79
3.3 Cloning of Aminoglycoside-Resistance Determinants from M.purpurea

4. DISCUSSION

CHAPTER 6 BIOCHEMICAL CHARACTERISATION OF AMINOGLYCOside RESISTANCE IN CLONES OF STREPTOMYCES LIVIDANS CONTAINING DNA FROM STREPTOMYCES TENTHARIENSIS

1. INTRODUCTION

2. METHODS
   2.1 Preparation of Ribosomal Subunits
   2.2 Preparation of RNA and Proteins from 30S Ribosomal Subunits
   2.3 Reconstitution of 30S Ribosomal Subunits
   2.4 Preparation of Aminoglycoside-Resistance Methylase

3. RESULTS
   3.1 Ribosomal Resistance to Aminoglycosides
   3.2 Determination of the Ribosomal Component(s) Responsible for Aminoglycoside Resistance in S.lividans TSK41.
   3.3 Subcloning of Aminoglycoside-Resistance Determinant(s) in S.lividans TSK41.
   3.4 Origin of the Aminoglycoside-Resistance Determinant in S.lividans TSK412.
   3.5 Biochemical Basis of Aminoglycoside Resistance in S.lividans TSK412.

4. DISCUSSION

CHAPTER 7 AMINOGLYCOside RESISTANCE IN CLONES OF STREPTOMYCES LIVIDANS CONTAINING DNA FROM MICROMONOSPOREA PURPUREA

1. INTRODUCTION
2. RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Ribosomal Resistance to Aminoglycosides</td>
<td>117</td>
</tr>
<tr>
<td>2.2 Biochemical Basis of Aminoglycoside Resistance in <em>S. lividans</em></td>
<td>118</td>
</tr>
</tbody>
</table>

3. DISCUSSION

CHAPTER 8 AMINOGLYCOSIDE RESISTANCE IN CLONES OF
*STREPTOMYCES LIVIDANS* CONTAINING DNA FROM *STREPTOMYCES TENEBRARIUS*

1. INTRODUCTION

2. RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Aminoglycoside Resistance in <em>S. lividans</em> TSK31</td>
<td>125</td>
</tr>
<tr>
<td>2.2 AminoglycosideResistance in <em>S. lividans</em> TSK51</td>
<td>127</td>
</tr>
<tr>
<td>2.3 Aminoglycoside Resistance Methylases and their Sites of Action.</td>
<td>128</td>
</tr>
<tr>
<td>2.4 Homology Between Aminoglycoside-Resistance Determinants Cloned from Different Producing Organisms.</td>
<td>131</td>
</tr>
</tbody>
</table>

3. DISCUSSION

CHAPTER 9 GENERAL DISCUSSION

1. Functional Importance of Ribosomal RNA in the Translation Process

2. Structure-Function Relationships in 23S Ribosomal RNA

3. Structure-Function Relationships in 16S rRNA

3.1 Initiation of Protein Synthesis and mRNA Binding

3.2 Binding of tRNA and Decoding

4. Aminoglycoside-Resistance Methylases and Predicted Sites of Action Within 16S rRNA

Concluding Remarks
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_x$</td>
<td>absorbance at a wavelength of $x$ nm in a 1cm light path</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>di-methyl sulphoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DSM</td>
<td>Deutsche Sammlung von Mikroorganismen</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminoothanol tetraacetic acid</td>
</tr>
<tr>
<td>EF G</td>
<td>elongation factor G</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>NCIB</td>
<td>National Collection of Industrial Bacteria</td>
</tr>
<tr>
<td>NRRL</td>
<td>Northern Regional Research Laboratories</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
</tr>
<tr>
<td>poly(C)</td>
<td>polycytidylic acid</td>
</tr>
<tr>
<td>poly(U)</td>
<td>polyuridylic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNA 70</td>
<td>total ribosomal RNA derived from 70S ribosomes</td>
</tr>
</tbody>
</table>
mRNA, rRNA, tRNA : messenger, ribosomal and transfer RNA
S : Svedberg unit ($10^{-11}$ seconds)
SDS : sodium dodecyl sulphate
TCA : trichloroacetic acid
TES : N-tris [hydroxymethyl] methyl-2-aminoethane sulphonic acid
TEMED : N,N,N',N'-tetramethylethylenediamine
TP30 : total proteins derived from 30S ribosomal subunits
Tris : tris (hydroxymethyl) aminomethane
TTP : thymidine triphosphate
UTP : uridine triphosphate
CHAPTER 1

GENERAL INTRODUCTION
CHAPTER 1

More than half the natural antibiotics known today are produced by bacteria belonging to the genus *Streptomyces*. These drugs often inhibit primary metabolic processes such as nucleic acid or protein synthesis, but can also induce more physical aberrations, e.g. membrane damage. Therefore, most antibiotic-producing organisms are faced with the problem of defending themselves against their potentially toxic products. The survival strategies adopted by these bacteria have been studied extensively, since they are interesting not only for scientific reasons, but because of the industrial production and clinical importance of antibiotics. Unfortunately, it has been difficult to prove that a particular mechanism of antibiotic resistance which is known to exist in a given producer, does indeed contribute to the resistance phenotype of that organism. However, the recent development of a gene cloning system for *Streptomyces* has provided a definitive method of examining the properties of these putative resistance determinants in an antibiotic-sensitive host. Therefore, recent investigations on *Streptomyces* involving this recombinant DNA technology have generated invaluable data on the mechanisms of self-defence in several antibiotic-producing organisms.

1. Antibiotics as Secondary Metabolites.

The growth cycle of microorganisms can be broadly divided into two phases, according to the biosynthetic pathways required for metabolism. In the first phase, during primary metabolism, biosynthetic precursors are converted into essential macromolecules, e.g. nucleic acids, proteins, lipids and polysaccharides. The metabolic reactions involved are finely balanced and intermediates
CHAPTER 1

other than those necessary for cell survival rarely accumulate. During secondary metabolism, in the second (or stationary) phase of the growth cycle, specialised products, such as antibiotics, are synthesised. These compounds are not essential for the growth of the producing organism, although they might have survival functions in nature or play a role in the regulation of cell growth or differentiation (for a review see Katz and Demain, 1977).

Antibiotics form a special class of secondary metabolites, distinguished by their toxicity to other organisms. They are usually produced as families of closely related compounds, which contain unusual chemical structures (amino-sugars, epoxides, glycosides or macrolides) and often also include unusual chemical linkages, e.g. β-lactam rings and cyclic peptides synthesised from normal and modified amino acids. The proportion of each compound in a given family depends on genetic and environmental factors, apparently because of the low specificity of the enzymes involved in secondary metabolism.

An important characteristic of secondary metabolites is that generally they are produced only at low growth rates. Several hypotheses on the control of antibiotic biosynthesis have been reported (for a review see Martin and Demain, 1980). However, the most widely accepted idea is that a deficiency of nutritional, growth-limiting components regulates the onset of antibiotic synthesis, by arresting growth and inducing the synthesis of enzymes required for the production of secondary metabolites. That being so, it is possible to regulate the biosynthesis of an antibiotic (and perhaps resistance to that drug), simply by altering the growth conditions of a given producing organism. For example, in the
CHAPTER 1

controlled environment of a chemostat, antibiotics can be produced continuously under restricted growth conditions (Pirt and Righelato, 1967: Blanch and Roger, 1971). Conversely, antibiotic biosynthesis can be prevented completely by growth of the 'producer' in a defined, rich medium (Nakano et al., 1984).

2. Antibiotic Resistance in Producing Organisms

Streptomyces which synthesise antibiotics that act exclusively on eukaryotes, clearly do not have target sites for their drugs and, therefore, need no specific protection against their endogenous products. On the other hand, those organisms which produce potentially toxic antibiotics, such as peptidoglycan cell wall inhibitors, or drugs that inhibit prokaryotic nucleic acid or protein synthesis, might be expected to protect themselves against their toxic products. Originally, however, it was thought that even these organisms did not really require special defence mechanisms, since antibiotics are secondary metabolites and, therefore, would be synthesised after cell growth had been completed. However, as discussed in the previous section, under certain restricted growth conditions, antibiotic synthesis and cell proliferation may occur simultaneously, so that the producing organism is exposed to endogenous antibiotics during growth. Specific protection then becomes a necessity.

Studies on the survival strategies employed by antibiotic-producing organisms suggest that there are three principal resistance mechanisms whereby these bacteria can protect themselves against their endogenous products (for reviews see Vining, 1979: 

-3-
Cundliffe, 1984).(i) Antibiotic entering the cell might be enzymically converted to a biologically inert form. (ii) Transport of the drug into the cell might be partially or totally blocked. (iii) The target site for the antibiotic might be modified and thereby desensitised. Although each of these mechanisms could, in principle, render a producing organism totally refractory to the antibiotic product(s), provided exclusion of the drug was complete in (ii), producers sometimes achieve complete protection by possessing two complementary methods of protection. This is best explained by considering several examples of the protective measures undertaken by various antibiotic-producing organisms.

2.1 Resistance due to Antibiotic Inactivation.

In a high-level producing organism it is unlikely that antibiotic inactivation would represent the only mechanism of self-protection, because in addition to being energetically unfeasible, it would render the organism incapable of producing active drug. Where an antibiotic-inactivation system is thought to operate in producers, it is sometimes associated with a partially effective permeability barrier, so that only small amounts of drug reach the cytoplasm and require inactivation. This type of survival strategy is found in Streptomyces griseus, the streptomycin producer. Streptomycin is a bactericidal antibiotic which causes multiple effects on protein synthesis (for a review see Gale et al., 1981). Studies on the mechanism(s) of resistance operating in S.griseus revealed that as mycelia entered stationary phase, there was a specific decrease in the permeability to streptomycin (Cella and Vining, 1975). However,
radiolabelled drug was still detected in the cytoplasm and therefore, the change in permeability could not account for the high-level resistance observed in *S. griseus* during streptomycin production.

Furthermore, when ribosomes were prepared from this producer and shown to be sensitive to streptomycin *in vitro* (Hotta et al., 1981), this suggested that resistance could not be attributed to target-site modification. During these studies, however, the post-ribosomal supernatant, prepared from mycelium in late exponential growth, was shown to contain an enzyme capable of inactivating streptomycin.

These data were compatible with the results of previous investigations which demonstrated that as streptomycin was transported into stationary-phase mycelia, it was inactivated by phosphorylation (Miller and Walker, 1969; Cella and Vining, 1975). Therefore, this phosphorylating enzyme, designated aminoglycoside 6-phosphotransferase [APH (6)], was suggested to play a critical role in the protection of *S. griseus* during antibiotic production, by synthesis of an inactive intracellular precursor, 6-phosphoryl streptomycin. The final stage of streptomycin biosynthesis then involved dephosphorylation of this precursor, during transport out of the cell, thus allowing excretion of active antibiotic (Walker and Walker 1971). Evidence in support of this hypothesis was provided when streptomycin phosphate was accumulated by cultures of *S. griseus* grown in high phosphate medium (Miller and Walker, 1970). Presumably the final dephosphorylation reaction was inhibited by the high concentration of extracellular inorganic phosphate.

It also seems likely that APH (6) protects streptomycin producers by inactivating any drug which re-enters the cell after excretion, because APH-minus mutants of *Streptomyces glaucescens* the
hydroxystreptomycin producer, were sensitive to streptomycin throughout the growth cycle (Ono et al., 1983).

The presence of antibiotic-modifying enzymes in extracts of producer organisms provokes certain questions as to their roles. For example, these enzymes might normally act during the biosynthesis of the antibiotic, and play no part in conferring resistance in the producing organism. A definitive method of solving this problem is to isolate resistance determinants from producing organisms and introduce them into an antibiotic-sensitive host, using recombinant DNA technology. Biochemical characterisation of the resultant antibiotic-resistant clones, therefore, allows identification of the mechanism(s) of protection operating in the producer. In this way, the resistance determinant responsible for defending S. griseus against streptomycin has recently been isolated (Vallins and Baumberg, 1985). The streptomycin-resistant clones obtained, were shown to contain the gene encoding streptomycin phosphotransferase activity, APH (6). In conclusion, this enzyme does indeed play a role in protecting S. griseus against its toxic product, streptomycin.

2.2 Resistance due to Permeability Changes.

This mechanism could clearly render a producing organism completely resistant to its antibiotic products, provided exclusion was total. An example of a producing organism that defends itself, during antibiotic biosynthesis, by exclusion or excretion of the drug without apparent modification, is Streptomyces sp. 3022a (a strain of Streptomyces venezuelae), which synthesises chloramphenicol. This bacteriostatic antibiotic inhibits protein synthesis by binding to
prokaryotic 50S ribosomal subunits, thereby preventing peptidyl transferase activity (for a review see Gale et al., 1981). The ribosomes from Streptomyces sp. 3022a are inhibited by chloramphenicol, binding the drug to the same extent as ribosomes from sensitive bacteria (Malik and Vining, 1972). The antibiotic also inhibits incorporation of \[^{14}C\] phenylalanine in \textit{in vitro} poly U-directed protein synthesising systems, using extracts prepared at all stages of the growth cycle (Malik and Vining, 1972). Therefore, it appears that neither target alteration nor antibiotic inactivation is important in defending this producing organism \textit{in vitro}.

When \textit{Streptomyces sp.} 3022a is grown under conditions where antibiotic is not produced, although it is initially sensitive to added chloramphenicol, the inhibition is temporary. The level of resistance acquired is proportional to the concentration of drug to which the cells are exposed and is maintained as long as chloramphenicol is present in the medium (Malik and Vining, 1972). This increased resistance to chloramphenicol was associated with a decrease in permeability to the drug, induced by antibiotic entering the cell. However, exclusion was not complete, as a slow uptake of chloramphenicol still persisted and the intracellular drug was shown to be inactivated by hydrolysis and acetylation (Malik and Vining, 1970).

During growth of \textit{Streptomyces sp.} 3022a in medium supporting antibiotic synthesis, however, a slightly different mechanism of resistance is induced, which depends solely on exclusion of the drug. At the onset of antibiotic production, a decrease in permeability to chloramphenicol occurs which leads to termination of the uptake and concomitant hydrolytic degradation of the antibiotic.
CHAPTER 1

(Malik, 1972). The cultures are subsequently insensitive to added antibiotic and no effect on \textit{in vivo} protein biosynthesis is detectable. In conclusion, it appears that during chloramphenicol production, \textit{Streptomyces sp.} 3022a is protected from its endogenous antibiotic by an efficient excretion system, which simultaneously prevents the drug from re-entering the cell.

2.3 Resistance due to Modification of Antibiotic Target-Sites

The first case in which the mechanism of antibiotic resistance in a producing organism was satisfactorily explained at the molecular level, was that of \textit{Streptomyces azureus}, the thiostrepton producer. Thiostrepton normally binds very tightly to a single site on the 50S subunit of bacterial ribosomes, thereby inhibiting protein synthesis (for a review see Gale \textit{et al.}, 1981). However, the ribosomes from \textit{S. azureus} do not bind thiostrepton, thus rendering this organism insensitive to the drug. When the mechanism of ribosomal resistance was studied further, this producer was shown to possess a methylase enzyme, which can act \textit{in vitro} upon 23S rRNA from other bacteria, such that reconstituted ribosomes, containing this modified RNA, are rendered resistant to thiostrepton (Cundliffe, 1978).

A similar enzyme is responsible for resistance to erythromycin in the producer, \textit{Streptomyces erythraeus}. Erythromycin is a member of the macrolide group of antibiotics and inhibits bacterial protein synthesis by binding to 50S ribosomal subunits and (perhaps) interfering with the translocation reaction (for a review see Gale \textit{et al.}, 1981). In \textit{S. erythraeus}, however, the antibiotic does not bind to the ribosome (Teraoka and Tanaka, 1974) due to methylation of the
23S rRNA (Skinner and Cundliffe, 1982), (for further details on the thiostrepton and erythromycin-resistance methylases, see chapter 6, page 103 et seq.).

Another example of target-site modification in a producing organism, emerged from studies on Nocardia mediterranei. This organism produces the antibiotic rifamycin, which is a potent inhibitor of DNA-dependent RNA polymerase. Initial investigations showed that the in vitro synthesis of RNA by RNA polymerase from the producing organism, was more resistant to rifampicin (a semi-synthetic derivative of rifamycin) than that catalysed by RNA polymerases from S.griseus or E.coli (Watanabe and Tanaka, 1976). It was concluded that N.mediterranei relied upon a unique property of its RNA polymerase, for continued RNA synthesis in the presence of rifamycin. Evidence in support of this hypothesis has been obtained recently and similar conclusions have been extended to other producing organisms which synthesise inhibitors of RNA polymerase (Blanco et al., 1984). In these studies, RNA polymerases from N.mediterranei, Streptomyces spectabilis and Streptomyces lydicus were shown to be highly resistant, in vitro, to their antibiotic products, rifamycin, streptovaricin and streptolydigin, respectively. Furthermore, these investigations also showed that none of these producing organisms possessed any detectable antibiotic-inactivating enzymes.

In conclusion, from the examples discussed above, it is clear that producing organisms have evolved a variety of different survival strategies. Although there are many factors governing the choice of the resistance mechanism(s) employed by any one producer, if the level of resistance was the sole criterion, it may be that
CHAPTER 1

target-site modification would be the preferred mechanism.

3. Applications of Studies on Antibiotic-Resistance Determinants from Producing Organisms.

Many clinically resistant isolates contain mechanisms of antibiotic resistance that are essentially similar to those found in producing organisms. This observation has led to the proposal that the resistance determinants present in clinical isolates, and often located on R plasmids, might have originated in antibiotic-producing organisms (Walker and Walker, 1970). Therefore, by studying antibiotic resistance in producers, it might be possible to characterise novel resistance mechanisms before they arise in clinical situations and thus, in some cases, by therapeutic use of strategically modified antibiotics, prevent the emergence of clinical isolates.

Evidence in support of the hypothesis concerning the origin of antibiotic-resistance genes in clinical isolates, is that various aminoglycoside phosphotransferase and acetyltransferase enzymes in producing organisms have been shown to catalyse reactions identical to those which underlie resistance in clinical strains (Benveniste and Davies, 1973a). Moreover, considerably homology (36-40%) was observed when the amino acid sequence of the APH (3') from S. fradiae (the neomycin producer) was compared with those of the functionally related enzymes encoded by antibiotic-resistance transposons, Tn5 and Tn903. This suggested that these resistance determinants might have a common evolutionary origin (Thompson and Gray, 1983).
CHAPTER 1

Resistance determinants from certain producing organisms have also proved useful in studies on the genetics of antibiotic biosynthesis in these bacteria. This subject is of considerable commercial interest, with respect to improving the efficiency of industrial processes involved in antibiotic production. In some producers, the antibiotic resistance genes have been used to locate and isolate determinants involved in drug biosynthesis, since they are located adjacent, or close, to one another in the genome. For example, in *Streptomyces rimosus*, the oxytetracycline producer, the genes coding for the early steps of antibiotic biosynthesis and for drug resistance, were mapped to the same region of the chromosome of *S.rimosus*. Subsequently, the resistance determinant was used as a selectable marker, to clone the genes involved in antibiotic production (Rhodes et al., 1984).

Similarly, genetic studies on *Streptomyces coelicolor* A3(2), which produces methylenomycin A, revealed that genes for the biosynthesis of and resistance to this antibiotic, were located together (Kirby and Hopwood, 1977). In this case, the determinants were present on a piece of extrachromosomal DNA, designated plasmid SCP1.

Finally, antibiotic resistance genes which protect producing organisms from endogenous antibiotics by modification of the ribosomes, have provided valuable data on the relationship between certain ribosomal structures and their functions in protein synthesis. It is for this reason that the work presented here has been restricted to the study of resistance in bacteria which produce inhibitors of ribosome function. This particular application of investigations on antibiotic-resistance determinants is discussed more fully below.

Several approaches have been used to study the structure of the ribosome and its relationship to the mechanism of protein synthesis (for a review, see Chamblis et al., 1980). Of particular interest here, are the many antibiotics which selectively inhibit protein synthesis by binding directly to ribosomes and which, in principle, can be used as tools to investigate ribosome structure-function relationships. Such an approach depends on the assumption that the ribosomal component(s) involved in binding a given drug will also be involved in the processes inhibited by that antibiotic. Certainly this assumption has proved valid in studies on the action of the antibiotic thiostrepton, discussed below (see also Cundliffe, 1983).

Thiostrepton binds to bacterial ribosomes and prevents functional interaction with various protein factors involved in the initiation, elongation and termination stages of protein synthesis. The hydrolysis of GTP associated with the binding of such factors to ribosomes is powerfully inhibited in vitro, and, therefore, provides a convenient assay for the action of thiostrepton (for a review see Gale et al., 1981). Initial studies on the interaction of thiostrepton with E.coli ribosomes revealed that the drug could bind remarkably tightly to a single site on the 50S subunit (Highland et al., 1975a) and that this high affinity binding was dependent upon the presence of protein L11 from the 50S particle (Highland et al., 1975b). However, protein L11 alone does not constitute the entire binding site for thiostrepton, since ribosomal particles which are deficient of this protein, still bind the drug. Indeed, the primary binding site of thiostrepton was shown to be on 23S rRNA, which in the additional presence of protein
L11, could be induced to bind the antibiotic with an affinity approaching that observed with intact ribosomes (Thompson et al., 1979). Furthermore, when complexes of E. coli 23S rRNA with protein L11 were incubated with ribonuclease, an oligonucleotide (about 60 residues in length) was protected from digestion (Schmidt et al., 1981). Since this complex alone could bind thiostrepton with high affinity, it was assumed to contain the ribosomal binding site of the drug and, by inference, to constitute part, or all, of the domain involved in protein factor-dependent GTP hydrolysis.

Direct antibiotic binding studies, as discussed above, were complemented by investigations on thiostrepton-resistant mutants of Bacillus megaterium (Cundliffe et al., 1979) and Bacillus subtilis (Weinen et al., 1979) which had arisen spontaneously. Ribosomal 50S subunits from these mutants appeared to lack a single protein, which was serologically homologous with protein L11 of E. coli. These proteins, designated 'BM-L11' and 'BS-L11' respectively, were suggested, therefore, to play a role in the binding of thiostrepton. When ribosomes from the thiostrepton-resistant mutant of B. megaterium were analysed for their ability to support protein synthesis in vitro, they were only poorly active compared with ribosomes from wild-type B. megaterium. However, on reconstitution of the mutant particles with purified protein BM-L11 from the wild-type strain, full protein synthetic activity was restored, as was the sensitivity of the reconstituted ribosomes to thiostrepton (Cundliffe et al., 1979). That being so, it was possible to examine the biological role(s) of protein BM-L11. A study of partial reactions of protein synthesis revealed that protein BM-L11 was involved in promoting the ribosomal GTP hydrolysis dependent upon elongation factor G (EF G). In conclusion,
it was clear that there was a strong association between the mode of action of thiostrepton and the function of ribosomal protein L11, which was directly involved in drug binding. Since ribosomes contain only a single copy of protein L11, it was suggested that thiostrepton interferes with bacterial protein synthesis by altering or preventing functions of that protein. Thus, thiostrepton acts where it binds with no obvious propagation of its effects to distant sites on the ribosome.

Another approach to the investigation of the relationships existing between ribosomal structure and function, concerns studies on antibiotic-producing organisms. Of particular interest in this context, are those bacteria which synthesise inhibitors of protein synthesis, but are protected from their endogenous products as they possess antibiotic-resistant ribosomes. This approach has provided compatible data with that discussed above, on the functional binding site of thiostrepton. The thiostrepton producer, Streptomyces azureus has been shown to possess ribosomes which do not bind thiostrepton due to the action of a methylase enzyme which introduces a single methyl group at position 1067 of 23S rRNA (Thompson et al., 1982; see also Chapter 6, page 104 et seq., for further details). The methylated residue is situated within the fragment of 23S rRNA protected from nuclease activity in the presence of protein L11, and presumably indicates precisely where thiostrepton binds. Therefore, it appears that the site of action of the thiostrepton resistance methylase, the binding site of thiostrepton and the binding site of protein L11, all involve a common fragment of 23S rRNA, which is involved in the GTPase centre of the ribosome.

The latter approach to studying ribosomal structure-function relationships has been used in the work presented here and, therefore,
CHAPTER 1

those antibiotic-producing organisms which are rendered resistant to their toxic products by ribosomal modification, were chosen for investigation. It was hoped that the functional binding sites of specific antibiotics could be located, by determining the sites of alteration of the ribosome in the relevant producer.
CHAPTER 2

INTRODUCTION

AMINOGLYCOSIDE ANTIBIOTICS
CHAPTER 2

The producing organisms chosen for study were those which synthesise aminoglycoside antibiotics. Although the exact modes of action of these drugs are not clear (see below), they have all been shown to cause mistranslation of mRNA to varying extents. The ability to translate RNA transcripts accurately is clearly an important property of ribosomes, indeed the survival of a cell depends on it. However, little information is currently available on the ribosomal components which are involved in this critical step of protein synthesis. In this context, it seemed relevant that several aminoglycoside-producing organisms had recently been shown to possess ribosomes which were resistant to their endogenous antibiotics and to several other aminoglycosides. Thus, by determining the ribosomal sites that had been modified in these organisms, it was hoped to locate the domain(s) of the ribosome concerned with the fidelity of protein synthesis.

1. The Chemistry of Aminoglycoside Antibiotics.

The aminoglycoside antibiotics consistute a group of highly active antibacterial agents that are used in the treatment of severe Gram-negative infections (Phillips, 1982). These antibiotics all contain aminosugar residues which are glycosidically-linked to aminocyclitols, and can be divided into two groups according to whether the aminocyclitol constituent is streptidine (or a close relative) or 2-deoxystreptamine. The streptidine group includes streptomycin, whereas the 2-deoxystreptamine group is more complex, containing a large proportion of the aminoglycosides known today. The latter group can be further subdivided, according to whether the deoxystreptamine
nucleus is 4,5-disubstituted, e.g. neomycins, or 4,6-disubstituted, e.g. gentamicins and kanamycins. The research work discussed in this dissertation concerns only those antibiotics containing 2-deoxystreptamine and, therefore, the term 'aminoglycoside' is restricted here to this group of drugs.

In general, the nomenclature used to identify the various structural rings in these aminoglycosides is as follows: the 2-deoxystreptamine ring is known as Ring II, the aminosugar ring linked to C-4 of 2-deoxystreptamine is designated as Ring I and the aminosugar moiety linked to positions 5 or 6 of Ring II is known as Ring III. Any additional ring attached to Ring III, as in neomycins, is termed Ring IV. The elucidation of the chemical structures of aminoglycosides has been reviewed recently (see Cox et al., 1977). In general, the aminoglycosides can be classified into families according to the combination and orientation of the rings which make up the basic structure of the drugs. The kanamycins, for example, have aminosugars as Rings I and III (see Fig. 2.1), and the gentamicins are similar to members of the kanamycin family, except that they possess a different substitution pattern in Ring I and a unique aminosugar, garosamine, as Ring III (see Fig. 2.2). On the other hand, the neomycin, paromomycin and lividomycin families of aminoglycosides all have a pentosyl moiety as Ring III and aminosugars as Rings I and IV (see Fig. 2.3). Given these basic ring structures, or skeletons, the members constituting each family can be distinguished by the relative positions of the amino, hydroxyl and methyl groups (or their derivatives), which are substituted onto the rings. Finally, there is the novel aminoglycoside, apramycin (Fig. 2.4), which has a unique feature, an octadiose (8-membered ring) that exists as a rigid bicyclic system.
Legend to Fig. 2.1

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin A</td>
<td>NH₂</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>NH₂</td>
<td>NH₂</td>
<td>H</td>
</tr>
<tr>
<td>Kanamycin C</td>
<td>OH</td>
<td>NH₂</td>
<td>H</td>
</tr>
</tbody>
</table>

Tobramycin is 3’-deoxykanamycin B.

(The kanamycin sulphate used in this work typically contains 95% kanamycin A and 5% kanamycin B).
Fig 2.1

Kanamycin
Legend to Fig. 2.2.

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin C₁</td>
<td>CH₃</td>
<td>NHCH₃</td>
</tr>
<tr>
<td>Gentamicin C₁ₐ</td>
<td>H</td>
<td>NH₂</td>
</tr>
<tr>
<td>Gentamicin C₂</td>
<td>CH₃</td>
<td>NH₂</td>
</tr>
</tbody>
</table>

Sisomicin is 4', 5'-dehydrogentamicin C₁ₐ.

(The gentamicin C complex used in this work contains an approximately equimolar mixture of gentamicin C₁, gentamicin C₁ₐ and gentamicin C₂).
Fig 2.2

Gentamicin

$R_1^{CH-R_2}$
Legend to Fig. 2.3

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin B</td>
<td>NH₂</td>
<td>H</td>
<td>CH₂NH₂</td>
<td>H</td>
</tr>
<tr>
<td>Neomycin C</td>
<td>NH₂</td>
<td>CH₂NH₂</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

Paromomycins, R₁ = OH, otherwise as neomycins B and C.
Lividomycin B, R₁ = OH, otherwise as 3'-deoxyneomycin B.
Lividomycin A, R₄ = mannose, otherwise as lividomycin B.

(The neomycin complex used in this work typically contains 90-95% neomycin B and the remainder is neomycin C).
The molecule also contains 2-deoxystreptamine and an aminosugar.

2. The Mode of Action of Aminoglycosides.

In general, aminoglycoside antibiotics display a broad antimicrobial spectrum and are bactericidal in their action. Although neomycin and paromomycin have been shown to cause phenotypic suppression of nonsense mutations in yeast (Palmer et al., 1979; Singh et al., 1979), eukaryotic cells are generally resistant to most aminoglycosides. Therefore, the following discussion is restricted to the effects of aminoglycosides on prokaryotic systems.

The mechanisms of action of aminoglycoside antibiotics have been studied for more than 20 years, during which time a large amount of conflicting data has accumulated. Despite reports that aminoglycosides have a direct effect on the cell wall (Hancock, 1981) and that kanamycin and gentamicin inhibit the initiation of DNA replication (Tanaka et al., 1984), the most widely accepted hypothesis is that the primary effect of these drugs is on the ribosome, resulting in the inhibition of protein synthesis (for reviews see Gale et al., 1981; Tanaka, 1982). The lethal action of kanamycin can be reversed by various protein synthesis inhibitors, such as chloramphenicol and erythromycin, but is stimulated by puromycin (Yamaki and Tanaka, 1963; White and White, 1964). These effects of inhibitors of protein synthesis, therefore, favour the assumption that killing depends on the normal ribosomal cycle being in operation, for aminoglycosides to exert a bactericidal action. However, the detailed mechanism by which aminoglycoside antibiotics inhibit protein synthesis and the relationship to a variety of other effects on the cell (including
death), remain incompletely characterised. Although the primary mode of action of aminoglycosides is not clear, previous investigations have demonstrated that these antibiotics can induce several different effects on protein synthesis, including an increase in misreading, and an inhibition of the initiation and elongation phases. These effects are considered in more detail below.

2.1 Effect of Aminoglycosides on the Fidelity of Protein Synthesis.

Most aminoglycosides have been shown to cause mistranslation of mRNA in prokaryotic extracts. For example, kanamycin, gentamicin and neomycin induced misreading of synthetic templates in extracts of E. coli (Davies et al., 1965). However, the concentration of tRNA in these assays had a marked effect on the extent of misreading, so that with increasing amounts of tRNA the relative misreading became less prominent. It is perhaps significant, therefore, that even at the highest concentrations of tRNA used in those studies, the levels of misreading probably exceeded those occurring under physiological conditions in the cell. Nevertheless, aminoglycosides do cause misreading in vivo, as demonstrated by phenotypic suppression of nonsense and missense mutations (Gorini, 1974). The extent of misreading caused by various aminoglycosides in vitro, was shown to vary widely over a range of antibiotic concentrations (Davies and Davis, 1968). Thus, neomycin, kanamycin and gentamicin showed an increase in the level of misreading with increasing concentrations of antibiotic, whereas in the presence of paromomycin (Davies and Davis, 1968) or apramycin (Perzynski et al., 1979) there was almost no increase in misreading over the same concentration range. This
suggested that paromomycin and apramycin might interact with the ribosome at a single site, whereas kanamycin, gentamicin and neomycin might possess multiple target sites on the ribosome (for further details see section 2.4, below).

2.2 Effect of Aminoglycosides on the Initiation Step of Protein Synthesis.

The effects of kanamycin and gentamicin on the initiation of protein synthesis apparently vary, according to the mRNA being translated. Studies on f2 phage RNA-directed protein synthesis showed that both antibiotics inhibited the initiation of translation of the coat protein cistron, which forms a base-paired hairpin loop near the initiation complex. However, neither drug affected initiation of translation of the maturation protein cistron which had no equivalent secondary structure (Okuyama and Tanaka, 1972). Thus, it is possible that only when the ribosome is 'stalled' during initiation, as a consequence of secondary structure in the mRNA around the initiation codon, can kanamycin and gentamicin exert their inhibitory effects. On the basis of these results, it is unlikely that the inhibition of the initiation of protein synthesis is the primary mode of action of these antibiotics.

2.3 Effect of Aminoglycosides on the Translocation Step of Protein Synthesis.

The fact that neomycin, gentamicin and kanamycin can all inhibit the translocation step of bacterial protein synthesis, provides an explanation for why non-initiating systems (e.g. those directed by
poly U), are sensitive to aminoglycosides (e.g. Davies et al., 1965). The translocation reaction occurs as peptidyl-tRNA moves from the ribosomal acceptor (puromycin-unreactive) to the donor (puromycin-reactive) site, whilst the ribosome moves precisely one codon closer to the 3' end of mRNA. Translocation can be assayed in vitro by peptidyl-puromycin synthesis, enhanced by EF G and GTP. Kanamycin, gentamicin and neomycin did not inhibit the peptidyl transferase step of protein synthesis, represented in vitro by the reaction of puromycin with ribosome-bound acetyl phe-tRNA, although stimulation of this reaction by factor EF G and GTP was inhibited (Misumi et al., 1978; Cabañas et al., 1978a). Attempts to determine the mechanism of inhibition of translocation by aminoglycosides showed that kanamycin did not inhibit GTP hydrolysis catalysed by EF G and ribosomes (Misumi and Tanaka, 1980), but that it fixed peptidyl-tRNA into the acceptor site of the ribosome (Misumi and Tanaka, 1980; Semenkov et al., 1982). Although all these studies on the inhibition of translocation were performed using synthetic templates as mRNA, kanamycin, gentamicin and neomycin again inhibited polypeptide elongation when a more physiological system, containing endogenous polysomes of E.coli, was employed (Cabañas et al., 1978a). Moreover, the translocation step was shown to be inhibited in vivo by apramycin, and it was suggested that this was the primary inhibitory effect of this antibiotic (Perzynski et al., 1979).

In conclusion, since the extent of inhibition of protein synthesis with different aminoglycosides correlates well with their capacity to inhibit elongation (Cabañas et al., 1978a), it is reasonable to propose that this effect is significant in the overall inhibition of protein synthesis.

-21-
2.4 Aminoglycoside Binding Sites on the Ribosome.

Various attempts have been made to correlate the effects of aminoglycosides on translocation and misreading with specific antibiotic binding sites on the ribosome. Although no unequivocal conclusions have yet been drawn from such investigations, several hypotheses on this subject will be outlined briefly below.

Recent studies on the binding of gentamicin to wild type \textit{E. coli} 70S ribosomes have revealed that this aminoglycoside can interact with three classes of sites, based on the affinity of the antibiotic for the ribosomal site (Tangy \textit{et al.}, 1985). In this context, it is interesting to note that results of earlier investigations had suggested that gentamicin caused a multiphasic disturbance of \textit{in vitro} protein synthesis (Tai and Davis, 1979; Kühberger \textit{et al.}, 1979). It was proposed that at low concentrations, gentamicin acts as a potent inhibitor of protein synthesis, whereas at higher concentrations it allows a partial recovery of ribosome functions, which has been associated with misreading and read-through of termination codons (Zierhut \textit{et al.}, 1979). At still higher concentrations inhibition increases again (Tai and Davis, 1979). Although there was a correlation between the concentrations of gentamicin that could saturate the different classes of ribosomal sites and the concentrations that induced the various stages of the multiphasic effect on protein synthesis, there was little experimental evidence in support of this hypothesis.

A large amount of information on aminoglycoside binding sites on the ribosome has accumulated from studies on aminoglycoside-resistant mutants. For example, mutants of \textit{E. coli} that are resistant to
CHAPTER 2

gentamicin, have been isolated by stepwise exposure to increasing concentrations of the drug (Buckel et al., 1977). These gentamicin-resistant strains had acquired both defective antibiotic-uptake systems (Ahmad et al., 1980) together with ribosomes which possessed an altered L6 protein in the 50S subunit (Buckel et al., 1977). Results of studies on these ribosomes suggested that gentamicin might normally induce errors in translation by interaction with the 50S subunit, because the mutant ribosomes showed reduced levels of misreading in the presence of this aminoglycoside. However, in vitro protein synthesis was still as sensitive to gentamicin in the mutants as in the wild type (Kühberger et al., 1977). Using a similar isolation procedure, mutants of E.coli which are resistant to kanamycin have been generated (Thorbjarnardóttir et al., 1978). These mutants also possess antibiotic-uptake mutations co-operating with target site alterations. In this case, the ribosomal mutation was mapped to the rpsL gene, coding for protein S12, in the 30S subunit. On the basis of these results, it appears that kanamycin and gentamicin might possess functional binding sites on different subunits of the ribosome. On the other hand, at moderate inputs (about 10^{-5} M), [^{3}H]-kanamycin has been shown to bind to a single site on either subunit, or to two sites on the 70S ribosome, (Misumi et al., 1978). Moreover, these binding sites were suggested to be directly involved in the inhibitory effects induced by kanamycin, as a result of studies on kanamycin-resistant mutants of E.coli (Choi et al., 1980). Resistance in these mutants was attributed to alterations in either the 30S or the 50S ribosomal subunit and resulted in a concomitant loss of [^{3}H]-kanamycin binding. Therefore, it appears that both kanamycin and gentamicin possess functional binding sites on the 50S ribosomal
subunit. However, it is not yet clear whether these are the primary target sites of these aminoglycosides.

Other *E. coli* mutants, which have arisen spontaneously, are resistant to low levels of neamine. In some cases (*nea A* strains), the mutants possess ribosomes with an altered S17 protein (Cannon *et al*., 1974; Bollen *et al*., 1975), whereas in others the mutations have been mapped to *rps E* and *rps L* genes, coding for proteins S5 and S12, respectively (DeWilde *et al*., 1975). This suggests that proteins S17, S12 and S5 might be spatially related in the intact ribosome, and a mutation in any one of these is sufficient to affect neamine binding.

The gentamicin, kanamycin and neamine resistant mutants discussed above clearly have only limited use in elucidating the mode(s) of action or functional binding sites of aminoglycosides, since none of them are resistant to high levels of these antibiotics. Hence, the ribosomal alterations proposed to confer resistance on the mutants might only interfere with antibiotic molecules binding to low affinity sites on the ribosome.

Attempts to correlate directly, the effects of kanamycin and neomycin on translocation and misreading with specific binding sites on either the 30S or 50S ribosomal subunits, or both, have provided some interesting data (Campuzano *et al*., 1979). An increase in translational errors was observed when the 30S ribosomal subunit, but not the 50S subunit, was treated with neomycin B or kanamycin B and then supplemented with the complementary untreated subunit. In addition, treatment of either subunit with kanamycin B was shown to block translocation. Therefore, it was suggested that interaction of neomycin and related antibiotics with the 30S ribosomal subunit caused codon misreading and impaired translocation, whilst aminoglycoside
binding to the 50S subunit was responsible for inhibition of translocation. However, it is worth noting that these antibiotics might have exchanged between ribosomal subunits during reassembly of 70S ribosomes, especially as the subunits have been shown to possess different affinities (albeit slight) for kanamycin (Misumi et al., 1978). Indeed, since kanamycin increases the affinity of peptidyl-tRNA for the A site of the ribosomal 30S subunit, this strongly suggests that antibiotic which is bound to the small subunit is responsible for the inhibition of translocation (Semenkov et al., 1982).

In conclusion, the problems encountered during studies with most aminoglycosides are associated with their multiphasic effects on protein synthesis. It is difficult, therefore, to compare results from different research groups, carried out at various antibiotic concentrations, using a wide range of experimental cell-free systems. In particular, some assays contain drug to ribosome ratios that are much higher than those likely to be achieved in intact cells. On that basis, it is difficult to relate the situation in vivo to the in vitro results. Despite these caveats, it is clear that aminoglycosides interfere with both the fidelity and elongation steps of protein synthesis. In fact, these two effects may well be closely linked, such that a distortion of the ribosomal site involved in the recognition of aminoacyl-tRNA might also affect the translocation reaction (Perzynski et al., 1979).

3. Aminoglycoside Resistance in Clinical Isolates.

The continued therapeutic use of aminoglycosides has led to the development of antibiotic resistance in clinical isolates (for a
review, see Phillips and Shannon, 1984). In general, the determinants conferring aminoglycoside resistance in these strains are located on R-plasmids, a characteristic which facilitates the spread of resistance to other bacteria (Davies and Smith, 1978). These plasmids encode aminoglycoside-modifying enzymes, however the exact mechanisms whereby these enzymes determine resistance are currently unclear.

Numerous different kinds of modifying enzymes exist in Gram-positive and Gram-negative bacteria. These enzymes can be divided into three distinct groups (Davies, 1980): acetyltransferases, which act on amino groups in the aminoglycosides and use acetyl-CoA as the source of acetyl groups, and adenylyltransferases and phosphotransferases which modify hydroxyl groups and use ATP as the source of adenylyl and phosphoryl residues, respectively. The modifying enzymes can be further subdivided, according to the position and ring of the drug which is modified (Davies, 1980). For example, gentamicin, tobramycin and kanamycin can be adenylylated at the hydroxyl group at position 2 of Ring I (see Figs. 2.1 and 2.2), and the enzyme catalysing this reaction is known as 2'-0 adenylyl-transferase, AAD(2'). The same three aminoglycosides can also be acetylated at the hydroxyl group on carbon-3 of Ring II: this modifying enzyme is known as 3-0 acetyl-transferase, AAC(3). It is obvious from these two examples, that the aminoglycoside-modifying enzymes present in a given resistant strain, cannot safely be predicted solely on the basis of the resistance phenotype.

Investigations on the biochemical mechanisms of R-plasmid encoded aminoglycoside resistance suggested that either the drugs are inactivated by the modifying enzymes and, thereby, prevented from interacting with their target site(s) and/or antibiotic transport into
the cell is blocked (Bryan, 1980). However, it is difficult to distinguish between these possibilities, as binding to ribosomes is believed to be an essential component of the uptake of aminoglycosides into bacteria. Studies on E. coli revealed that the presence of a R-plasmid was associated with a large decrease in the uptake of [³H]-gentamicin (Kagan and Davies, 1980) and that antibiotic in the culture medium was not inactivated (Davies and Kagan, 1981). The mechanism of aminoglycoside resistance operating in these bacteria is, therefore, restricted to protecting those cells which contain R-plasmid. Aminoglycosides were still transported into resistant strains, but were modified and retained inside the cell (Bryan, 1980; Davies and Kagan, 1981). Since enzymically-modified aminoglycosides have a reduced affinity for their ribosomal binding sites (Benveniste and Davies, 1973b), it appears that a strain could remain resistant to the drug, provided the rate of inactivation of the antibiotic exceeds the rate of aminoglycoside uptake.

A model to explain the mechanisms of aminoglycoside transport and resistance in clinical isolates has been proposed, and suggests that the uptake of these antibiotics occurs in three phases (Bryan and Vanden Elzen, 1977). First, aminoglycosides bind to the exterior of the cell in an energy-independent phase. This is followed by an energy-dependent phase (EDP-I), which represents the initial transport of the drugs across the cytoplasmic membrane. This phase occurs at a very slow rate and competes with the rate of aminoglycoside inactivation by intracellular antibiotic-modifying enzymes. The final stage of aminoglycoside transport, energy-dependent phase II (EDP-II), only occurs in sensitive strains, and is initiated by, and associated with, the binding of aminoglycosides to ribosomes. Although convincing
proof for this model is lacking, proton-leaky bacterial mutants and uncoupling reagents have been shown to stop or decrease the accumulation of aminoglycosides, confirming that transport of these antibiotics is energy-dependent (Bryan, 1980). Also, ribosomes were shown to participate in drug uptake, since if they were rendered incapable of binding active drug (by mutation or by the enzymic inactivation of aminoglycosides), antibiotic uptake was greatly reduced (Kagan and Davies, 1980), in particular EDP-II is delayed or prevented (Ahmad et al., 1980).


Aminoglycoside-modifying activities similar to those present in clinical isolates, are of wide distribution among bacteria known to produce these antibiotics (Davies et al., 1979). Investigations into the roles of these enzymes in producing organisms have led to the suggestion that they are involved either in the protection of the producer, or as part of the biosynthetic pathway of the drug. For example, the neomycin producer, Streptomyces fradiae, possesses neomycin-sensitive ribosomes (Hotta et al., 1981), but contained 3'-0 phosphotransferase, APH (3'), and 3-N acetyltransferase, AAC(3), activities, which can both inactivate neomycin in vitro (Davies et al., 1979). Previously, data which supported the hypothesis that APH(3') activity is concerned with neomycin resistance in S. fradiae were reported (Komatsu et al., 1981). These results showed that resistant mutants of this organism, which had arisen spontaneously, displayed an increase in APH(3') activity. On the other hand, AAC(3) activity was detected in both producing and non-producing strains of S. fradiae.
suggesting that this enzyme may not have a biochemical role in aminoglycoside production (Davies et al., 1979). However, investigations involving another neomycin producer, *Micromonospora chalcea*, revealed that this organism contained similar AAC(3) and APH(3’) activities as the generically unrelated *S. fradiae* (Davies et al., 1979). This suggested that *M. chalcea* may require both enzymes for neomycin production and/or resistance.

Further evidence that both neomycin-modifying enzymes are concerned with resistance in *S. fradiae*, was generated by studies involving recombinant DNA technology and a non-producing, neomycin-sensitive host, *Streptomyces lividans* (Thompson et al., 1982a). When fragments of *S. fradiae* DNA were cloned in *S. lividans*, several neomycin-resistant clones were obtained. Some of these strains were shown to contain APH(3’), and others AAC(3) activity. However, it was surprising to find that all of these clones displayed only low-level resistance to neomycin, despite the fact that those containing APH(3’) activity produced large amounts of the enzyme. Interestingly, high-level resistance was observed when both APH(3’) and AAC(3) activities were present together in *S. lividans*, as in *S. fradiae*. In conclusion, it appears that both of these aminoglycoside-modifying enzymes are required by the producing organism to maintain adequate protection against neomycin.

Several other aminoglycoside-producing organisms have been reported to protect themselves solely with the aid of drug-modifying enzymes and to possess ribosomes which are sensitive to their toxic products, (Hotta et al., 1981: 1983). Recently, however, it was found that resistance may also occur at the level of the ribosomes. The first example reported was that of *Streptomyces tenjimariensis*, the producer
of istamycin (Okami et al., 1979), which possessed ribosomes resistant
to istamycin and kanamycin, but not to various other aminoglycosides,
including gentamicin (Yamamoto et al., 1981a:b). Furthermore, no
antibiotic-inactivating enzymes have been detected in this organism
(Yamamoto et al., 1981b), suggesting that ribosomes have the main role
in the self-resistance mechanism. Subsequent studies revealed that
several other aminoglycoside producers also possess ribosomes that are
resistant to their endogenous antibiotics. These include
Micromonospora purpurea (Piendl and Böck, 1982), Streptomyces
tenebrarius (Yamamoto et al., 1982) and Streptomyces kanamyceticus
(Nakano et al., 1984). Respectively, these organisms produce the
gentamicin C complex, the nebramycin complex (including kanamycin B and
its derivatives, plus apramycin) and kanamycin. Studies involving
M. purpurea have demonstrated that this organism is highly resistant to
gentamicin and to several other aminoglycosides, including kanamycin,
during all phases of the growth cycle. Although specific gentamicin-
modifying enzymes could not be detected in this producer, the in vivo
resistance pattern was shown to be associated with some property of the
ribosomes (Piendl and Böck, 1982). Therefore, it appears that
M. purpurea is another example of an aminoglycoside producer in which
ribosomes play a prominent role in the mechanism(s) of self defence.
However, in other producers, such as S. kanamyceticus and S. tenebrarius,
the situations are more complicated, as these organisms contain
ribosomes which are refractory to the relevant antibiotic products, in
addition to possessing aminoglycoside-modifying enzymes. On the one
hand, it was possible that both mechanisms of resistance were required
in order to render the producer resistant to high levels of
aminoglycoside. On the other hand, however, it was feasible that one
or other of these resistance mechanisms was redundant in terms of protecting the producing organism. This hypothesis was recently confirmed for *S. kanamyceticus*, by cloning the kanamycin-resistance determinant from this producer in *S. lividans*. The gene was shown to provide resistance to kanamycin by altering ribosomes (Nakano *et al.*, 1984), and this topic is discussed in further detail below (see section 5, page 38).

High-level resistance to a wide range of aminoglycosides was also observed in *S. tenebrarius* (Higgins and Kastner, 1968), although the relative contributions of ribosomes and modifying enzymes to the resistance phenotype have not been fully established. Ribosomes from this producer are resistant to various aminoglycosides, including kanamycin, gentamicin and istamycin, whereas cell-free extracts of this organism possess phosphotransferase and N-acetyltransferase activities (Yamamoto *et al.*, 1982). The former enzyme was classed as APH(6'), as it inactivated streptomycin, and the latter as AAC(2'), due to its substrate specificity for kanamycins, however, it did not acetylate gentamicins as do similar enzymes in clinical isolates (Benveniste and Davies, 1973a). Therefore, it was suggested that *S. tenebrarius* might possess either a novel type of AAC(2') activity, or an acetyltransferase enzyme that had not been characterised previously (Yamamoto *et al.*, 1982).

Recently, ribosomes from *S. tenjimariensis*, *S. tenebrarius* and *M. purpurea* have been analysed biochemically, in an attempt to determine which ribosomal components were responsible for conferring the various patterns of aminoglycoside resistance on these producers (Piendl *et al.*, 1984). These experiments involved the use of ribosomal components (RNA and proteins) from resistant and sensitive organisms to
reconstitute various hybrid particles \textit{in vitro}. In each case, resistance was associated with the 16S rRNA present in the 30S subunits (Piendl et al., 1984). Moreover, since each of these producers possessed a different pattern of aminoglycoside resistance, at least three distinct mechanisms of resistance must be operating in these organisms, each involving 16S rRNA.

The work described in this dissertation attempts to further the analysis of the mechanisms of resistance in \textit{S.tenjimeriensis}, \textit{S.tenebrarius} and \textit{M.purpurea} and to ascertain their relationships to one another. To do this, recombinant DNA technology has been used to transfer the aminoglycoside-resistance determinant(s) present in each of these producers, into a host organism i.e. \textit{Streptomyces lividans} TK21. This aminoglycoside-sensitive strain of \textit{S.lividans} provides a common, clean background, in which subsequent alterations in ribosomal behaviour can be readily assessed and compared.

5.1 Gene Cloning in \textit{Streptomyces}.

Despite the great progress that has been made in the development of recombinant DNA technology in \textit{Escherichia coli} host-vector systems, this organism is not an advisable choice for the cloning of \textit{Streptomyces} genes. The main reason for this is that many of these genes are not expressed in \textit{E.coli}, probably since \textit{E.coli} RNA polymerase cannot recognise the sequences which normally act as transcriptional control signals in \textit{Streptomyces} (see Bibb and Cohen, 1982). In this context, it is perhaps relevant to note that \textit{Streptomyces} DNA has an extremely high guanosine plus cytosine (G + C) content (on average 73\% G + C: Enquist and Bradley, 1971) which might be reflected in the
composition of its promoters, whereas the promoter regions identified in other prokaryotes, such as E. coli, are known to contain adenine and thymine (A + T) rich sequences (Rosenberg and Court, 1979).

Recent advances in the streptomycetes gene cloning system (Chater et al., 1982) have provided ideal hosts for the cloning and study of antibiotic-resistance determinants, namely Streptomyces coelicolor A3(2) and Streptomyces lividans. The organism used in the work presented here is S. lividans TK21, which was derived from a wild-type strain, S. lividans 66, by the elimination of two naturally occurring plasmids from the latter organism (Kieser et al., 1983). This host strain, S. lividans TK21, is particularly useful since it is sensitive to aminoglycosides, does not produce any antibiotics, is experimentally easy to handle as it grows and sporulates vigorously and reproducibly, and is a host for all the plasmid and phage vectors discussed below (for reviews, see Chater et al., 1982; Hopwood and Chater, 1982; Hopwood et al., 1985). Furthermore, S. lividans is not known to restrict DNA from any other streptomycete, nor does it restrict plasmid or phage DNA which has previously been grown in E. coli.

Several plasmids have been developed for use as cloning vectors in Streptomyces (for a review see Hopwood et al., 1985). Initial studies involved plasmid SLP1.2 (see Fig. 2.5 for restriction map), which is the largest naturally occurring member of the SLP1 family of plasmids. These plasmids are derived from the chromosome of S. coelicolor A3(2) (Bibb et al., 1981) and can replicate autonomously in S. lividans with a low copy number of about 4-5 per chromosome (Bibb et al., 1980a). An important characteristic of SLP1.2 is its ability to transfer from a plasmid-bearing strain of S. lividans to a plasmid-free strain, during which it causes inhibition of the recipient's growth. This effect is
known as the 'lethal zygosis' (Ltz*) phenotype (although there is no
evidence to suggest that the inhibition of growth is lethal), and is
visible during growth on agar, as a narrow zone of delayed sporulation
(a 'pock') around the donor strain. These pocks are useful in the
detection of transformants and in the isolation of recombinant
plasmids. There are several useful enzyme restriction sites that lie
within a dispensable region of SLP1.2, notably a unique Bam HI site and
three Pst I sites, which are available for insertion of foreign DNA.
These sites were employed in initial shotgun cloning experiments
performed in Streptomyces, in which antibiotic-resistance determinants
were cloned from several species into S.lividans (Bibb et al., 1980b:
Thompson et al., 1980). For example, neomycin and thiostrepton
resistances have been cloned from Streptomyces fradiae and Streptomyces
azureus, respectively and these resistance determinants have been
utilised in the development of several useful cloning vectors. One of
these, plasmid pIJ61 (see Fig. 2.6 for restriction map), is a
derivative of SLP1.2 containing the neomycin and thiostrepton
resistance genes (Thompson et al., 1982c). Unique Bam HI and Pst I
sites allow detection of clones by insertional inactivation of neomycin
resistance. Other useful derivatives of SLP1.2 include a shuttle
vector for Streptomyces and E.coli, which contains SLP1.2 and pBR322
replicons (Thompson et al., 1982c) and promoter-probe plasmids, for
investigations of gene expression in Streptomyces (Bibb and Cohen,
1982: Horinouchi and Beppu, 1985). The major disadvantage of
SLP1.2-derived vectors is their narrow host-range, which is so far
confined to S.lividans and Streptomyces reticuli (Bibb et al., 1981:
Hopwood et al., 1984a).

Several naturally occurring low copy number plasmids, including
Legend to Fig. 2.6.

Simplified Restriction Map of pIJ61.

This plasmid was derived by replacement in vitro of a segment of SLP1.2 DNA (see Fig. 2.5) with DNA carrying the neomycin phosphotransferase (aph) determinant from *S. fradiae* and the thiostrepton resistance (tsr) determinant from *S. azureus*. 
Fig 2.6

plJ61
15.7kb

Bcl I
Hind III
Cla I
Eco RI
Hind III
Sph I
Pst I
Bam HI
aph
tsr
Cla I
Bgl II
Hind III
SCP2 and SCP2* (each of 31kb in length), exist in \textit{S. coelicolor} A3(2) (Bibb \textit{et al.}, 1977). Although both plasmids act as sex factors in \textit{S. coelicolor}, the ability to promote chromosomal recombination and to display the \text{Ltz}^+ phenotype is enhanced in SCP2*, which is a spontaneous variant of SCP2. Using SCP2*, several vectors have been developed recently, which can carry stable inserts of DNA larger than 30kb (Lydiate \textit{et al.}, 1985). For example, fragments of \textit{S. coelicolor} DNA up to 35kb in length, carrying some or all of the genes for actinorhodin biosynthesis have been cloned using one of these SCP2*-derived vectors, pIJ922 (Malpartida and Hopwood, 1984). These vectors also have the advantage over SLP1.2-derived plasmids, in that they have a broad host range within the genus \textit{Streptomyces} (Lydiate \textit{et al.}, 1985).

Another family of plasmids which has been used in the development of cloning vectors, was first discovered in \textit{Streptomyces lividans} ISP 5434, as naturally occurring plasmid species (Kieser \textit{et al.}, 1982). The largest of these, pIJ101 (see Fig. 2.7 for a restriction map) has been studied, after transformation into \textit{S. lividans} 66, where it exists at a high copy number of between 40 and 300 copies per chromosome, depending on the age or physiological state of the culture (Kieser \textit{et al.}, 1982). The high copy number of this plasmid offers the potential advantage of enhanced product formation of cloned genes, facilitating further biochemical analysis. Another advantage of pIJ101 and its derivatives is their broad host-range, since 13 out of 18 wild type strains of \textit{Streptomyces} tested, were successfully transformed.

Plasmid pIJ101 is self-transmissible by conjugation and displays the \text{Ltz}^+ phenotype. However, this characteristic is not recommended for high copy number vectors if they are to be used in shotgun cloning experiments, since conjugative plasmids can hamper the purification of
Legend to Fig. 2.8.

Simplified Restriction Map of pIJ702.

This plasmid has been used as the cloning vector during the course of this study. The plasmid contains the thioestrepton-resistance (\textit{tsr}) determinant from \textit{S. azureus} and the tyrosinase (\textit{mel}) determinant from \textit{S. antibioticus}. Unique restriction sites within pIJ702 are available for insertion of foreign DNA (e.g. \textit{Kpn I}, \textit{Pst I}, \textit{Bgl II}, \textit{Sph I} and \textit{Sst I}).
Fig 2-8

plJ702
5.7kb
presumed clones. Therefore, several non-transmissible (Lt"7")
derivatives of pIJ101 have been constructed for use as DNA cloning
vectors. An early example of such a vector is plasmid pIJ350, which
was derived from pIJ102 (a naturally occurring deletion-derivative of
pIJ101) by replacement of a dispensable region of the plasmid with the
thiostrepton resistance gene (Kieser et al., 1982). A derivative of
pIJ350, containing the tyrosinase gene (mel) from Streptomyces
antibioticus has been constructed (Katz et al., 1983), and designated
pIJ702 (see Fig. 2.8 for a restriction map). This plasmid has been
used as the vector in the cloning experiments described in this
dissertation. It is non-transmissible, with a high copy number and
displays a broad host range within Streptomyces (Katz et al., 1983).
The thiostrepton resistance determinant provides a good selectable
marker, because very few streptomycetes are naturally resistant to
thiostrepton (Kieser et al., 1982) and because resistant mutants rarely
arise spontaneously. This gene is transcribed in a clockwise direction
as represented in the restriction map of pIJ702* in Figure 2.8 (Bibb et
al., 1985). The tyrosinase gene catalyses the production of melanin, a
brown pigment, from tyrosine and is a particularly useful marker, since
it contains unique restriction sites for Bgl II, Sph I and Sst I, that
are absent from the rest of the plasmid. Insertion of DNA fragments at
any one of these sites usually abolishes the mel+ phenotype, allowing
easy identification of non-melanin producing colonies, which presumably
contain recombinant plasmids (Katz et al., 1983). Unique restriction
sites for Pst I and Kpn I, present within a dispensable region of
pIJ702, can also be used for cloning purposes.

Other useful derivatives of the pIJ101 series of plasmids include
those which serve as shuttle vectors between Streptomyces and E.coli,
since they contain two functional replicons, one from pBR322 and one from pIJ101 (Kieser et al., 1982). Also, a series of versatile promoter-probe vectors has been developed, some of which are bifunctional plasmids (Bibb et al., 1984).

Finally, the temperate phage, \( \Phi C31 \), has been developed recently as a useful cloning vector in Streptomyces (Chater et al., 1982). This phage has a wide host range within streptomycetes, and derivatives have been constructed which can replicate in E. coli as multicopy plasmids, or in Streptomyces as phages (Chater et al., 1982). Phage vectors have not been used in the cloning experiments described in this dissertation, however, they are mentioned here, since they provide important and, sometimes, preferential alternatives to plasmids as cloning vectors. The development of \( \Phi C31 \)-derived vectors and their applications, have been reviewed recently (see Harris et al., 1983: Rodicio et al., 1984).

The cloning vectors discussed above have been used extensively in the isolation of a variety of streptomycete genes. In particular, vector pIJ702 has been used successfully to clone genes involved in antibiotic biosynthesis (Fietelson and Hopwood 1983: Gil and Hopwood, 1983: Jones et al., 1984) and in antibiotic resistance (Thompson et al., 1982b: Nakano et al., 1984: Pérez-González and Jiménez, 1984: Vallins and Baumberg, 1985).

5.2 Cloning of Aminoglycoside-Resistance Genes in Streptomyces.

The development of a gene cloning system in Streptomyces has provided an invaluable approach to the study of antibiotic-resistance mechanisms that operate in producing organisms. In particular, it has
been used as a definitive method of establishing whether or not an aminoglycoside-modifying enzyme which has been detected in a given producer, does indeed contribute to the resistance phenotype of that organism. For example, paromomycin resistance has been cloned from Streptomyces rimosus forma paromomycinus, the paromomycin producer, and the resistant clones were shown to contain a phosphotransferase (PPH) which was capable of inactivating paromomycin (Pérez-González and Jiménez, 1984). These data implied that the PPH activity which had been detected in the producer, was involved in conferring paromomycin resistance. Similar cloning experiments have also revealed that the neomycin producer, Streptomyces fradiae, owes its resistance properties to the action of aminoglycoside-modifying enzymes (see section 4, above, page 28, for further details). However, in this case, both neomycin phosphotransferase and acetyltransferase activities were required in the same clone to provide high level resistance to neomycin (Thompson et al., 1982b). This example serves as a warning, because even though a given producer might contain an enzyme which inactivates specific antibiotics in vitro, this enzyme alone might not be sufficient to confer complete protection from the endogenous antibiotic in vivo.

In contrast, the kanamycin-producing strain of Streptomyces kanamyceticus contains an acetyltransferase, AAC(6’), which inactivates this aminoglycoside in vitro, and was thought to play a major role in conferring kanamycin resistance (Hotta et al., 1981). However, when attempts were made to clone the AAC(6’) activity from S. kanamyceticus in S. lividans, some kanamycin-resistant transformants were selected that contained ribosomes which were refractory to the action of kanamycin (Nakano et al., 1984). Furthermore, the latter transformants
of *S. lividans* were also more resistant to certain other aminoglycosides when compared with the producer. The reason for the apparent discrepancies in resistance phenotypes was that the ribosomal resistance in the clones was constitutive, whereas in *S. kanamyceticus* it was inducible and was only 'switched on' during antibiotic production. Previous reports had suggested that ribosomes from *S. kanamyceticus* were sensitive to kanamycin (Hotta *et al.*, 1981). However, these results were obtained using ribosomes derived from cultures of *S. kanamyceticus* that had been grown in a medium which did not support Kanamycin production and, therefore, ribosomal resistance to this aminoglycoside had not been induced. Thus, by cloning antibiotic-resistance determinants away from the DNA sequences which normally control their expression in producing organisms, it might be possible, in the future, to uncover resistance mechanisms which would otherwise have remained undetected. The gene conferring ribosomal resistance to kanamycin in *S. kanamyceticus* is the only inducible aminoglycoside-resistance determinant reported to date and it provides a unique opportunity for investigating the regulation of expression of antibiotic-resistance genes in streptomycetes.

In conclusion, the aminoglycoside-resistance determinants cloned so far, have provided confirmation of previous hypotheses, for example aminoglycoside-modifying enzymes have been shown to be involved in conferring resistance to aminoglycosides in *S. rimosus forma paromomycinus* (Pérez-González and Jiménez, 1984) and in *S. fradiae* (Thompson *et al.*, 1982b). In addition, data from cloning experiments have revealed some surprising discoveries on the mechanisms of self-defence present in antibiotic-producing organisms. For example, both neomycin-modifying enzymes detected in *S. fradiae* are required for high-level resistance to...
neomycin (Thompson et al., 1982b) and S. kanamyceticus contains kanamycin-resistant ribosomes during antibiotic production (Nakano et al., 1984).

The cloning experiments undertaken in this work were designed to isolate aminoglycoside-resistance genes from S. tenjimariensis and S. tenebrarius, to investigate the mechanisms of self-defence employed by these producers. By analysis of the resistant clones, it was hoped to confirm that the resistance phenotypes of these producing organisms could be attributed to some property of their 16S rRNA, as suggested previously (Piendl et al., 1984), and to determine the nature of the rRNA modifications involved. Finally, it was hoped that resistance determinants cloned from these producers would provide novel selectable markers, which could be used in the construction of new cloning vectors. These vectors would be especially useful, because aminoglycoside antibiotics are readily available.
CHAPTER 3

Table 3.1

Sources of Microorganisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotics Produced</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces tenimariensis</em></td>
<td>Istatamycin</td>
<td>ATCC 31603</td>
<td>Prof. A. Bock, University of Munich.</td>
</tr>
<tr>
<td><em>Streptomyces tenebrarius</em></td>
<td>Nebramycin</td>
<td>NCIB 11028</td>
<td>NCIB</td>
</tr>
<tr>
<td><em>Streptomyces vinaceus</em></td>
<td>Viomycin</td>
<td>NCIB 8852</td>
<td>NCIB</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em></td>
<td>None</td>
<td>TK21</td>
<td>Prof. D. A. Hopwood, John Innes Institute, Norwich.</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em></td>
<td>None</td>
<td>TSK 1</td>
<td>This work: Strain TK21 containing plasmid pIJ702.</td>
</tr>
<tr>
<td><em>Micromonospora purpurea</em></td>
<td>Gentamicin</td>
<td>DSM 43036</td>
<td>Prof. A. Bock, University of Munich.</td>
</tr>
</tbody>
</table>
CHAPTER 3

This chapter describes methods which have been used throughout the study. Techniques specific to one part of the work will be found in the appropriate chapter, as will details of individual experiments.

1. Origin and Maintenance of Bacterial Strains.

The organisms used in this work are listed in Table 3.1 together with their strain number, source and antibiotic products, where relevant.

Spores of Streptomyces (or samples of broth culture in the case of S. tenebrarius) were stored in 20% (v/v) glycerol at -20°C. Strains were maintained on 2% (w/v) agar plates containing NE medium. This comprised (per litre) 10g glucose, 2g yeast extract (Difco), 2g casamino acids (Difco), 1g beef extract (Lab Lemco, Oxoid). The pH was adjusted to 7.0 with KOH prior to autoclaving. Strains of S. lividans containing aminoglycoside-resistance determinants were grown in the presence of kanamycin (20µg ml⁻¹ final concentration), to ensure that antibiotic resistance was retained. (There was no evidence, however, to suggest that resistance determinants were unstable in the absence of antibiotics).


Spores from one plate of the organism were suspended in 10 ml of 0.1% (v/v) 'Synperonic' (detergent obtained from ICI Ltd) by gentle scraping with a wire loop, and used to inoculate 1 l of liquid medium in a 2l baffled conical flask. Cultures were incubated at 30°C with vigorous shaking to provide adequate aeration. (For growth of
**S. tenebrarius**, a 5 ml overnight culture grown in TSB, was used as inoculum.

**S. tenjimariensis**, **S. tenebrarius** and all strains of **S. lividans** were grown in TSB (Difco). **S. vinaceus** was grown in liquid YEME medium supplemented with 34% (w/v) sucrose and 5mM MgCl<sub>2</sub>. Basic YEME medium contained 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone and 1% (w/v) glucose.

3. Determination of Minimum Inhibitory Drug Concentrations (MIC Values).

Spores were streaked on NE agar plates containing different concentrations of antibiotics. Growth was monitored after incubation at 30°C for 3-5 days.

4. Preparation of Total DNA.

Cells from 40-48h cultures were harvested and washed twice by resuspension in and centrifugation from 10% (v/v) glycerol. Centrifugation was at 10,000 rev.min<sup>-1</sup> for 10 min at 4°C in the Beckman JA-10 rotor. Mycelia (2g wet weight) were resuspended in 50mM Tris-HCl, pH 8.0 at 20°C, containing 25% (w/v) sucrose, to 6 ml final volume and then incubated at 30°C for about 40 min. in the presence of lysozyme (1mgml<sup>-1</sup> final concentration). After addition of Na<sub>2</sub>EDTA and pronase (75mM and 1 mgml<sup>-1</sup> final concentrations, respectively), the incubation was continued for a further 5 min. (Pronase was previously predigested by incubation at 37°C for 1 h). The cells were then lysed by treatment with SDS (0.3% (w/v) final concentration) and incubated at 37°C for
CHAPTER 3

2 h. Solubilised nucleic acids were extracted with phenol which contained 0.1% (w/v) 8-hydroxyquinoline and was saturated with TE buffer plus 0.1M NaCl. (TE buffer contained 10mM Tris-HCl, pH 8.0 at 20°C, 1mM Na₂EDTA).

Following removal of RNA by incubation at 37°C for 1 h with RNase (40µg/ml final concentration, pre-heated to 80°C for 15 min to remove DNase) genomic DNA was precipitated overnight at 4°C with 1M NaCl and 10% (w/v) PEG (mwt.6000, final concentrations). The DNA was collected by centrifugation at 8,000 rev min⁻¹ for 5 min at 4°C, dissolved in TE buffer and reprecipitated with sodium acetate (0.3M final concentration) and 3 volumes of ethanol by incubation at -20°C overnight. Total DNA was finally pelleted by centrifugation and redissolved in TE buffer. The concentration of DNA was determined by measurement of the absorbance at 260nm, given that 1 A₂₆₀ unit corresponds to a DNA concentration of 50 µg ml⁻¹. Typical yields of about 0.5mg DNA per gram cells (wet weight) were obtained. (This method followed conventional techniques as published by Chater et al., 1982).

5. Large Scale Preparation of Plasmid DNA.

Plasmid DNA was isolated by conventional techniques using a caesium chloride density gradient (see Chater et al., 1982). The purification relies on the equilibration of circular plasmid DNA at a higher buoyant density in the caesium chloride gradient than linear chromosomal DNA.

Plasmid pIJ702 was used as vector in the cloning experiments described below and was prepared from Streptomyces lividans TSK1. Cells were harvested from 48h cultures, when growth yields of about 6g
per litre were obtained. After washing twice in 10% (v/v) glycerol, mycelia were resuspended in 50 ml TE buffer containing 34% (w/v) sucrose and then incubated at 30°C for 30 min in the presence of Na₂EDTA (40 mM final concentration, pH 8.0) and lysozyme (4 mg ml⁻¹ final concentration in 10 mM Tris-HCl, pH 8.0 at 20°C). The cells were then lysed using SDS (0.25% (w/v), final concentration) and chromosomal DNA precipitated with NaCl (1M, final concentration) at 4°C for 2 h. This DNA was removed by centrifugation at 16,000 rev min⁻¹ for 30 min at 4°C. Plasmid DNA was precipitated from the resultant supernatant by addition of PEG (m.wt.6000) to 10% (w/v) final concentration and the mixture was left at 4°C overnight. Plasmid DNA was collected by centrifugation at 4,000 rev min⁻¹ for 5 min at 4°C and redissolved in buffer containing 30 mM Tris-HCl, pH 8.0 at 20°C, 5 mM Na₂EDTA and 50 mM NaCl. Caesium chloride (1.05 g per ml DNA solution) and ethidium bromide (500 μg ml⁻¹ final concentration) were added to the DNA solution and the refractive index adjusted to 1.3925 at 20°C. Contaminating protein was removed by centrifugation at 8,000 rev min⁻¹ for 5 min at 4°C, and plasmid DNA then separated from any remaining chromosomal DNA by centrifugation at 40,000 rev min⁻¹ for 18 h at 20°C, in the vertical Sorvall TV850 rotor. Bands of DNA in the gradient were then visualised using U.V. light and the lower plasmid band carefully collected. Ethidium bromide was removed by extraction with caesium chloride-saturated isopropanol and the plasmid solution dialysed against 4 x 100 volumes of TE buffer at 4°C. Finally, plasmid DNA was precipitated using sodium acetate (0.3M final concentration) and 3 volumes of ethanol, and redissolved in TE buffer. Average yields of 70 μg plasmid DNA per gram cells (wet weight) were obtained.
6. Religation of Vector and Genomic DNA.

In a typical shotgun cloning ligation, genomic DNA fragments (5μg; generated by partial digestion with an appropriate restriction enzyme) were mixed with vector plasmid pIJ702 (1μg; linearised with the same restriction enzyme) and heated to 75°C for 10 min. After precipitation, the DNA mixture was resuspended at about 40μg ml\(^{-1}\) in ligation salts (150μl of 66mM Tris-HCl, pH 7.5 at 20°C, 6.6mM MgCl\(_2\), 1mM DTT, 0.4mM ATP) and incubated at 22°C for 1 h with T\(_4\) DNA ligase. (The amount of enzyme used was sufficient to circularise 3μg of linearised plasmid pIJ702). Ligation was continued overnight at 4°C and the DNA then precipitated and finally redissolved in 20μl TE buffer, ready for transformation of \(S.\) lividans TK21 protoplasts. (Digestions and ligations of DNA were routinely monitored by agarose gel electrophoresis).

7. Analysis of DNA by Agarose Gel Electrophoresis.

Generally, horizontal submerged gels (24 cm x 12.5 cm x 0.4 cm) were used for electrophoresis of DNA. These were prepared using agarose (Litex), at either 1.4% (w/v) or 0.7% (w/v) final concentration (see below) dissolved in TEA buffer (40 mM Tris-acetate, pH 8.0 at 20°C, 2 mM Na\(_2\)EDTA). The gels were loaded with DNA samples containing 10% (v/v) sample buffer, which consisted of TEA buffer supplemented with 50% (v/v) glycerol, 0.01% (w/v) xylene cyanol FF and 0.01% (w/v) bromophenol blue. Electrophoresis was carried out at a constant voltage of approximately 6 Vcm\(^{-1}\) for about 3 h in TEA buffer. After staining in ethidium bromide (1 μg ml\(^{-1}\) final concentration) for about
20 min, the DNA fragments were visualised and photographed on a U.V. transilluminator. The sizes of the DNA fragments were estimated by comparison with fragments of known size, generated by Hind III digestion of bacteriophage lambda DNA (Daniels et al., 1980).

For general purposes, 0.7% (w/v) agarose gels were used, however, for analysis of religated vector and genomic DNAs, gels containing 1.4% (w/v) agarose were required, to separate the more slowly migrating open circular plasmid DNA (indicative of a good religation), from religated linear chromosomal DNA fragments.


(Acid-washed glassware or, where possible, sterile plastic apparatus was used in the generation and subsequent manipulation of protoplasts, to avoid contamination of preparations with traces of detergent).

Protoplasts were routinely prepared from mycelia of S. lividans TK21, the antibiotic-sensitive, plasmid-free host. The growth stage of these mycelia is an extremely important determinant for generating protoplasts with an optimal transformation efficiency (Thompson et al., 1982a) and, therefore, a filtered suspension of S. lividans TK21 spores was used as the inoculum. This was prepared by collecting spores from a confluent plate of S. lividans, in 10 ml of distilled water, and filtering the suspension through a cotton wool plug to remove mycelia. (If the latter are present in the inoculum, they tend to grow more rapidly than germinating spores and the resulting culture contains cells at various stages of growth). After filtration, spores were harvested by centrifugation and resuspended in 1 ml of 20% (v/v)
glycerol, for storage at -20°C. Prior to inoculation, these suspensions were vortexed vigorously, to disrupt spore chains.

In general, about 100 μl spore suspension was used to inoculate 25 ml YEME medium, supplemented with 34% (w/v) sucrose, 5 mM MgCl₂ and 0.5% (w/v) glycine (final concentrations). Cultures were grown in 250 ml flasks, containing coiled springs to aid dispersal of mycelial clumps. The cultures were shaken at 250 rev min⁻¹ at 30°C for about 4 h, and then periodically examined under the microscope, using phase-contrast optics. When mycelial clumps had developed lengthy peripheral branches (40 - 44 h cultures), and before these branches fragmented to begin the formation of new clumps, the cultures were harvested by centrifugation at 4,000 rev min⁻¹ for 15 min at 20°C. Cell pellets which were generally pale orange in colour, were washed twice in 10.3% (w/v) sucrose and resuspended in 4 ml L medium containing 1 mg ml⁻¹ lysozyme. (L medium contained 10.3% (w/v) sucrose, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 1.4 mM K₂SO₄, 0.4 mM KH₂PO₄, trace elements and 25 mM TES-NaOH, pH 7.2). The mycelia were incubated for 1 h at 30°C, during which time protoplasts were generated by cell wall digestion.

The cells were examined under the microscope to ascertain when all the mycelial clumps had been dispersed and an equal volume of P medium was then added. (P medium contained the same components as L medium except that the concentrations of magnesium and calcium were raised to 10 mM MgCl₂ and 25 mM CaCl₂, final concentrations, to osmotically stabilise protoplasts). All subsequent steps in the procedure were carried out at room temperature. Protoplasts were filtered through a cotton wool plug to remove any remaining mycelial clumps, pelleted by centrifugation at about 3,000 rev min⁻¹ for 7 min, and finally
resuspended in 2.5 ml P medium.

Initially, the concentration of the protoplast suspension was calculated using a haemocytometer slide and was related to an A<sub>oo</sub> measurement of the suspension. The standard conversion factor thus obtained, (1 A<sub>oo</sub> unit is equivalent to 1.5 x 10<sup>5</sup> protoplasts), was used to estimate protoplast yields in subsequent preparations. On average, each 25 ml culture of <i>S. lividans</i> TK21 generated about 5 x 10<sup>10</sup> protoplasts.

Freshly prepared protoplasts were used for shotgun cloning experiments, when it was important to have optimal transformation efficiencies. Protoplasts were also stored in aliquots at -20°C, by slow freezing, however, after rapid thawing, the efficiency of transformation was reduced by 10 - 100 fold.


Samples of 4 x 10<sup>5</sup> protoplasts were washed in 5 ml P medium, pelleted and resuspended in about 100 μl of this buffer. Plasmid or religated DNA (maximum volume 20 μl in TE buffer) was then mixed with the protoplasts and DNA uptake assisted by the addition of 0.5 ml T medium containing 25% (w/v) PEG (Mr.1000). (T medium contained 2% (w/v) sucrose, 1 mM K<sub>2</sub>SO<sub>4</sub>, 75 mM CaCl<sub>2</sub>, 35 mM Tris-maleate, pH 8.0 at 20°C, trace elements). Within 30 s of addition of T medium, transformation was terminated by adding 5 ml of P medium. Protoplasts were pelleted, resuspended in 1 ml of P medium and finally spread on ten plates of R2YE agar medium, i.e. 4 x 10<sup>4</sup> protoplasts per plate, (which was the optimal density for protoplast regeneration: Thompson et
al. 1982a). R2YE agar medium contained 10.3% (w/v) sucrose, 1.4 mM 
K₂SO₄, 50 mM MgCl₂, 1% (w/v) glucose, 0.4 mM KH₂PO₄,
20 mM CaCl₂, trace elements, 0.3% (w/v) L-Proline, 0.5% (w/v) yeast 
exttract (Difco), 0.01% (w/v) casamino acids (Difco), 25 mM TES-NaOH, 
pH 7.2, 5 mM NaOH, 2.2% (w/v) agar (Difco). Prior to use, the plates 
were dried in a laminar flow hood for about 4 h, until they had lost 
about 18% of their weight.

After 18 - 20 h incubation at 30°C to allow for expression of the 
thiostrepton-resistance gene, the regeneration plates were overlaid 
with 0.8% (w/v) soft NE agar, containing thiostrepton and tyrosine 
(20 g ml⁻¹ and 0.0075% (w/v) respective final concentrations per 
plate). Thiostrepton selected for primary transformants, (i.e. those 
cells which contained plasmid DNA) and tyrosine induced melanin 
production in cells possessing an intact tyrosinase gene. After a 
further 4 - 5 days incubation at 30°C these colonies had sporulated and 
were replica-plated onto NE plates containing thiostrepton (20 μg ml⁻¹ 
final concentration) and the antibiotic for which resistance 
determinants were being cloned. Colonies growing on these plates were 
potential clones and were maintained on double-drug plates for further 
investigation.

10. Small Scale (Mini-lysate) Preparation of Plasmid.

This method was used for rapid analysis of recombinant plasmids 
present in antibiotic-resistant transformants (i.e. potential clones). 
Plasmids isolated by this procedure were routinely used:
(i)    to retransform S.lividans TK21 protoplasts to check antibiotic 
resistance was mediated by the plasmid.
(ii) to determine the size of the insert, by brief restriction analysis of the plasmid.

The method outlined below, was previously designed for the preparation of plasmids from *Escherichia coli* (Holmes and Quigley, 1981) and has been adapted here for use with *Streptomyces*.

Spore suspensions were prepared from single colonies, and used to inoculate 25 ml YEME medium containing 34% (w/v) sucrose, 5 mM MgCl₂, and 0.5% (w/v) glycine, in 250 ml baffled flasks. Cells were harvested from 40 h cultures by centrifugation at 4,000 rev min⁻¹ for 10 min at 20°C. The mycelia were then washed twice in 10% (v/v) glycerol and resuspended in 400 µl of 50 mM Tris-HCl (pH 8.0 at 20°C) containing 10% (w/v) sucrose. A portion of this cell suspension (400 µl) was incubated for 5 min at room temperature in the presence of lysozyme (2 mg ml⁻¹ final concentration), Na₂EDTA (85 mM final concentration, pH 8.0) was then added and the incubation continued at 37°C for 1 h. Addition of 160 ml 'TIM buffer' (which contained 0.3% (v/v) Triton X-100, 200 mM Na₂EDTA, 150 mM Tris-HCl, pH 8.0 at 20°C) was followed by further incubation at 37°C for 5 min. The temperature was subsequently raised to 65°C for 5 min, at which stage the cells readily lysed.

Chromosomal DNA, together with some protein, was pelleted by centrifugation for 15 min in a bench microfuge, and the remaining protein was removed from 400 µl of the supernatant by extraction with an equal volume of phenol/chloroform (1:1 (v/v)). Plasmid DNA was precipitated from 200 µl of the resulting aqueous phase by addition of 2 volumes of ethanol, and incubation for 30 min in a methanol-CO₂ ice bath, and finally collected by centrifugation. After rinsing the DNA pellet with 70% (v/v) ethanol, it was dried and redissolved in approximately 50 µl TE buffer. Contaminating RNA was removed by
*digestion with RNase A (40\(\mu g\) ml\(^{-1}\), final concentration), during incubation at 37°C for 30 min. Average yields of 20-30 µg plasmid DNA per 25 ml culture were obtained.


Cultures of *Streptomyces* were generally grown with vigorous shaking for 24 - 48 h at 30°C. (All subsequent manipulations were carried out at 4°C). Cells were harvested by centrifugation at 10,000 rev min\(^{-1}\) for 5 min in the Beckman JA-10 rotor and then washed twice by resuspension in and centrifugation from 'high salt' buffer (10 mM Hepes-KOH, pH 7.5 at 20°C, 30 mM MgCl\(_2\), 1 M NH\(_4\)Cl, 3 mM 2-mercaptoethanol). After a further two washes in 'HRS buffer' (10 mM Hepes-KOH, pH 7.5 at 20°C, 10 mM MgCl\(_2\), 50 mM NH\(_4\)Cl, 3 mM 2-mercaptoethanol), mycelia were resuspended in similar buffer at 2 g wet weight ml\(^{-1}\). The cells were broken by passage through a chilled French pressure cell at 70-80 MPa (10,000-12,000 psi) and DNase I added to a final concentration of 5\(\mu g\) ml\(^{-1}\). After pressing the extract a second time, cell wall debris and any unbroken cells were pelleted by centrifugation at 18,000 rev min\(^{-1}\) for 30 min in the Beckman JA-21 rotor. The resulting supernatant was then layered over an equal volume of high salt buffer (see above) containing 20% (w/v) sucrose and salt-washed ribosomes were sedimented by centrifugation at 40,000 rev min\(^{-1}\) (120,000 x g) for 5 h in the Beckman 75Ti rotor. The post-ribosomal supernatant (designated S100*, to indicate that it contained materials released from ribosomes by salt washing), was dialysed against 4 x 100 volumes of HRS buffer. Small aliquots of S100* were frozen rapidly and stored at 70°C. (In general the extract
was frozen and thawed only once).

The salt-washed ribosomes were resuspended in HRS buffer by gentle agitation with a glass rod and subsequently centrifugated through an equal volume of HRS buffer containing 40\% (w/v) sucrose at 40,000 rev min\(^{-1}\) for 5 h in the Beckman 75Ti rotor. After resuspending ribosomes in HRS buffer, the suspension was clarified by centrifugation in a bench microfuge for 1 min. The concentration of ribosomes was determined by measurement of the absorbance at 260 nm, given that 1 A\(_{260}\) unit corresponds to 29.4 pmol 70S ribosomes. Ribosomes were stored as small samples at -70°C, after fast freezing.


The system chosen has been widely used in the study of antibiotic-resistance \textit{in vitro} and involves synthesis of polyphenylalanine, directed by the synthetic messenger, polyuridylic acid (poly U). This assay system only measures the ability of a ribosome to carry out the elongation phase of protein synthesis, since there are no initiation or termination sequences in poly U messenger. In order to allow efficient initiation, protein synthesis is carried out at unphysiologically high magnesium concentrations (15 mM), and consequently, the system cannot be used to study antibiotics whose primary effect is inhibition of the initiation steps of protein synthesis. Despite these caveats, however, all the antibiotics used in the investigations described later, are potent inhibitors of poly U directed cell-free protein synthesis. Furthermore, it is relatively
easy to prepare active systems from a wide variety of organisms and the methods available for the reconstitution of ribosomes (Traub et al., 1971) generate particles that are active in this assay system.

12.2. Polyphenylalanine Synthesis.

Reaction mixtures (50µl) contained ribosomes or their subunits (native or reconstituted) present at 5 pmol each, together with post-ribosomal supernatant (S100*: 10-20% (v/v) final concentration) and 25 µl 'poly U cocktail' (see below). In general, where protein synthesis was resistant to an antibiotic due to a property of the ribosomes, the drug (or HRS buffer in controls) and ribosomes were mixed and incubated at 30°C for 10 min, before adding S100* and poly U cocktail to initiate the reaction. (Poly U cocktail contained 40 mM Hepes-KOH, pH 7.6 at 20°C, 0.75 mM GTP, 5 mM ATP, 10 mM PEP, 19 common amino acids (minus phenylalanine) at 75 µM each, 200 units ml⁻¹ pyruvate kinase, 1 unit ml⁻¹ phenylalanine-specific tRNA from E.coli, 1 mg ml⁻¹ poly U, 5 µl L-[U¹⁴C] phenylalanine (518 mCi mmol⁻¹, 10 µCi ml⁻¹). Solutions of GTP, ATP and PEP were neutralised with KOH before use. The final concentrations of Mg²⁺ and total monovalent cations (NH₄⁺ and K⁺) in the assays, were 15 mM and 125 mM, respectively. Assay mixtures were incubated at 30°C and at various time intervals, 10µl samples were removed into 1 ml of 0.1 M KOH. Samples were then heated for 7 min at 90°C to hydrolyse tRNA, an excess of 10% (w/v) TCA added to each sample, and acid-precipitable material collected on glass fibre discs (Whatman GF/C). After extensive washing with 5% (w/v) TCA, filters were dried under infra-red light and the radioactivity retained was estimated by liquid scintillation spectrometry, in a toluene-based
scintillation fluid (Fisofluor No.3, obtained from Fisons).

To check that preparations of SlO0* were not contaminated with ribosomes (and vice versa), each component was incubated in the absence of the other, in a standard poly U-directed protein synthesis assay. Ribosomal 30S subunits were incubated in the presence of SlO0* only, to check for possible contamination with 50S particles and the reciprocal assay carried out to ensure 50S subunit preparations were clean. In all cases, each preparation gave time-independent, low levels of incorporation (about 200 cpm). In all assays, an incorporation of 1000 cpm represents approximately one residue of [14C] phenylalanine polymerised per ribosome.

The results from studies on the effects of aminoglycosides on poly U-dependent cell-free protein synthesis, were generally represented as dose-response curves, in which antibiotic concentration was plotted against '% activity'. Most protein synthesis assays showed a linear rate of incorporation of [14C]-phenylalanine up to about 30 min incubation and, therefore, to calculate '% activity' values, the linear rates determined from assays carried out in the presence of various concentrations of the relevant aminoglycoside, were expressed as a percentage of the rate in the control (zero drug) assay.

13. Southern Transfer and Hybridisation of DNA.

This method essentially involved separation of DNA fragments according to size (by electrophoresis in an agarose gel), followed by denaturation of the DNA to allow transfer on to a nylon membrane (Gene Screen Plus, obtained from New England Nuclear). After immobilising the DNA on the membrane, it was incubated with 32P-labelled probe DNA
and autoradiography used to locate the position of any DNA fragments on
the membrane which were complementary with the radioactive probe.

This technique was used during the course of this work, for two
reasons:

(i) to check that a particular cloned resistance-gene was indeed
present within the genomic DNA of the producing organism from
which it was presumed to have originated:

(ii) to ascertain whether, in a preliminary investigation, there was
any detectable homology between antibiotic-resistance
determinants that had been cloned from different
aminoglycoside-producing organisms.

13.1. DNA Transfer.

Restriction digests were analysed on a 0.7% (w/v) agarose gel by
electrophoresis under standard conditions (Section 7, this chapter). In
order to visualise a single hybridising band, about 5 μg of genomic
DNA, digested to completion with a restriction enzyme, were loaded on
to a single gel track. When probing plasmid digests, the objective was
to load sufficient DNA such that each track contained 1 ng of the
fragment expected to hybridise with the radioactive probe.

After electrophoresis, the gel was stained with ethidium bromide
(1 μg ml⁻¹), photographed, and any unused areas of the gel trimmed
away. To allow more efficient transfer of large fragments in genomic
digests, the DNA was depurinated by incubation in 100 ml of 0.25 N HCl,
for 15 min at room temperature. The gel was subsequently soaked in 200
ml of 0.4 N NaOH plus 0.6 M NaCl, for 30 min at room temperature with
gentle agitation, to denature the DNA, and then neutralised by replacing the alkali solution with 1.5 M NaCl plus 0.5 M Tris-HCl (pH 7.5 at 20°C) and incubating under similar conditions. Gene Screen Plus membrane was cut to the exact size of the gel, rinsed in distilled water and finally soaked for 15 min at room temperature in 10 x SSC solution (containing 1.5 M NaCl and 0.15 M sodium citrate).

The blotting apparatus consisted of a piece of Whatman 3MM paper (forming the wick) over a glass plate which was elevated above a tray containing about 500 ml of 10 x SSC solution. The gel was placed onto the wick, which had been soaked in 10 x SSC, and then the pre-soaked membrane positioned over the gel. Care was taken to ensure that no air bubbles were trapped between the gel and the membrane. Several pieces of dry 3MM paper, cut to the size of the gel, and then a stack of absorbent paper towels (2-3 in high) were placed on to the membrane. Finally a small weight (500 g) was placed on top of the towels and the transfer of DNA allowed to proceed overnight at room temperature. The objective of this apparatus was to set up a flow of 10 x SSC solution from the tray, through the gel and the membrane so that DNA fragments were eluted from the gel and deposited on the membrane. To prevent 10 x SSC solution being absorbed directly from the wick to the towels, the gel was surrounded by a water-proof border (e.g. Saran Wrap).

When transfer was complete, the towels and filter papers were carefully removed and the positions of the wells of the gels marked on to the membrane. To ensure that immobilised DNA was completely denatured, the membrane was soaked in 100 ml of 0.4N NaOH for 30 sec and then transferred to 100 ml of 0.2M Tris-HCL (pH 7.5 at 20°C) plus 2 x SSC solution (containing 0.3 M NaCl and 0.03 M sodium citrate). Finally, the membrane (with transferred face uppermost) was dried at
room temperature on a piece of filter paper. The filter was stored in a heat-sealed plastic bag at room temperature. (To ensure that transfer of genomic DNA was complete after blotting, the gel was soaked in ethidium bromide (1 μg ml⁻¹) and examined using a U.V. transilluminator).

13.2. Preparation of Radioactive Probe.

Plasmid DNA containing the gene(s) to be used as probe, was purified on a caesium chloride gradient (section 5, this chapter), and cleaved with the appropriate restriction enzyme(s) to generate the required fragment for labelling. These fragments were separated by electrophoresis in a 0.7% (w/v) low gelling temperature agarose gel, which was then stained with ethidium bromide (1 μg ml⁻¹) so that DNA bands could be visualised using a U.V. transilluminator. The required band was carefully excised, with the minimum amount of extraneous agarose and 1.5 ml water added per gram of gel. (By estimating the amount of fragment excised, the approximate concentration of DNA was calculated). The mixture was then incubated at 90°C for 7 min to melt the agarose and denature the DNA, and left at 37°C for 10-60 min prior to initiating the labelling reaction. (DNA was occasionally stored at -20°C, and when required the gel was reboiled for 3 min prior to incubation at 37°C).

The labelling of DNA fragments involved the use of hexadeoxynucleotide primers, which were extended by DNA polymerase I (large fragment) activity, thereby incorporating radioactive nucleotides. The labelling reaction (25 μl total volume) contained reagents added in the following order: 5μl OLB (see below), 1 μl BSA
(enzyme grade, 10 mg ml⁻¹), DNA fragment (25 ng), 2.5 µl ³²P α-dCTP (10 µCi µl⁻¹) and 0.5 µl Klenow (large fragment) DNA polymerase I (2 units). After an overnight incubation at room temperature, the reaction was terminated by the addition of 100 µl 'stop buffer' (containing 20 mM NaCl, 20 mM Tris-HCl, pH 7.5 at 20°C, 2 mM Na₂EDTA, 0.25% (w/v) SDS, 1 µM unlabelled dCTP). (OLB buffer consisted of three solutions A, B and C, mixed in the ratio 10:25:15, respectively. Solution A contained 1.25 M Tris, pH 8.0 at 20°C, 125 mM MgCl₂, 25 µM 2-mercaptoethanol, dATP, dGTP and dTTP each at 0.5 mM. Solution B was 2 M Hepes, triturated to pH 6.6 with 4N NaOH, and solution C contained hexadeoxynucleotides at a concentration of 90 A₂₆₀ units ml⁻¹).

The number of counts incorporated were then determined by transferring a sample (1 µl) of the reaction mixture into 1 ml of 10% (w/v) TCA containing 30 mg salmon sperm DNA. After incubation on ice for 20 min, the DNA was collected by filtration through glass fibre filters (Whatman GF/C) and washed extensively with ice-cold 5% (w/v) TCA. After drying the filters, the radioactivity retained was estimated by liquid scintillation spectrometry.

Unincorporated nucleotides were removed from the labelling reaction using a Sephadex-G50 column. A siliconised gilson tip (1 ml) was plugged with polyallomer wool, and packed with Sephadex-G50 which had been pre-soaked in T'E⁻¹ buffer (containing 3 mM Tris-HCl, pH 7.0 at 20°C, plus 0.2 mM Na₂EDTA). The column was rinsed with two volumes of T'E⁻¹ buffer and allowed to drain. After loading the reaction mixture onto the column it was washed through using T'E⁻¹ buffer and finally collected in 200 µl fractions. Samples (1 µl) of each fraction were then counted in a xylene-based scintillation fluid (Fisofluor No.1 obtained from Fisons). Labelled DNA passed straight through the column.
and was generally present in fraction numbers 2-4, which were subsequently pooled. (The efficiency of labelling was calculated and on average, about $10^8$ cpm per µg of DNA were incorporated).

13.3. Hybridisation of DNA.

The hybridisation of radioactive probe with DNA on the membrane was carried out at a relatively low stringency to allow a high rate of hybridisation and this was subsequently followed by a series of washes of increasing stringency (i.e. at lower ionic strengths).

The membrane was treated with prehybridisation solution containing 1% (w/v) SDS, 1 M NaCl and 10% (w/v) dextran sulphate in a heat-sealed plastic bag and incubated with constant agitation at 65°C for 6 h. Radioactive probe (25 ng) and salmon sperm DNA (1 mg) were denatured by heating at 90°C for 10 min and then quickly cooled on ice. The mixture of denatured DNA was subsequently added to the bag containing prehybridisation buffer and membrane. After careful resealing, the bag was incubated with constant agitation for about 18 h at 65°C to allow hybridisation of the radioactive probe with DNA immobilised on the membrane. (Dextran sulphate was included to increase the rate of hybridisation, possibly due to the promotion of a network formed by overlapping probe sequences). The unlabelled salmon sperm DNA was included in the hybridisation solution to saturate sites on the membrane that bound single or double-stranded DNA non-specifically.

After hybridisation, the membrane was removed from the bag, submerged in 100 ml of 2 x SSC, and incubated with constant agitation at room temperature for 5 min. This washing procedure was then repeated and essentially removed any DNA which was bound
non-specifically. The membrane was then given two further washes in 200 ml of 2 x SSC solution plus 1% (w/v) SDS at 65°C for 30 min. Any probe remaining bound to the filter had at least 63% homology with the immobilised DNA. After drying the membrane at room temperature, this radioactivity was detected by autoradiography at -70°C, using Fuji RX film. A final set of washes was then performed using 0.1 x SSC solution (15 mM NaCl: 1.5 mM sodium citrate) plus 1% (w/v) SDS at 65°C for 30 min. This removed probe that had less than 85% homology with the DNA on the membrane. Radioactivity retained was located by autoradiography, as before. (SDS was included in the washing procedures to aid the removal of any probe which was bound non-specifically).
CHAPTER 3

14. MATERIALS.

14.1. Enzymes.

The following enzymes were obtained from Sigma Chemical Co: pyruvate kinase (E.C. 2.7.1.40) Type III, deoxyribonuclease I (E.C. 3.1.21.1) from bovine pancreas, pancreatic ribonuclease A (E.C. 3.1.27.5) Type 1-A, pronase Type XIV from *Streptomyces griseus*, lysozyme (E.C. 3.2.1.17) Grade I. Calf intestinal alkaline phosphatase (E.C. 3.1.3.1) and micrococcal nuclease (E.C. 3.1.4.7) from *Staphylococcus aureus* were obtained from Boehringer Mannheim.

Restriction endonuclease enzymes and $T_4$ DNA ligase were obtained from BRL and DNA polymerase I was from PL Biochemicals.

14.2. Biochemicals.

The following biochemicals were obtained from Sigma: PEP (trisodium salt), ATP (disodium salt), CTP (sodium salt), GTP (sodium salt), UTP (sodium salt), poly-U (potassium salt), $\gamma$-adenosyl-L-methionine chloride, L-amino acids, 2-mercaptoethanol, HEPES, TES, TEMED, TRIS-HCl. Low melting point agarose and BSA were both from BRL, SDS for use in gels was from Serva Ltd. and Sephadex G-50 was from Pharmacia Fine Chemicals.

14.3. Radiochemicals.

All radiochemicals were obtained from the Radiochemical Centre, Amersham, UK.
14.4. Antibiotics.

The source of antibiotics used in this work were as follows: thiostrepton (Squibb, Princeton, NJ), tobramycin and apramycin sulphate (Lilly, Windlesham, UK), sisomicin sulphate, lividomycin and paromomycin (J. Davies, Biogen SA, Geneva), kanamycin sulphate, neomycin sulphate and gentamicin sulphate (Sigma).

14.5. Other Chemicals.

Standard reagents were of 'AR' grade, or otherwise of the highest purity commercially available.

All dialysis tubing was autoclaved before use in 5% (w/v) NaHCO₃/10 mM Na₂EDTA and washed extensively in sterile water. 'Visking' dialysis tubing (molecular weight cut off: 14,000) was used for dialysis of plasmid solutions, Spectropor No.1 (molecular weight cut off: 6,000) was employed for dialysis of ribosomes or S100* preparations and Spectropor No.3 (molecular weight cut off: 3,500) was used for microdialysis of ribosomal protein preparations.

Deionised water, with a conductivity of about 2µmho, was used for the preparation of all solutions involved in the manipulation of subcellular components.
CHAPTER 4

CLONING OF THE VIOMYCIN-RESISTANCE DETERMINANT FROM "STREPTOMYCES VINACEUS"
CHAPTER 4

1. INTRODUCTION.

Viomycin is a cyclic peptide antibiotic that has been claimed to inhibit the initiation and elongation phases of bacterial protein synthesis (Liou and Tanaka, 1976, Misumi and Tanaka, 1980). However, the organism which produces viomycin, Streptomyces vinaceus, clearly protects itself against its endogenous product, since it is highly resistant to this antibiotic. When the mechanism(s) of self-defence in S. vinaceus were investigated, although ribosomes from the producer were shown to be sensitive to viomycin, the organism contained an enzyme which could inactivate the drug in vitro by phosphorylation (Skinner and Cundliffe, 1980). However, these results did not reveal whether the phosphotransferase enzyme contributed to the mechanism of resistance in vivo. Evidence in support of this was provided when the viomycin-resistance determinant was transferred from S. vinaceus into S. lividans, using plasmid SLP1.2 as vector (Thompson et al., 1982a). Several clones were obtained which were highly resistant to viomycin and extracts of these strains were shown to contain viomycin phosphotransferase activity (Thompson et al., 1982b). Therefore, it appears that this antibiotic-modifying enzyme does indeed play a role in vivo, in rendering S. vinaceus resistant to viomycin. The recombinant plasmids that were isolated from these clones contained the viomycin resistance gene (vph) on a 1.9kb Bam HI fragment of S. vinaceus DNA. This fragment was subsequently transferred into pBR322, to facilitate further restriction mapping and subcloning (Thompson et al., 1982c). Finally, the vph gene was subcloned into pIJ702 on a Sph I fragment, of about 1 kb in length (Katz et al., 1983). This suggested that all of the essential coding sequences of the viomycin-resistance
determinant were located within this fragment of *S. vinaceus* DNA.

Before attempting to clone aminoglycoside-resistance determinants from the relevant producing organisms, several preliminary experiments were performed to ensure that the required transformation efficiencies could be achieved. To do this, attempts were made to clone the *Sph I* fragment encoding the *vph* gene, from *S. vinaceus* into the host strain, *Streptomyces lividans* TK21, using vector pIJ702. Since this resistance determinant had already been isolated and shown to be stable on the high copy number plasmid, pIJ702 (Katz *et al.*, 1983), any difficulties encountered during the cloning experiment attempted here, could thus be attributed to purely technical problems.
CHAPTER 4

2. METHODS.

2.1. Preparation of Cell Extracts from Streptomyces lividans TK21 for Coupled Transcription-Translation.

A coupled transcription-translation system has been developed recently using extracts of S. lividans TK21 (Thompson et al., 1984) and was used here for analysis of the products of cloned genes.

Spores of S. lividans TK21 were used to inoculate 6 x 11 YEME medium containing 0.5% (w/v) PEG (Mr.6000) plus 5 mM MgCl₂. The cultures were grown with vigorous shaking, for 16 h at 30°C and then harvested by centrifugation at 10,000 rev min⁻¹ for 10 min at 4°C in the Beckman JA-10 rotor. Cells were washed twice in buffer containing 10 mM Hepes-KOH (pH 7.5 at 20°C), 10 mM MgCl₂, 1M KCl, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol and then given two further washes in 'S30 buffer' (50 mM Hepes-KOH, pH 7.5 at 20°C: 10 mM MgCl₂, 60 mM NH₄Cl: 10% (v/v) glycerol: 5 mM 2-mercaptoethanol). Mycelia were finally collected by filtration through Whatman No.1 paper in a Buchner funnel and resuspended in S30 buffer using 2.5 ml buffer per gram wet weight. Cells were broken by passage through a chilled French pressure cell at 70-80 MPa (10,000-12,000 psi) and the cell extract centrifuged at 15,000 rev min⁻¹ for 30 min at 4°C in the Beckman SW27 rotor. The supernatant was removed and recentrifuged under similar conditions. The resultant supernatant, designated 'S30', had an absorbance of about 1 A₂₆₀ unit per 5 μl, and was subsequently assayed for activity in the coupled transcription-translation system (see below). However, before the extract could be used to analyse protein products of cloned genes, it was necessary to reduce plasmid-independent background activity.

-65-
CHAPTER 4

This was achieved using micrococcal nuclease, a Ca^{2+}-dependent endonuclease which degrades both DNA and RNA.

To establish the conditions for nuclease treatment, a small portion of fresh extract (20 A_{260} units) was treated with 75 units micrococcal nuclease (made up in 50 mM glycine-KOH, pH 9.2 at 20°C, 5 mM CaCl_2) in the presence of 1 mM CaCl_2 (final concentration). After incubation at 30°C for varying times, samples were removed and the nuclease silenced by addition of 2 mM EGTA-KOH, pH 7.0 (final concentration). By assaying the activity of these nuclease-treated extracts in coupled transcription-translation, in the absence of plasmid, the minimal treatment required to eliminate the background activity was established. The bulk of the S30 extract, previously stored at 0°C, was subsequently treated with nuclease under the appropriate conditions and stored at -70°C as small samples.

2.2. Assay of Coupled Transcription-Translation Activity.

The activity of the coupled transcription-translation system was followed by the incorporation of [^{35}S]-methionine into hot TCA-precipitable material. Assays performed for subsequent analysis of protein products on gels, included [^{35}S]-methionine (specific activity 30 TBq mmol^{-1}: 800 Ci mmol^{-1}, from Amersham International). However, when following the incorporation of radioactivity into proteins (directed by an E.coli plasmid, pBR322) as a measure of the activity of the system, the radiolabelled material was diluted 100 fold with unlabelled methionine. Incubations were carried out at 30°C in 30 µl S30 buffer, except that the final concentration of MgCl_2 was adjusted to 12 mM. Each assay contained 2 A_{260} units of S30 extract, 2µg
plasmid DNA (added last), 8 μl synthesis mix (see below) and 2 μl
['S]-methionine. Synthesis mix contained 200 mM Hepes-KOH, (pH 8.2 at
20°C) 48 mM ammonium acetate; 95 mM potassium acetate, 7 mM
dithiothreitol, 5 mM ATP, 3.4 mM (each) GTP, CTP, UTP, 100 mM PEP, 19
amino acids (zero methionine) each at 1.4 mM, 7.5% (w/v) PEG (Mr.6000),
folinic acid, CaCl₂ salt 0.14 mg ml⁻¹ (final concentration). Stock
solutions containing nucleotides or PEP were adjusted to pH 7.0 using
KOH or acetic acid respectively. The mix was stored at -70°C as small
aliquots.

2.3. Analysis of Protein Products on Polyacrylamide Gels.

After incubation of assay mixtures for 20 min, small samples (2μl)
were removed for estimation of hot TCA-precipitable material.
Unlabelled methionine (250 fold excess over ['S]-methionine) was then
added to the remainder of the assay mixtures and incubation continued
for a further 10 min to allow completion of all radiolabelled
polypeptides. Samples for gel analysis containing 100,000-200,000 cpm
were mixed with one-third volume 'SDS-buffer' and incubated at 100°C
for 10 min. (SDS buffer contained 375 mM Tris-HCl, pH 8.8 at 25°C, 4%
(w/v) SDS, 35% (v/v) glycerol, 2.86 M 2-mercaptoethanol, 0.01% (w/v)
bromophenol blue). Radiolabelled proteins for use as molecular weight
markers (obtained from Amersham International), together with the
samples, were loaded onto 10% (w/v) polyacrylamide gels (14.5 cm x 10
cm x 1.5 mm), containing 0.1% (w/v) SDS, prepared according to a
standard method (Laemmli, 1970). Essentially, the lower (separating)
gel contained 10% (w/v) acrylamide 0.3% (w/v) methylene bisacrylamide,
0.1% (w/v) SDS plus 375 mM Tris-HCl, pH 8.8 at 25°C. The upper
(stacking) gel contained 3% (w/v) acrylamide, 0.08% (w/v)
bisacrylamide, 0.1% (w/v) SDS, 0.06% (w/v) ammonium persulphate, 0.2% (v/v) TEMED plus 125 mM Tris-HCl, pH 6.8 at 25°C. After electrophoresis for 4-5 h at a constant current of 20 mA, gels were fixed using 30% (v/v) methanol, 10% (w/v) TCA and 10% (v/v) acetic acid, then treated with En3Hance (New England Nuclear) and finally dried on to Whatman 3MM chromatography paper. Protein products were subsequently visualised after fluorography using Fuji RX film at -70°C for 24-48 h.
CHAPTER 4

Table 4.1

Efficiency of Transformation of *S. lividans* TK21 Protoplasts Using Vector pIJ702.

<table>
<thead>
<tr>
<th>Protoplasts spread per plate (a)</th>
<th>Primary Transformants per plate (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncut (native) plasmid (100 ng)</td>
<td>$4 \times 10^8$</td>
</tr>
<tr>
<td>Religated (open circular)</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>plasmid (100 ng)</td>
<td>$4 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$</td>
</tr>
</tbody>
</table>

(a) A density of $4 \times 10^8$ protoplasts per plate allows optimal regeneration efficiency (Thompson *et al.*, 1982a).

(b) Numbers were calculated from serial dilutions of transformed protoplasts, which had been spread on regeneration plates and overlaid with soft agar containing thiostrepton (20 μg ml$^{-1}$, final concentration).
3. RESULTS AND DISCUSSION.

3.1. Construction of Viomycin-Resistant Clones.

The cloning strategy used to isolate the viomycin-resistance determinant involved complete digestion of *S. vinaceus* total DNA with *Sph I* and ligation of these restriction fragments into the *Sph I* site of plasmid pIJ702 (Fig. 2.8). The ligation mixture, containing 0.5 µg pIJ702 and 2.5 µg *S. vinaceus* DNA, was subsequently introduced into *S. lividans* TK21 protoplasts. Primary transformants (i.e. those containing plasmid DNA), were selected by overlaying the protoplasts with thiostrepton, and confluent growth was observed on all the regeneration plates. After these colonies had sporulated, they were replica-plated onto agar containing thiostrepton plus viomycin (20 µg ml\(^{-1}\) and 50 µg ml\(^{-1}\) final concentrations, respectively, and a total of 12 viomycin-resistant isolates were obtained. As expected, these potential clones were all non-pigmented, since the ligation of foreign DNA into the *Sph I* site in pIJ702 usually results in insertional inactivation of the tyrosinase gene and, therefore, a loss of melanin production.

To estimate the efficiency of transformation, protoplasts of *S. lividans* TK21 were transformed with native pIJ702 and also with plasmid that had been linearised with *Sph I* and subsequently religated. The results (Table 4.1) showed that the frequency of transformation with religated plasmid was about 100 fold less than that with uncut pIJ702. This was probably due to inefficiencies in both the recircularisation of the plasmid during ligation (linear plasmid DNA being less efficient in transformation than religated circular DNA).
and the topological problems associated with the introduction of relaxed circular molecules into protoplasts. Nevertheless, these figures were compatible with those obtained using plasmid SLP1.2 (Thompson et al., 1982a).

As a useful guideline for future cloning experiments, it is possible to predict the number of primary transformants required from a shotgun ligation, to contain a statistically representative population of an entire streptomycete genome. The genome from these organisms is about 9 x 10^9 kb in length (Benigni et al., 1975) and is composed of just over 1,000 Sph I fragments, averaging about 8kb in length. Previous studies indicated that under the ligation conditions used above, at least 10% of the vector molecules should contain inserted foreign DNA after transformation (Thompson et al., 1982a). One clone in about 10^4 transformants could, therefore, be expected. Although it was impossible to determine the exact yield of primary transformants obtained in the cloning experiment carried out here to isolate the vph gene, the number of viomycin-resistant clones obtained appeared to be within these predictions. When plasmid DNA was isolated from the presumed clones and used to retransform S. lividans protoplasts, all the transformants obtained were viomycin resistant, confirming that resistance to this antibiotic was plasmid-mediated. Preliminary restriction mapping of the recombinant plasmid, identified a Sph I fragment of about 1 kb in length, inserted into the tyrosinase gene in pIJ702. Furthermore, the inserted DNA contained a central Sst I restriction site, as predicted from the restriction map of the vph gene (Thompson et al., 1982c). One of these viomycin-resistant clones, designated S. lividans TSK12, contained the recombinant plasmid pLST12, and was chosen for further investigation. A restriction map of pLST12
CHAPTER 4

and a photograph of an agarose gel containing restriction digests of this plasmid are shown in Figs. 4.1 and 4.2, respectively.

3.2. Biochemical Analysis of a Viomycin-Resistant Clone.

To determine whether the viomycin-resistance observed in vivo in S. lividans TSK12 was reflected in the properties of cell-free protein synthesising systems, ribosomes and post-ribosomal supernatant (S100*) were prepared from S. lividans TSK12 and from the parental strain (S. lividans TSK1, which is S. lividans TK21 containing native vector pIJ702). For details on the preparation of ribosomes and S100*, refer to Chapter 3, page 51 et seq. These components were then combined in all four possible modes and assayed for their response to viomycin in poly U-directed protein synthesising systems. In these assays, viomycin (or HRS buffer in controls) and S100* were incubated in the presence of 5 mM ATP (final concentration) as cofactor for any phosphotransferase enzymes, prior to initiating protein synthesis (see Chapter 3, page 53 et seq., for details). The results are summarised in Fig. 4.3. Resistance to viomycin was observed in assays containing ribosomes and S100* from S. lividans TSK12, confirming that the resistance phenotype of strain TSK12 was reflected in vitro. Moreover, the results also showed that resistance was determined exclusively by the S100* preparation from S. lividans TSK12, and suggested that the mechanism of resistance operating in this strain might involve enzymic-inactivation of viomycin. In conclusion, the data presented so far in this chapter strongly implied that the vph gene present in S. vinaceus had been cloned successfully in S. lividans, using vector pIJ702. On that basis, it appeared that the transformation
Legend to Fig. 4.1.

Restriction Map of pLST12.

The plasmid was derived from pIJ702 (see Fig. 2.8) by insertion of a Sph I fragment of DNA from *S. vinaceus*, encoding the viomycin-resistance (*vph*) gene, into the tyrosinase (*mel*) gene. The recombinant plasmid also contained the thiostrepton-resistance (*tsr*) determinant from *S. azureus*.
Fig 4.1

pLST12
6.7kb
Legend to Figure 4.2.

Restriction Analysis of pLST12 and pIJ702.

DNA restriction digests were electrophoresed in a 0.7% (w/v) agarose gel. The gel tracks contain Sph I digests of (a) pIJ702 and (b) pLST12 and 
Pci I digests of (d) pLST12 and (e) pIJ702. Track (c) contains size markers (Hind III digest of bacteriophage lambda DNA: 23.5, 9.6, 6.8, 4.5, 2.3, 1.95 kb).
Legend to Figure 4.3

Post-Ribosomal Supernatant (S100*) from \textit{S. lividans} TSK12 Determines Viomycin Resistance.

Polyphenylalanine synthesis assays contained various combinations of ribosomes and S100* prepared from a viomycin-resistant clone, \textit{S. lividans} TSK12, and the parental strain, \textit{S. lividans} TSK1. (●) control: (○) 1 μg viomycin ml^{-1}; (A) Ribosomes and S100* from \textit{S. lividans} TSK12, (B) Ribosomes from \textit{S. lividans} TSK1 and S100* from \textit{S. lividans} TSK12, (C) Ribosomes and S100* from \textit{S. lividans} TSK1, (D) Ribosomes from \textit{S. lividans} TSK12 and S100* from \textit{S. lividans} TSK1.
efficiencies required for successful shotgun cloning experiments in *Streptomyces*, had been achieved.

3.3. Analysis of the Product(s) of the Viomycin-Resistance Determinant.

Whilst the investigations described above were in progress, the nucleotide sequence of the *vph* gene had been determined (Bibb et al., 1985). The sequence data revealed that the *Sph* I fragment (equivalent to that cloned in pLST12), contained a single open reading frame of 861 nucleotides, that could encode a protein of molecular weight 30,500 daltons. As confirmation of this prediction, the protein product of the gene cloned on pLST12 was analysed using an in vitro coupled transcription-translation system from *S. lividans* TK21 (Thompson et al., 1984). A cell-free extract was prepared from *S. lividans* TK21 and treated with micrococcal nuclease to remove plasmid-independent background activity, (see methods section, page 65 et seq.). This extract was used to synthesise proteins both from intact and linearised forms of plasmids pLST12 and pIJ702. The restriction enzyme, *Cla* I, was used to cleave both plasmids in the thiostrepton-resistance gene (*tsr*), which is known to encode a protein of slightly less than 30,000 daltons (Thompson et al., 1984). When the protein products were analysed on a 10% SDS-polyacrylamide gel (see Fig.4.4), pLST12 was shown to yield a protein of molecular weight slightly greater than 30,000 daltons, in agreement with the predicted size of the *vph* gene product. (This result has since been confirmed by in vitro coupled transcription-translation of pBR322 derivatives carrying the *vph* gene: Mervyn J.Bibb, unpublished results).

During the preparation of the products of the *tsr* and *vph* genes,
Legend to Figure 4.4.

Electrophoretic analysis of the products of coupled transcription-translation directed by pIJ702 and a recombinant derivative, pLIST12, which contains the viomycin-resistance determinant.

Products were subjected to electrophoresis on SDS-polyacrylamide gels, together with $[^{14}C]$ proteins as molecular weight markers (track a). Proteins were synthesised from DNA as follows: uncut (b) and linearised (c) forms of pLIST12, uncut (d) and linearised (e) forms of pIJ702, background activity i.e. zero DNA (f). (Plasmids were linearised using Cla I, resulting in the loss of the tsr gene product, of about 29,000 daltons.)
Fig 4.4

- pLST12 uncut
- pLST12 + ClaI
- plJ702 uncut
- plJ702 + ClaI
- zero DNA

Mr x10^{-3}

200 92 69 46 30

a b c d e f
protein synthesis was monitored by the incorporation of \(^{35}\)S-methionine into TCA-precipitable material. From sequence analysis (Bibb et al., 1985), it was clear that the \(vph\) gene encoded only half the number of methionine residues compared with the \(tsr\) gene. This is one possible explanation for the intriguing difference in the intensities of the protein bands relating to the products of the \(tsr\) and \(vph\) genes (see Fig. 4.4). Another, perhaps more likely, explanation is that the resistance genes were being expressed with different efficiencies. Gene expression can be controlled either at the level of transcription, or at the level of translation.

Considering the latter possibility, translation can be regulated by the binding affinity of ribosomes for available mRNA. In \(E. coli\) and \(B. subtilis\), ribosome binding sites within mRNA are characterised by a degree of complementarity to a region near the 3' end of 16S rRNA (Shine and Dalgarno, 1974). In this context, it was interesting to note that when the nucleotide sequences preceding the two resistance determinants were compared, the \(vph\) gene had a sequence of potentially stronger interaction with 16S rRNA from \(S. lividans\) than did the \(tsr\) gene. This evidence is, therefore, contrary to the hypothesis that the \(tsr\) gene is expressed more efficiently than the \(vph\) gene and suggested that the regulation of expression of these genes might be controlled by other factors, possibly at the level of transcription.

The most feasible hypothesis for the apparent differences in expression of the two resistance determinants, involves regulation at the transcriptional level and is supported by recent reports on the location of the promoter sequences of the \(tsr\) and \(vph\) genes (Bibb et al., 1985). Attempts to isolate these sequences were carried out using promoter-probe plasmid vectors. The promoter sequence upstream from
CHAPTER 4

the translational start of the \textit{tsr} gene was located within the \textit{Bcl I} fragment present in plasmids pIJ702 and pLST12. However, although the essential coding sequence of the \textit{vph} gene had been shown to lie within the \textit{Sph I} fragment cloned in pLST12 (Katz et al., 1983), it was clear that some, if not all, of the transcription of this resistance determinant originated at a point outside this region of \textit{S.vinaceus} DNA. Expression of the viomycin resistance gene in pLST12, therefore, might rely, at least partially, on transcriptional read-through from promoters on vector pIJ702, whereas the \textit{tsr} gene could be transcribed from its own promoter. Hence, this hypothesis might explain why the \textit{vph} gene product was apparently produced in such small amounts compared with the protein encoded by the \textit{tsr} gene. It would be interesting to determine the orientation of the viomycin-resistance determinant in pLST12 and from the direction of transcription of the \textit{vph} gene (see Bibb et al., 1985), to ascertain which region of pIJ702 might be promoting transcription of this gene.

In conclusion, the viomycin-resistance gene from \textit{S.vinaceus} has been cloned successfully in \textit{S.lividans}, using vector pIJ702. Analysis of the viomycin-resistant clone, \textit{S.lividans} TSK12, strongly implied that it contained the \textit{vph} gene which had been cloned and characterised previously (Thompson et al., 1982a,b,c). Furthermore, using an \textit{in vitro} coupled transcription-translation system from \textit{S.lividans}, the \textit{vph} gene was shown to encode a protein with an apparent molecular weight of about 30,000 daltons, in agreement with the size predicted from the nucleotide sequence of the gene (Bibb et al., 1985). In view of the data presented in this chapter, it seemed feasible to attempt novel shotgun cloning experiments, designed to isolate antibiotic-resistance determinants from aminoglycoside-producing organisms.
CHAPTER 5

CONSTRUCTION OF AMINOGLYCOSIDE-RESISTANT CLONES OF STREPTOMYCES LIVIDANS.
1. INTRODUCTION.

*Streptomyces tenkimariensis*, *Streptomyces tenebrarius* and *Micromonospora purpurea* are all resistant to their own aminoglycoside antibiotic products: istamycin, nebramycin complex and gentamicin C complex, respectively (Yamamoto *et al.*, 1981b, 1982; Piendl and Böck, 1982). These producing organisms are also insensitive to a variety of other aminoglycosides, although they possess different phenotypic patterns of resistance. In particular, *S. tenkimariensis* is resistant to kanamycin but sensitive to gentamicin, whereas *S. tenebrarius* and *M. purpurea* are refractory to both of these aminoglycosides (Yamamoto *et al.*, 1982; Piendl and Böck, 1982). This chapter describes how recombinant DNA technology has been used to isolate in *Streptomyces lividans*, the genes that are responsible for aminoglycoside resistance in these producing organisms.
2. METHODS.

2.1. Size-Fractionation of Genomic DNA from *S. tenimariensis* and *S. tenebrarius*.

Partial restriction digests of genomic DNA were size-fractionated to isolate 4-10kb fragments, in an attempt to increase the likelihood of generating antibiotic-resistant clones. Size-fractionation removes small pieces of DNA, which might have preferentially religated with the vector plasmid, but may not have been large enough to encode the resistance determinant(s).

The conditions for partial digestion of total genomic DNA with *Sau* 3A, were adjusted to give the optimal yield of fragments in the 4-10kb range, as determined by agarose gel electrophoresis. The size-fractionation was carried out using 10-40% (w/v) sucrose-density gradients (17 ml each), made up in buffer containing 10 mM Tris-HCl, pH 8.0 at 20°C, 10 mM Na₂EDTA; 200 mM sodium acetate. Approximately 100 µg of partially digested DNA was loaded on each gradient and centrifugation was for 42 h at 24,000 rev min⁻¹ in the Beckman SW27 rotor at 4°C. Fractions of about 1 ml each, were collected dropwise and 10µl samples from alternate fractions were analysed by electrophoresis in 0.7% (w/v) agarose gels, together with size markers (Fig. 5.1). Fractions containing 4-10kb DNA fragments were pooled and the DNA was precipitated with ethanol, dried and redissolved in TE buffer. About 20 µg of size-fractionated DNA was obtained from each gradient. These genomic fragments were used in subsequent religation reactions (see Chapter 3, page 45).
Legend to Figure 5.1

Size-Fractionation of Genomic DNA.

Partial *Sau* 3A digests of genomic DNA were size-fractionated using 10-40% (w/v) sucrose gradients (see Methods Section for details). Samples of gradient fractions were analysed by electrophoresis in a 0.7% (w/v) agarose gel, (see tracks b-k). Track (a) contains size markers (Hind III digest of bacteriophage lambda DNA: 23.5, 9.6, 6.8, 4.5, 2.3, 1.95kb).
2.2. Phosphatase Treatment of Vector DNA.

In some shotgun cloning experiments, after vector pIJ702 had been linearised with the appropriate restriction enzyme, it was treated with calf intestinal alkaline phosphatase (CIAP). The latter enzyme removes terminal phosphate groups, thus preventing religation of native vector molecules. After incubation of plasmid DNA with CIAP for 1h at 37°C (1 unit of CIAP was sufficient to treat several μg of DNA), the enzyme was inactivated by incubation at 75°C for 15 min, in the presence of 0.1% (w/v) SDS, 10 mM Tris-HCl (pH 8.0 at 20°C), 100 mM NaCl, 1 mM Na₂EDTA, final concentrations. Phosphatase was then removed by phenol extraction and the DNA precipitated and finally redissolved in TE buffer. The CIAP-treated vector was mixed with genomic DNA fragments and religated using standard procedures (as described in Chapter 3, page 45). (A small sample of the CIAP-treated vector was also taken through the religation reaction and subsequently analysed on an agarose gel, to check that the DNA remained in the linear form, i.e. that the phosphatase treatment had worked efficiently).
CHAPTER 5

3. RESULTS.

3.1. Cloning of Aminoglycoside-Resistance Determinant(s) from *S. tenjimariensis*.

The following shotgun cloning experiment was carried out to isolate the kanamycin-resistance determinant(s) from *S. tenjimariensis*. Genomic DNA from *S. tenjimariensis* was partially digested with Sau 3A, and the products size-fractionated on a sucrose gradient to isolate 4-10kb DNA fragments (see this Chapter, page 76, for details). These genomic fragments were ligated with vector pIJ702 that had been linearised using *Bgl* II, and the DNA mixture introduced into protoplasts of *S. lividans* TK21 (see Chapter 3, page 48 et seq., for details on preparation, transformation and regeneration of protoplasts). When the resultant primary transformants were replica-plated onto agar containing thiostrepton and kanamycin (20 µg ml\(^{-1}\) and 50 µg ml\(^{-1}\), final concentrations, respectively), three antibiotic-resistant colonies were selected. To ensure that kanamycin resistance was plasmid-mediated in these strains, plasmid preparations were made from each and used to transform *S. lividans* TK21 protoplasts. In each case, resistance to thiostrepton and kanamycin was acquired concurrently, confirming that the plasmids did indeed confer aminoglycoside resistance. Following restriction analysis, the kanamycin-resistant clone containing the smallest recombinant plasmid (pLST41: 9.2kb), was chosen for further investigation and was designated *S. lividans* TKS41. Plasmid pLST41 contained about 3.5kb of *S. tenjimariensis* DNA inserted into the *Bgl* II site of pIJ702 (see Fig.5.2 for a preliminary restriction map).
Legend to Fig. 5.2.

Preliminary Restriction Map of pLST41.

This recombinant plasmid is a derivative of pIJ702 (see Fig. 2.8) which contains the kanamycin-apramycin resistance (kan-apr) determinant from S.tenjimariensis. Plasmid pLST41 also contains the tyrosinase (mel) gene from S.antibioticus and the thiostrepton-resistance (tsr) gene from S.azureus.
Fig 5.2

pLST41
9.2kb

kan-apr
Bgl II/Sau 3A
mel
Sph I
Bcl I
Bgl II/Sau 3A
tsr
Bcl I
mel
### Table 5.1

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>S. lividans</em> TSK1 (parental)</th>
<th><em>S. lividans</em> TSK41 or TSK412 (clones)</th>
<th><em>S. tenjimariensis</em> TSK51 (clone)</th>
<th><em>S. lividans</em> TSK31 (clone)</th>
<th><em>S. tenebrarius</em> JR14 (clone)</th>
</tr>
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<td>100-300</td>
<td>300-1000</td>
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<td>5-10</td>
<td>10-30</td>
<td>1000</td>
</tr>
<tr>
<td>Sisomicin</td>
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<td>100-300</td>
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<td>Neomycin</td>
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<td>10-30</td>
<td>10-30</td>
<td>1-3</td>
<td>1000</td>
</tr>
<tr>
<td>Lividomycin</td>
<td>30-100</td>
<td>30-100</td>
<td>1-3</td>
<td>30-100</td>
<td>1000</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>3-10</td>
<td>10-30</td>
<td>1-3</td>
<td>10-30</td>
<td>30-100</td>
</tr>
</tbody>
</table>

Minimal Inhibitory Concentration of Aminoglycosides for *S. tenjimariensis*, *S. tenebrarius*, and *S. lividans* strains (μg ml⁻¹).
3.2. Cloning of Aminoglycoside-Resistance Determinant(s) from *S. tenebrarius*.

A similar series of experiments was carried out to isolate the gentamicin-resistance gene(s) from *S. tenebrarius*. Genomic fragments of *S. tenebrarius* DNA were size-fractionated, religated with vector pIJ702 and introduced into protoplasts of *S. lividans* TK21. Several gentamicin-resistant colonies were selected on plates containing thiostrepton plus gentamicin (20 \( \mu \text{g ml}^{-1} \) and 50 \( \mu \text{g ml}^{-1} \) final concentrations, respectively) and each of these strains contained a recombinant plasmid capable of conferring gentamicin resistance when transformed into *S. lividans* TK21. This confirmed that the aminoglycoside-resistance phenotype was indeed plasmid-dependent. Further investigations were carried out with one of these clones, designated *S. lividans* TSK31. This strain contained plasmid pLST31 (approximately 8.7kb), in which about 3kb of *S. tenebrarius* DNA had been inserted into the *Bgl* II site of vector pIJ702 (see Fig. 5.3 for restriction map).

The MIC values of various aminoglycosides for *S. lividans* strains TSK31 and TSK41 were determined and compared with those for the parental strain (*S. lividans* TSK1) and the corresponding aminoglycoside-producing organisms (see Table 5.1). It was interesting to observe that the resistance profiles of strain TSK41 and *S. tenjimariensis* were similar, and, on that basis, it was possible that all the aminoglycoside-resistance determinants present in this producer had been cloned in *S. lividans* TSK41. However, it is important to note that the antibiotic produced by *S. tenjimariensis*, istamycin, was not included in these investigations, since it was unavailable and,
Legend to Fig. 5.3.

Preliminary Restriction Map of pLST31.

This recombinant plasmid is a derivative of pIJ702 (see Fig. 2.8) which contains the kanamycin–gentamicin resistance (kan, gen) determinant(s) from *S. tenebrarius*. Plasmid pLST31 also contains the tyrosinase (mel) gene from *S. antibioticus* and the thiostrepton-resistance (tsr) gene from *S. azureus*. 
**Fig 5.3**

Diagram of the pLST31 plasmid:
- **kan-gen**
- **mel**
- **tsr**

Enzyme sites:
- **Bcl I**
- **Bgl II/Sau 3A**

Size: 8.7 kb
therefore, it is not yet clear whether strain TSK41 is also resistant to this aminoglycoside. Nevertheless, it was obvious that the determinant(s) of resistance to several aminoglycosides had been cloned in S. lividans TSK41. Since this strain had been selected on kanamycin, it was not too surprising to observe collateral resistance to tobramycin (another member of the kanamycin family). However, it was interesting that strain TSK41 resembled S. tenjimariensis in being highly resistant to the novel aminoglycoside, apramycin, and also to sisomicin, but not to the gentamicin C complex (see also Piendl et al., 1984).

A comparison of the MIC data for strain TSK31 and for S. tenebrarius revealed that the patterns of resistance were different (see Table 5.1). Although strain TSK31 had been selected on gentamicin, it proved to be cross-resistant to another antibiotic of the gentamicin family, sisomicin, as well as to tobramycin (one of the antibiotic products of S. tenebrarius) and to kanamycin. However, unlike strain TSK31, S. tenebrarius was also resistant to high levels of apramycin (another of the sub-components of the nebramycin complex) and to neomycin and the related antibiotics, paromomycin and lividomycin. Therefore, another shotgun cloning experiment was carried out, in an attempt to isolate the determinant(s) in S. tenebrarius responsible for resistance to apramycin and neomycin-like antibiotics.

Partial Sau 3A digests of S. tenebrarius genomic DNA were ligated with vector pIJ702 which had been cleaved with Bgl II and treated with CIAP to prevent religation of native plasmid molecules. Protoplasts of S. lividans TK21 were transformed with the ligation mixture and the thiostrepton-resistant primary transformants were replica-plated on to agar containing thiostrepton plus apramycin (20 µg ml⁻¹ and 50 µg ml⁻¹
final concentrations, respectively). Six apramycin-resistant strains were obtained, and each of them contained a recombinant plasmid which was capable of conferring resistance to this aminoglycoside when introduced into \textit{S.\lividans} TK21. One of these clones, designated \textit{S.\lividans} TSK51 was chosen for further study. It contained plasmid pLST51, in which about 4kb of DNA had been inserted into the \textit{Bgl} II site of pLJ702 (see Fig.5.4 for restriction map).

The primary transformants (initially selected on thiostreppton plus apramycin), were also replica-plated onto agar containing thiostrepton plus neomycin (20 \( \mu \text{g ml}^{-1} \) and 50 \( \mu \text{g ml}^{-1} \) final concentrations, respectively), however, no colonies with high-level resistance to neomycin were obtained.

When MIC determinations were carried out with \textit{S.\lividans} TSK51, it was interesting to observe that this strain was not only resistant to high levels of apramycin, but was also cross-resistant to kanamycin, tobramycin and sisomicin (see Table 5.1). Also, unlike strain TSK31, \textit{S.\lividans} TSK51 proved to be relatively sensitive to gentamicin. In conclusion, it appears that the gene(s) responsible for high-level resistance to gentamicin in \textit{S.tenebrarius}, had been cloned separately from that (those) conferring apramycin resistance. Therefore, this producer must possess at least two independent aminoglycoside-resistance determinants.

3.3. Cloning of Aminoglycoside-Resistance Determinant(s) from \textit{M.purpurea}.

The gentamicin-resistance gene(s) in \textit{M.purpurea} were isolated in \textit{S.lividans} following a shotgun cloning experiment similar to those
Legend to Fig. 5.4.

Preliminary Restriction Map of pLST51.

This recombinant plasmid is a derivative of pIJ702 (see Fig. 2.8) which contains the kanamycin-apramycin resistance (kan-apr) determinant(s) from *S. tenebrarius*. Plasmid pLST51 also contains the tyrosinase (*mel*) gene from *S. antibioticus* and the thiostrepton-resistance (*tsr*) gene from *S. azureus*. 
Fig 5-4

pLST51 9.7kb

Bgl II/Sau3A
Bcl I
mel
tsr
kan-apr
Bgl II/Sau3A
Bcl I
described in the previous section. These studies were carried out in this laboratory by Dr. Jill Thompson, who has kindly given permission for the results to be outlined in this dissertation (see Thompson et al., 1985, for further details).

When fragments of genomic DNA from *M. purpurea* were cloned into *S. lividans* TK21, using vector pIJ702, several gentamicin-resistant strains were produced. One of these, designated *S. lividans* JR14, was chosen for further analysis and contained a recombinant plasmid (pLST14: 8.7 kb), in which about 3 kb of *M. purpurea* DNA had been inserted into the *Sph I* site of pIJ702 (see Fig. 5.5 for restriction map). Although strain JR14 had been selected on gentamicin, it proved to be cross-resistant to kanamycin and sisomicin, but sensitive to apramycin (see Table 5.1). This multiple-resistance pattern was mirrored by *M. purpurea* (data not shown, see Thompson et al., 1985) and, therefore, suggested that perhaps all the aminoglycoside resistance determinants from the producing organism had been cloned in *S. lividans* JR14. (All the experiments described in the above paragraph were carried out by Dr. Jill Thompson).
Legend to Fig. 5.5.

Preliminary Restriction Map of pLST14.

This recombinant plasmid is a derivative of pIJ702 (see Fig. 2.8) which contains the kanamycin-gentamicin resistance (kan-gen) determinant(s) from *M. purpurea*. Plasmid pLST14 also contains the tyrosinase (*mel*) gene from *S. antibioticus* and the thiostrepton-resistance (*tsr*) gene from *S. azureus*.

The DNA probe that was used in the hybridisation experiments referred to in Chapter 8 (page 130 *et seq.*) is represented by the Sph I/Bgl II fragment of *M. purpurea* DNA (indicated by asterisks).
4. DISCUSSION.

The results presented in this chapter demonstrated that the determinants of resistance to various aminoglycosides had been cloned successfully in *S. lividans*. However, at that stage of the investigations, it was not clear how many resistance determinants were present in each of the clones, since all the recombinant plasmids contained sufficient DNA to encompass several genes. Nevertheless, by analysis of the MIC data (Table 5.1), it was interesting to observe that the clones of *S. lividans* could be divided into two groups, according to their patterns of collateral aminoglycoside resistance. Thus, one group was resistant to kanamycin, apramycin and sisomicin, and included *S. lividans* strains TSK41 and TSK51, whereas the other group contained *S. lividans* strains TSK31 and JR14, which were resistant to kanamycin, gentamicin and sisomicin. Therefore, it was logical to suggest that *S. lividans* strains belonging to a given group might possess similar mechanisms of aminoglycoside resistance and on that basis, the resistance genes cloned in such strains might be expected to show a significant degree of sequence homology. Also, a more general prediction of this hypothesis would be that different aminoglycoside-producing organisms might contain common resistance genes. In this context, it is tempting to speculate that *S. tenebrarius* is cross-resistant to a wide range of aminoglycosides (Yamamoto et al., 1982), as it possesses the resistance determinant(s) present in *S. tenjimariensis* (and *S. lividans* TSK41) in addition to that (those) in *M. purpurea* (and *S. lividans* JR14) and possibly other, as yet uncharacterised, resistance gene(s).

The failure to clone the neomycin-resistance determinant(s) from
S. tenebrarius presents an intriguing problem. It is possible that this gene is lethal to the cell, when present on a high copy number vector, such as pIJ702, so that the resultant strains of S. lividans are non-viable. An alternative suggestion relates to the mechanism of neomycin-resistance present in the neomycin producer, Streptomyces fradiae. Investigations have revealed that both AAC(3) and APH(3') activities had to be introduced into the same strain of S. lividans, in order to generate clones that were highly resistant to neomycin (Thompson et al., 1982b). Perhaps a conceptually similar situation exists in S. tenebrarius, and this producer is rendered resistant to high levels of neomycin owing to multiple resistance genes, each conferring partial resistance to the antibiotic. (These resistance determinants need not necessarily encode neomycin-inactivating enzymes). In this context, it might be interesting to introduce pLST31 and pLST51 together into S. lividans and to examine the possible consequences with regard to the level of neomycin resistance.

In conclusion, it was hoped that biochemical analysis of the mechanism(s) of resistance that exist in the aminoglycoside-resistant clones discussed above, would provide experimental evidence in support of some of the hypotheses and speculations put forward in this chapter.
CHAPTER 6

BIOCHEMICAL CHARACTERISATION OF AMINOGLYCOSIDE RESISTANCE IN CLONES OF STREPTOMYCES LIVIDANS CONTAINING DNA FROM STREPTOMYCES TENJIMARIENSIS
CHAPTER 6

1. INTRODUCTION.

Initial investigations concerning the mechanisms of aminoglycoside resistance in the clones described in the previous chapter, were restricted to S.lividans TSK41. This particular aminoglycoside-resistant strain was chosen for several reasons. First, since similar profiles of antibiotic resistance were observed in vivo for S.tenimariensis and S.lividans TSK41, it appeared that strain TSK41 contained all the determinants from this producer that were involved in conferring resistance to the aminoglycosides tested. On that basis, it was hoped to determine a complete story of how S.tenimariensis defends itself against such antibiotics. Secondly, aminoglycoside-inactivating enzymes have not been detected in this producer and its ribosomes are known to be resistant in vitro to various aminoglycosides (Yamanoto et al., 1981 a,b). Therefore, it seemed likely that the aminoglycoside-resistance gene(s) present in S.lividans TSK41 might be involved in ribosomal modification and hence, would be of particular interest here.

The resistance phenotype of S.lividans TSK41 revealed that it was refractory to a range of aminoglycosides and therefore, it was possible that more than one aminoglycoside-resistance determinant had been cloned in pLST41. Indeed, the piece of S.tenimariensis DNA present in this recombinant plasmid was about 3.5kb in length and therefore, was large enough to encompass several genes. Accordingly, it was necessary to subclone portions of it, in order to determine the number of resistance determinants present. Initially, however, the biochemical basis of resistance in S.lividans TSK41, the primary clone, was examined in the hope that it would not be necessary to repeat the
entire analysis with each subsequent subclone. The nature of the results discussed in this chapter clearly vindicated this decision.
2. METHODS.

2.1. Preparation of Ribosomal Subunits.

Salt-washed 70S ribosomes (prepared as described in Chapter 3, Page 51 et seq.) were separated into 30S and 50S subparticles by dialysis at 4°C against 2x1000 volumes of buffer containing 10mM Hepes, pH 7.5 at 20°C, 1mM MgCl₂, 150mM NH₄Cl, 3mM 2-mercaptoethanol. Subunits were purified by layering portions (50 Aₜₐₜ units) over 35 ml sucrose density gradients [linear concentration range 10-30% (w/v) made up in similar buffer] and centrifuged at 40,000 rev min⁻¹ for 70 min at 4°C in the Sorvall TV850 vertical rotor. The gradients were pumped through an Isco UA-5 analyser and by monitoring absorbance at 254nm, those fractions containing 30S and 50S particles were pooled separately. Preparations of 50S subparticles were sometimes contaminated with 30S subunits and were refractionated. After raising the magnesium chloride concentration to 10mM, both sets of subunits were collected by centrifugation, resuspended in HRS buffer (see Chapter 3, page 51), and stored as small portions at -70°C. The concentration of subunits was determined by measurement of absorbance at 260nm, given that 1 Aₜₐₜ unit corresponds to 87pmol 30S subunits or 46.2 pmol 50S subunits. Preparations of ribosomal particles were preincubated for 10 min at 30°C prior to use in cell-free protein synthesis.

2.2. Preparation of RNA and Proteins from 30S Ribosomal Subunits.

For the preparation of 16S rRNA, 30S subunits (prepared as described in the previous section) were diluted to 50 Aₜₐₜ units ml⁻¹
in HRS buffer containing 0.1% (w/v) SDS. Ribosomal proteins were removed by extraction with an equal volume of phenol, saturated with TE buffer. The aqueous phase was re-extracted with phenol and 16S rRNA precipitated using magnesium acetate (0.3M final concentration) and ethanol. After drying, the RNA pellet was finally dissolved at about 25 $A_{260}$ units ml$^{-1}$ in 10mM Hepes, pH 7.5 at 20°C, plus 0.2mM MgCl$_2$.

The integrity of each preparation of 16S rRNA was routinely checked after heating in the presence of formamide, by electrophoresis in a completely denaturing system (Lehrach et al., 1977). The gels contained 1% (w/v) agarose and were run for 40 min at 8Vcm$^{-1}$, in buffer containing 20mM Na$_2$HPO$_4$, 2mM NaH$_2$PO$_4$, and 16% (v/v) formaldehyde. After fixing in 10% (w/v) TCA (which also removed formaldehyde), gels were finally stained in 0.5M ammonium acetate plus 1µg ml$^{-1}$ ethidium bromide, to allow visualisation of rRNA with UV light.

Total proteins from 30S ribosomal subunits (TP30) were extracted by modification of the method of Traub et al., (1971). Equal volumes (100 µl) of 30S subunits (3.5 $A_{260}$ units) and a solution containing 8M urea and 4M LiCl were mixed and left at 4°C for 48 h. Urea/LiCl solutions were previously treated with bentonite to remove any contaminating ribonucleases. After pelleting rRNA by centrifugation at 48,500 rev min$^{-1}$ for 10 min at 4°C in the Beckman airfuge, the supernatant, containing TP30, was dialysed extensively at 4°C against buffer containing 30mM Hepes-KOH, pH 7.5 at 20°C, 20mM MgCl$_2$, 1M KCl, 6mM 2-mercaptoethanol. The flow rate of the dialysis buffer was about 150 ml h$^{-1}$. Such preparations of TP30 were used directly in the reconstitution of 30S ribosomal particles. One $A_{260}$ equivalent of TP30 was defined as those proteins obtained from 1 $A_{260}$ unit of 30S ribosomal subunits.
2.3. Reconstitution of 30S Ribosomal Subunits.

For reconstitution of 30S ribosomal particles, 2 A_{30} units of 16S rRNA and 3.5 A_{30} equivalents of TP30 (the latter always freshly prepared) were diluted to 1.0 ml with buffer, so that the final mixture contained 30mM Hepes-KOH, pH 7.5 at 20°C, 20mM MgCl2, 292mM KCl, 6mM 2-mercaptoethanol. The ionic strength was, therefore, about 0.37, as recommended by Traub et al., (1971). After incubation at 40°C for 20 min, reconstituted particles were collected by centrifugation overnight at 40,000 rev min⁻¹ at 4°C in the Beckman 75Ti rotor and subsequently resuspended in 150μl of HRS buffer. Reconstituted 30S subunits were incubated at 30°C for 10 min prior to use in cell-free protein synthesis. As necessary, these particles were stored at -70°C, after fast freezing, without apparent loss in activity.

2.4. Preparation of Aminoglycoside-Resistance Methylase.

Crude extracts (S100) of S. lividans TSK412 (an aminoglycoside-resistant clone) were used as the source of the 'kanamycin-apramycin' resistance methylase. The cofactor for methylation was S-adenosyl methionine.

The methylase extracts were prepared from cells that had been grown, harvested, washed and broken as described for the preparation of ribosomes and S100* (see Chapter 3, page 51 et seq.) After treatment with DNase I and centrifugation for 30 min at 18,000 rev min⁻¹ at 4°C in the Beckman JA-21 rotor, the resulting supernatant was centrifuged at 40,000 rev min⁻¹ for 3 h at 4°C in the Beckman 75Ti rotor. The resultant supernatant (designated S100), was dialysed against 3x1000
volumes of HRS buffer at 4°C and subsequently stored, after fast-freezing, as small aliquots at -70°C. Methylation incubations (100 µl total volume) were carried out at 35°C and contained 20 pmol substrate prepared from *S. lividans* TSK1 (parental strain), together with various amounts of S100, prepared from *S. lividans* TSK412 and 2.5µCi [methyl-³H] SAM (500mCi mmol⁻¹: 18.5 GBq mmol⁻¹) in buffer containing 50mM Hepes-KOH, pH 7.5 at 20°C, 7.5mM MgCl₂, 37.5mM NH₄Cl, 3mM 2-mercaptoethanol. At intervals, samples (20µl) were removed into ice-cold 5% (w/v) TCA and held at 0°C for 20 min. Precipitates were collected on glass fibre discs (Whatman GF/C) and washed extensively with 5% (w/v) TCA. The discs were dried and the radioactivity retained was estimated by liquid scintillation spectrometry, using a standard toluene-based liquid scintillant.

The scintillation counter was calibrated and revealed that [³H] S-adenosyl-methionine was counted with about 30% efficiency. Therefore, from the specific activity of radiolabelled cofactor, 1pmol [³H] S-adenosyl-methionine was equivalent to 300 cpm. This conversion factor was used to calculate the stoichiometries of methylation of 30S ribosomal particles, by the aminoglycoside-resistance enzyme prepared from *S. lividans* TSK412.

The methylation assay system described above was used to determine the best ribosomal substrate (prepared from the parental strain, *S. lividans* TSK1) for the aminoglycoside-resistance enzyme present in strain TSK412. The ribosomal derivatives tested for their suitability as substrates included:

(i) Total RNA extracted from 70S ribosomes using LiCl plus urea.

For the preparation of total rRNA from 70S particles (RNA70), equal volumes of a solution containing 8M urea and 4M LiCl, (pretreated with
bentonite to remove any contaminating ribonucleases), and 70S ribosomes (50 Asso units), were mixed and left at 4°C for about 48 h. Ribosomal RNA was subsequently collected by centrifugation for 2 min in a bench microfuge and redissolved in HRS buffer. Finally, urea and LiCl were removed from the rRNA solution by dialysis at 4°C, against 2 x 1000 volumes of HRS buffer.

(ii) Ribosomal core particles.

Core particles were generated by treatment of salt-washed 70S ribosomes (prepared as described in Chapter 3, page 51 et seq.) with solutions containing LiCl, which caused removal of some ribosomal proteins. Using different concentrations of LiCl, it was possible to remove ribosomal proteins from the 70S particles. Portions of 70S ribosomes (50 Asso units 2 mg ml⁻¹ final concentration), were mixed with buffer, to give final concentrations of 10mM Tris-HCl, pH 8.0 at 20°C: 10m M MgCl₂, and various concentrations of LiCl. For example, to generate '1M core particles', LiCl was present at a final concentration of 1M. LiCl solutions were pretreated with bentonite to remove any contaminating ribonucleases. The particles were left in LiCl overnight at 4°C, then collected by centrifugation at 45,000 rev min⁻¹ for 6 h at 4°C in the Beckman 75Ti rotor. Core particles were resuspended in HRS buffer and finally dialysed at 4°C against 2 x 2000 volumes of similar buffer to remove any remaining LiCl. Core particles generated using 0.2M, 0.5M, 1M and 2M LiCl solutions were prepared using this technique.

To investigate a possible correlation between methylation and resistance, 30S ribosomal subunits prepared from S. lividans TSK1 (parental strain), were preincubated for 20 min at 35°C as above, but in the presence, or absence, of unlabelled S-adenosyl-methionine (0.5mM final concentration). The particles were then precipitated with
ethanol, resuspended in HRS buffer and introduced into poly U-directed, cell-free protein synthesising systems. These assays contained 50S ribosomal subunits (5 pmol) and S100* both from S.lividans TSK1 (parental strain). The response of the systems to aminoglycoside antibiotics was then assessed.
3. RESULTS.

3.1. Ribosomal Resistance to Aminoglycosides.

Previous studies had shown that ribosomes from *S. tenjimariensis* were resistant *in vitro* to kanamycin, but not to gentamicin or neomycin (Yamamoto *et al.*, 1981b; 1982). However, the response of these ribosomes to apramycin and sisomicin had not been reported. It was, therefore, interesting to observe (Figs. 6.1 and 6.2) that ribosomes from *S. tenjimariensis* and from *S. lividans* TSK41 were highly resistant to these two drugs and also to kanamycin, but they showed only low level resistance to gentamicin C complex and neomycin. In these experiments, 70S ribosomes from the various organisms were supplemented with S100* from *S. lividans* TSK1 (parental strain) and assayed in cell-free protein synthesising systems directed by poly U (as described in Chapter 3, page 53 et seq.). These findings are compatible with reports by Piendl and co-workers (1984), although they do not entirely agree with those of Yamamoto *et al.*, 1981b; 1982, who propose that *S. tenjimariensis* was sensitive to gentamicin and neomycin, as were its ribosomes *in vitro*. However, when the MIC values of aminoglycosides for *S. tenjimariensis* and *S. lividans* TSK41 (clone) were compared with those of *S. lividans* TSK1 (parental strain), it was clear that the former two organisms were resistant to low concentrations of gentamicin and neomycin (see Table 5.1, page 79). Thus, there was a qualitative correlation between the resistance phenotype of *S. tenjimariensis* (and strain TSK41) and the pattern of ribosomal resistance. In agreement with previous data (Yamamoto *et al.*, 1981b), this observation also suggested that there was no obvious requirement in *S. tenjimariensis* for
Legend to Fig. 6.1

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine.

Assays contained S100* from \textit{S. lividans} TSK1 together with 5 pmol 70S ribosomes from: (O) \textit{S. lividans} clone TSK41 (100% activity = 33 phe per ribosome h\(^{-1}\)); (■) \textit{S. tenjimariensis} (100% activity = 21 phe per ribosome h\(^{-1}\)); (●) \textit{S. lividans} parental strain TSK1 (100% activity = 23 phe per ribosome h\(^{-1}\)). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h\(^{-1}\)).
Legend to Fig. 6.2

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine.

Assays contained S100* from \textit{S. lividans} TSK1 together with 5 pmol 70S ribosomes from: (○) \textit{S. lividans} clone TSK41 (100% activity = 33 phe per ribosome h\(^{-1}\)); (■) \textit{S. tenjimariensis} (100% activity = 21 phe per ribosome h\(^{-1}\)); (●) \textit{S. lividans} parental strain TSK1 (100% activity = 23 phe per ribosome h\(^{-1}\)). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h\(^{-1}\)).
Fig 6.2

Neomycin

Gentamicin

Antibiotic Concentration (µg/ml)

Activity (%)
aminoglycoside-inactivating enzymes.

3.2. Determination of the Ribosomal Component(s) Responsible for Aminoglycoside Resistance in \textit{S.\textit{lividans TSK41}}.

The next stage in the biochemical analysis of \textit{S.\textit{lividans TSK41}} was to ascertain whether the aminoglycoside resistances could be ascribed to either one of the ribosomal subunits. Previous reports had shown that \textit{S.\textit{tenjimariensis}} ribosomes were resistant to high levels of kanamycin or low levels of gentamicin, as a result of some property of their 30S subunits, (Piendl \textit{et al.}, 1984). In those experiments, 70S particles were formed by re-association of 50S and 30S ribosomal subunits from \textit{S.\textit{tenjimariensis}} and \textit{S.\textit{lividans}} wild type, to give all four possible combinations of subunits. The hybrid ribosomes were then assayed in cell-free, protein synthesising systems in the presence and absence of aminoglycosides. Analogous experiments were performed here to determine whether the 30S ribosomal subunits from \textit{S.\textit{lividans TSK41}} were responsible for the resistance phenotype of this strain.

Ribosomal subunits from this clone, together with those from the parental strain (\textit{S.\textit{lividans TSK1}}), were used to generate hybrid 70S ribosomes with heterologous and homologous combinations of subunits. The 70S particles were then assayed in the presence of S100* from the parental strain. Their responses to various aminoglycosides are shown in Fig. 6.3. Resistance to kanamycin, apramycin and sisomicin was clearly associated with 30S subunits from the clone. Moreover, the levels of resistance observed with hybrid ribosomes containing 30S particles from strain TSK41 were similar to those obtained with native 70S particles from this strain. Therefore, these results suggested
CHAPTER 6

Legend to Fig. 6.3.

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine by Homologous or Heterologous Combinations of Ribosomal Subunits.

Assays contained S100* from S. lividans TSK1 (parental strain) together with 5 pmol 30S and 5 pmol 50S subunits: (○) 30S and 50S particles both from S. lividans clone TSK41 (100% activity = 25 phe per ribosome h⁻¹); (●) 30S particles from S. lividans TSK41 and 50S particles from S. lividans TSK1 (100% activity = 14 phe per ribosome h⁻¹); (■) 30S particles from S. lividans TSK1 and 50S particles from S. lividans TSK41 (100% activity = 10 phe per ribosome h⁻¹) (□) 30S and 50S particles both from S. lividans TSK1 (100% activity = 15 phe per ribosome h⁻¹). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h⁻¹).
that the 50S ribosomal subunits play no role in determining the response of ribosomes from \textit{S. lividans} TSK41 to aminoglycosides. The data presented in Fig. 6.3 were entirely compatible with previous reports (Piendl \textit{et al}., 1984) and also with the results of subsequent studies in which hybrid ribosomes containing 30S subunits from \textit{S. tenjimariensis} were found to be resistant to apramycin and sisomicin (Piendl, 1984).

The final stage of the analysis of ribosomes from \textit{S. lividans} TSK41 was to determine whether the resistance properties of 30S subunits from this strain could be ascribed to 16S RNA or to one or more of the subunit proteins. In these experiments, total proteins (TP30) and 16S RNA were prepared from 30S ribosomal subunits from \textit{S. lividans} TSK41 and \textit{S. lividans} TSK1 (parental strain) and subsequently recombined in all four possible modes to reconstitute 30S subparticles (see this Chapter, page 90 \textit{et seq.}) These reconstituted particles were then supplemented with 50S ribosomal subunits and S100* (both from the parental strain), and assayed in a protein synthesising system to determine their response to aminoglycosides (see Fig. 6.4). Resistance to kanamycin and apramycin was clearly linked to the presence of 16S rRNA from the clone. Sisomicin was not included in these studies for purely logistical reasons, however, further analysis of the biochemical basis of resistance to this antibiotic is described later. These results are consistent with and extend data obtained previously from conceptually similar experiments, in which 16S rRNA from \textit{S. tenjimariensis} was shown to render reconstituted ribosomes resistant to kanamycin (Piendl \textit{et al}., 1984).

In conclusion, it appeared that the gene(s) involved in ribosomal resistance to aminoglycosides in \textit{S. tenjimariensis}, had been cloned
Legend to Fig. 6.4

Effect of Aminoglycosides on Synthesis of Polyphenylalanine in Systems Containing Reconstituted 30S Ribosomal Particles.

Assays contained 5 pmol 50S subunits plus S100* from *S. lividans* TSK1 (parental strain), together with 5 pmol reconstituted 30S particles. These contained: (○) 16S rRNA and TP30 both from *S. lividans* TSK41 (100% activity = 11 phe per ribosome h⁻¹); (●) 16S rRNA from *S. lividans* TSK41 and TP30 from *S. lividans* TSK1 (100% activity = 11 phe per ribosome h⁻¹); (■) 16S rRNA from *S. lividans* TSK1 and TP30 from *S. lividans* TSK41 (100% activity = 8 phe per ribosome h⁻¹); (□) 16S rRNA and TP30 both from *S. lividans* TSK1 (100% activity = 8 phe per ribosome h⁻¹).

Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h⁻¹).
successfully in \textit{S.\textit{lividans}.} The next objective was, therefore, to extend the observations of Piendl \textit{et al.}, (1984), discussed above, by determining the biochemical mechanism(s) of ribosomal resistance to aminoglycosides, present in \textit{S.\textit{lividans TSK}41}. However, prior to this, attempts were made to determine the number of resistance genes that were responsible for the observed multiple aminoglycoside-resistance phenotype of \textit{S.\textit{lividans TSK}41}.

3.3. Subcloning of Aminoglycoside-Resistance Determinant(s) in \textit{S.\textit{lividans TSK}41}.

The aim of the following series of experiments was to subclone portions of the \textit{S.\textit{tenjimariensis} DNA} present in \textit{pLST}41, to ascertain how many aminoglycoside-resistance determinants had been cloned in \textit{S.\textit{lividans TSK}41}.

Plasmid \textit{pLST}41 was purified on a caesium chloride density gradient (as described in Chapter 3, page 43 et seq). A small portion of the plasmid (1μg) was then partially digested with \textit{Sau} 3A, so that no intact plasmid was evident when the DNA was analysed on an agarose gel. This digested DNA was ligated with an equal amount of \textit{pIJ}702, which previously had been linearised with \textit{Bgl II} and then treated with CIAP to prevent regeneration of intact \textit{pIJ}702. The ligation mixture was introduced into \textit{S.\textit{lividans TK}21} protoplasts and selections made on plates containing either kanamycin or apramycin. Numerous resistant colonies were obtained, although restriction analysis of plasmid preparations from several of these strains revealed that only about 50% of the plasmids contained inserts of DNA that were smaller than the original fragment cloned in \textit{pLST}41. (In retrospect, the \textit{Sau} 3A
fragments of pLSr41 should have been treated with phosphatase to prevent reconstruction of this plasmid). The smallest recombinant plasmid that was isolated from this subcloning procedure was designated pLSr411 (see Fig. 6.5 for restriction map), and contained about 1.8kb of DNA inserted into the Bgl II site of pIJ702. Although this plasmid could confer resistance to kanamycin, apramycin and sisomicin in *S. lividans*, the inserted fragment of DNA was still big enough to include more than one complete gene. Therefore, a further series of subcloning experiments was necessary to determine whether the multiple aminoglycoside-resistance phenotype conferred by pLSr411 could be attributed to a single gene. Fortunately, a restriction site for Sph I was found towards the centre of the 1.8kb cloned insert in pLSr411 (see Fig. 6.5). Therefore, attempts were made to subclone the fragments of *S. tenuimariensis* DNA located on either side of this Sph I site.

After purification of pLSr411 on a caesium chloride density gradient, the plasmid was digested to completion with Sph I, and religated with vector pIJ702 that had been terminally dephosphorylated using CIAP, after cleavage with Sph I. Using a similar selection procedure to that employed in the previous subcloning experiment, several resistant colonies were obtained. Plasmid DNA was isolated from several of these strains and by restriction analysis, a construct was found which appeared to consist of pIJ702 plus a piece of extraneous DNA, that was about 0.9kb in length. This recombinant plasmid was designated pLSr412 (see Fig. 6.6 for a restriction map) and the strain containing this plasmid, designated *S. lividans* TSK412, proved to be highly resistant to kanamycin, apramycin and sisomicin (see Table 5.1, page 79 for MIC data). A detailed restriction map of pLSr411 was not constructed as part of the present work and so the
Legend to Fig. 6.5.

Restriction Map of pLST411.

This recombinant plasmid is a subclone of pLST41 (see Fig. 5.2) and contains the kanamycin-apramycin resistance (kan-apr) determinant(s) from *S. tenimariensis*. Plasmid pLST411 also contains the tyrosinase (mel) gene from *S. antibioticus* and the thiostrepton-resistance (tsr) gene from *S. azureus*. 
Fig 6.5

pLST411
7.5kb

kan-apr

mel
tsr

Bgl II/Sau 3A
Sph I
Pst I
Bcl I

Bgl II/Sau 3A
Sph I
Pst I
Bcl I
Legend to Fig. 6.6.

Restriction Map of pLST412.

This recombinant plasmid is a subclone of pLST411 (see Fig. 6.5) and contains the kanamycin-apramycin resistance (kan-apr) determinant from *S. tenjimariensis*. Plasmid pLST412 also contains the tyrosinase (mel) gene from *S. antibioticus* and the thiostrépton-resistance (tsr) gene from *S. azureus*.

The DNA probe that was used in the hybridisation experiments referred to in Chapter 8 (page 131 et seq.) was thought to be contaminated with trace amounts of the *Bcl I* fragment of DNA, indicated by asterisks.
precise relationship between it and pLST412 remained unclear. Recently, however, it has become apparent that pLST412 must have arisen by deletion of the Sph I fragment (about 1kb in length) from pLST411 and not via subcloning of that Sph I fragment into pIJ702. I am grateful to David Holmes of this laboratory for permission to use his restriction map of pLST411 (Fig. 6.5).

Since the cloned insert in pLST412 is unlikely to encompass more than one complete gene, this determinant must be responsible for the multiple aminoglycoside resistance in S. lividans TSK412 and, by implication, in the primary clone (S. lividans TSK41). Further evidence in support of this hypothesis was provided when the ribosomes from the subclone S. lividans TSK412 and the primary clone (S. lividans TSK41) were shown to be indistinguishable in their responses in vitro to various aminoglycosides (see Fig. 6.7). It was, therefore, unnecessary to repeat with the ribosomes from strain TSK412, the detailed analysis previously carried out with those from strain TSK41. (For a comparative analysis of restriction digests of pIJ702, pLST41, pLST411 and pLST412, see Fig. 6.8).


To relate the mechanism of aminoglycoside resistance in S. lividans TSK412 to that in S. tenjimariensis, it was necessary to confirm the origin of the aminoglycoside-resistance gene present in pLST412. To do this, DNA hybridisation experiments were carried out (as described in Chapter 3, page 54 et seq.) Southern blots were prepared from agarose gels containing S. tenjimariensis genomic DNA, digested to completion.
Legend to Figure 6.7.

Effect of Aminoglycosides on Poly U Dependent Protein Synthesis.

Assays contained S100* from \textit{S. lividans} TSK1, together with 5 pmol 70S ribosomes from (○) \textit{S. lividans} TSK1 (100% activity = 23 phe per ribosome h$^{-1}$); (□) \textit{S. lividans} TSK41 (100% activity = 33 phe per ribosome h$^{-1}$); (■) \textit{S. lividans} TSK412 (100% activity = 32 phe per ribosome h$^{-1}$).

Activities in drug free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h$^{-1}$).
Fig 6-7

Kanamycin

Apramycin

Sisomicin

Gentamicin

Activity (%)

Antibiotic Concentration (μg/ml)

0 0.01 1 100

0 0.01 1 100
Legend to Fig. 6.8

Restriction Analysis of Recombinant Plasmids Generated During Subcloning of the Kanamycin-Apramycin Resistance Determinant.

Restriction fragments of DNA were separated by electrophoresis in a 0.7% (w/v) agarose gel.

a. Cla I digest of pLST41
b. Cla I digest of pLST411
c. Cla I digest of pLST412
d. Cla I digest of pIJ702
e. Bcl I digest of pLST41
f. Bcl I digest of pLST411
g. Bcl I digest of pLST412
h. Bcl I digest of pIJ702
i. Sph I digest of pLST411

Size markers are represented by fragments generated by a Hind III digest of bacteriophage lambda DNA.
with Bam HI, together with Bcl I digests of plJ702 and plST412 as negative and positive controls, respectively. The probe DNA was isolated from a low melting point agarose gel, as a Pst I/Sph I restriction fragment of plST412 (see Fig. 6.6 for restriction map). It consisted of about 0.8kb of the cloned insert and was radiolabelled using random hexanucleotide primers to direct the incorporation of \(^{[\text{P}}\)dCTP. A single Bam HI fragment of _S. tenjimariensis_ DNA (of about 3.5kb in length) and the largest Bcl I fragment of plST412 (consisting of the tyrosinase gene plus the DNA presumed to have originated from _S. tenjimariensis_), were shown to hybridise with the radioactive probe, even at the highest level of stringency employed (i.e. the sequences had greater than about 85% homology, see Fig. 6.9). No hybridisation was observed with the tyrosinase gene in the gel-track containing the plJ702 digest, however, a faint band was visible in each track containing digests of plJ702 and plST412, which represented hybridisation of the radioactive probe with the 1.7kb Bcl I fragment common to both these plasmids (see Fig. 6.6). These observations suggested that the probe DNA was contaminated with small amounts of some, or all, of this plJ702-derived Bcl I fragment and, therefore, complicated the interpretation of the autoradiogram shown in Fig. 6.9. Nevertheless, the intensity of the hybridising band in the track containing _S. tenjimariensis_ DNA, strongly implied that it was complementary to the cloned fragment in plST412 rather than a piece of DNA derived from vector plJ702. In retrospect, and given a clean preparation of probe DNA, it would have been more conclusive to demonstrate that when _S. tenjimariensis_ genomic DNA was digested to completion with Pst I and Sph I restriction enzymes, a hybridising fragment was generated, which was the same length as the
Legend to Fig. 6.9

Southern Hybridisation of DNA Samples to $^{32}$P-labelled Kanamycin-Apramycin Resistance Determinant from pLST412.

Gel tracks contained the following samples:

a. Bcl I digest of pLST412
b. Bam HI digest of S. tenjimariensis total DNA
c. Bcl I digest of pIJ702.

The radiolabelled probe DNA consisted of a Pst I/Sph I restriction fragment from pLST412 (see text for details).
Fig 6.9

a  b  c

-3.5 kb
-2.4 kb
-1.7 kb
Pst I/Sph I fragment from pLST412 used as the radioactive probe. This experiment was attempted but, unfortunately, the results were inconclusive as problems were encountered in generating complete digests of S.tenjimariensis DNA with both Sph I and Pst I concurrently.

Taken collectively, the data presented so far suggested that S.tenjimariensis contained an aminoglycoside-resistance gene that could account for the observed phenotype of this producer. However, reservations about whether the gene was responsible for resistance to istamycin must be emphasised. Also, it remains possible that S.tenjimariensis might possess other resistance determinants, although there is currently no evidence that demands such an hypothesis.

3.5. Biochemical Basis of Aminoglycoside Resistance in S.lividans TSK412.

At this stage of the investigations, it was apparent that some property of 16S rRNA was responsible for multiple resistance to aminoglycosides in S.lividans TSK412. The next objective was to determine the nature of the alteration(s) in the rRNA. There were two possibilities, either of which could have been responsible for part or all of the resistance phenotype of the clone. First, the 16S rRNA might have undergone post-transcriptional modification, such that the ribosomes were refractory to the action of certain aminoglycosides. Secondly, the clone might have possessed a peculiarity in the nucleotide sequence of the RNA, in which case a rRNA gene must have been cloned. The latter possibility was unlikely, however, since the subcloning experiments described in the previous section implied that the S.tenjimariensis DNA present in pLST412 was too small to encode an
entire 16S rRNA gene (about 1.5kb of DNA would have been required). This hypothesis was supported by a series of concomitant experiments carried out by Dr. Jill Thompson in Professor A. Dahlberg's laboratory at Brown University, Providence, Rhode Island. Briefly, Southern blots of restriction fragments of plST41 were incubated with the rrn B operon of E. coli, which had been radiolabelled using random hexanucleotide primers. No hybridisation was detected, even at the lowest levels of stringency employed. However, in control experiments, when the rrn B operon was used to probe Northern blots carrying 16S rRNA from strain TSK41, strong hybridisation was observed at the highest level of stringency. It was concluded from these results, that plST41 did not contain a ribosomal RNA gene.

The principle conclusion so far was that multiple resistance to aminoglycosides in S. lividans TSK412 was due to post-transcriptional modification of 16S rRNA by the product of a single gene. The next objective was, therefore, to identify the type of modification involved and to establish a causal connection between that event and resistance. Previous examples of rRNA modification leading to antibiotic resistance involved exclusively methylation. For example, ribosomal resistance to erythromycin (a member of the macrolide group of antibiotics), thiostrepton and kasugamycin was, in each case, shown to result from changes in the extent of methylation of the rRNA. Given such precedents, it seemed logical to examine the possibility that methylation of 16S rRNA might underlie resistance to aminoglycosides in S. lividans TSK412.

Previously, resistance to the 'MLS' (macrolide, lincosamide and streptogramin B) group of antibiotics had been observed in certain clinical strains of Staphylococcus and Streptococcus (for a review, see
CHAPTER 6

Weisblum, 1975). Investigations showed that the resistance was inducible and could be correlated with the appearance of $N^\delta,N^\epsilon$-dimethyl-adenine in 23S rRNA (Lai and Weisblum, 1971). Furthermore, when reconstituted ribosomes were prepared using rRNA from induced and uninduced cells of *Staphylococcus aureus* together with ribosomal proteins from *Bacillus stearothermophilus*, the presence of dimethyladenine was shown to be directly responsible for resistance.

Following this work, *Streptomyces erythraeus*, the producer of erythromycin, was also shown to display the MLS-resistance phenotype, and resistance to erythromycin proved to be a property of the ribosomes (Teraoka and Tanaka, 1974). When the 23S rRNA of these particles was shown to contain $N^\delta,N^\epsilon$-dimethyladenine (Graham and Weisblum, 1979), this led to the suggestion that similar mechanisms of MLS-resistance might be present in *S.erythraeus* and in the clinical strains of staphylococci, mentioned above. Evidence in support of this hypothesis was provided when a methylase enzyme was found in *S.erythraeus* which could catalyse dimethylation of a single adenine residue within naked 23S rRNA from *Bacillus stearothermophilus* (Skinner and Cundliffe, 1982). The functional significance of this methylation was established using 50S ribosomal subunits which had been reconstituted in vitro. Only when such particles contained 23S rRNA that had previously been modified by the *S.erythraeus* methylase, were they resistant to specific MLS antibiotics. Thus, it appeared that methylation of 23S rRNA was responsible for ribosomal resistance to MLS antibiotics in the erythromycin producer and in clinical isolates. Recently, the site of action of the *S.erythraeus* resistance methylase within *B.stearothermophilus* 23S rRNA has been determined and shown to correspond to residue 2058 in *E.coli* 23S rRNA (Skinner et al., 1983).
A conceptually similar mechanism is responsible for resistance to thiostrepton in the producer, *Streptomyces azureus*. This organism avoids suicide by methylating 23S rRNA, so that its ribosomes do not bind the drug (Cundliffe, 1978). This methylase, which is apparently produced constitutively, acts on free rRNA, but not on 50S ribosomal subunits (Thompson and Cundliffe, 1981). Presumably, methylation of 23S rRNA occurs at an early stage during, or after, transcription, but before assembly of the 50S ribosomal subunit is complete. The enzyme was shown to produce a single residue of 2′O methyl adenosine (Cundliffe and Thompson, 1979), which was located at position 1067 of *E.coli* 23S rRNA (Thompson *et al.*, 1982d).

The ability of both the thiostrepton and erythromycin resistance methylases to confer resistance *in vivo* has been confirmed by cloning DNA fragments from the respective producing organisms, in *S.lividans* (Thompson *et al.*, 1982a), followed by biochemical analysis of the resistance mechanisms in the clones (Thompson *et al.*, 1982b).

The mechanisms of resistance to erythromycin and to thiostrepton, both concern an 'over-methylation' of 23S rRNA and have been fully characterised in the relevant antibiotic-producing organisms. However, a variant of this type of post-transcriptional modification, namely 'under-methylation' of 16S rRNA, has been studied in kasugamycin-resistant mutants of *E.coli*.

Kasugamycin is an inhibitor of the initiation step of bacterial protein synthesis. One particular resistant mutant, containing the *ksgA* mutation, possesses a modified target, in that the 30S ribosomal subunits of the mutant have altered 16S rRNA (Helser *et al.*, 1971). This was demonstrated by total reconstitution *in vitro* of 30S particles using 16S rRNA and ribosomal proteins from *ksgA* and wild-type strains.
in all four possible combinations. Further analysis showed that 16S rRNA from the wild type contained two residues of N^\text{6},N^\text{6}'-dimethyladenine, at positions 1518 and 1519, which were unmethylated in RNA from the \textit{ksgA} strain. Also, extracts of wild type strains were shown to introduce four methyl groups into ribosomal core particles of \textit{ksgA} strains. Moreover, when ribosomes were reconstituted from the resultant methylated core particles, by adding back the missing 'split-proteins', they proved to be sensitive to kasugamycin (Helser \textit{et al.}, 1972). This confirmed the connection between the state of methylation of two adenine residues within 16S rRNA and the kasugamycin phenotype.

Guided by these examples of post-transcriptional modification which led to antibiotic resistance, it seemed logical to investigate whether methylation of 16S rRNA was responsible for resistance to aminoglycosides in \textit{S.lividans} TSK412. To do this, post-ribosomal supernatant (S100) from strain TSK412 was used as the source of the putative resistance methylase and S-adenosyl-methionine was included as cofactor. Methylation of 16S rRNA was indeed shown to be responsible for kanamycin, apramycin and sisomicin resistance in strain TSK412 (see Fig. 6.10). In these experiments, 30S ribosomal subunits from the parental strain (\textit{S.lividans} TSK1) were preincubated with an S100 preparation from strain TSK412, both in the presence and absence of S-adenosyl-methionine, before being introduced into protein synthesising systems. These also contained 50S ribosomal subunits plus S100*, both from the parental strain, and protein synthesis was followed in the presence and absence of aminoglycosides. The results were unequivocal: kanamycin, apramycin and sisomicin resistance was observed only when 30S ribosomal particles had been preincubated with
Legend to Fig. 6.10

Correlation Between Methylation of 30S Ribosomal Subunits and Resistance to Aminoglycosides.

In panels A, B and C, ribosomal 30S subunits (5 pmol) from \textit{S.\,lividans TSK1} (parental strain) were pre incubated for 20 min at 35°C, in the presence or absence of S-adenosyl-methionine and/or 2.5% (v/v) S100 from \textit{S.\,lividans TSK412} (clone). Ribosomal particles were then ethanol-precipitated before being introduced into a cell-free protein synthesising system directed by poly U. In panel D, ribosomal 30S subunits (5 pmol) from \textit{S.\,lividans TSK1} were introduced directly into protein synthesising systems, without pre incubation or ethanol-precipitation. All protein synthesis assays also contained 5 pmol 50S subunits and S100*, both from the parental strain. Drugs were added at the following final concentrations: (●) drug-free controls: (○) kanamycin 100μg ml⁻¹: (□) sisomicin 10μg ml⁻¹: (■) apramycin 10μg ml⁻¹.
Table 6.1
Preliminary Studies of the Substrate Specificity of the 'Kanamycin-Apramycin' Resistance Methylase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol $[^{3}]H$ methyl groups incorporated per pmol 16S RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA 70</td>
<td>0.1</td>
</tr>
<tr>
<td>70S ribosomes</td>
<td>0.1</td>
</tr>
<tr>
<td>0.2M core particles</td>
<td>0.15</td>
</tr>
<tr>
<td>0.5M core particles</td>
<td>0.05</td>
</tr>
<tr>
<td>1M core particles</td>
<td>0.25</td>
</tr>
<tr>
<td>2M core particles</td>
<td>0.20</td>
</tr>
<tr>
<td>30S subunits</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Methylase assays were performed using 20% (v/v) S100 prepared from *S. lividans* TSK412 (as crude methylase extract), $[^{3}]H$-S-adenosyl methionine as cofactor together with various substrates, all prepared from the parental strain (*S. lividans* TSK1) as described in this chapter (page 92 *et seq.*) Incubations were carried out at 35°C for 90 min. Note: in subsequent experiments, stoichiometries of methylation significantly greater than those shown here were obtained with 30S ribosomal subunits (see Fig. 6.12).
S-adenosyl-methionine together with S100 from \textit{S. lividans} TSK412.

During the course of these studies, however, several problems were encountered. Initial investigations were carried out to determine the best ribosomal substrate for the resistance methylase in strain TSK412. Previous examples of rRNA modification, discussed above, had demonstrated that substrates for methylation varied from naked ribosomal RNA, as with the thiostrepton-resistance methylase (Thompson and Cundliffe, 1981) to intact 30S ribosomal subunits, as required by the methylase responsible for kasugamycin sensitivity (Poldermans \textit{et al.}, 1979). Thus, the S100 preparation from strain TSK412 was incubated in the presence of S-adenosyl-$[^{1}H$-methyl]-methionine, with a wide variety of ribosomal substrates prepared from \textit{S. lividans} TSK1 (parental strain). When the incorporation of radiolabelled methyl groups into these particles was assessed, it was clear that the optimal stoichiometries of methylation were obtained using 30S ribosomal subunits (see Table 6.1). However, neither intact 70S ribosomes, nor naked rRNA were suitable substrates for the methylase in \textit{S. lividans} TSK412. This suggested that the resistance enzyme might require a specific conformation of 16S rRNA for activity, and that the site(s) of modification might be located at the interface of the 30S and 50S subunits, within 70S particles. (The stoichiometries of methylation obtained in these preliminary experiments were rather low, however, higher levels of methylation were achieved subsequently, as discussed later).

To confirm that radiolabelled methyl groups were incorporated into 16S RNA and not ribosomal proteins, the 30S particles that had been methylated by S100 from strain TSK412, were treated with phenol. Essentially all the radioactivity was shown to partition into the
aqueous phase and proved to be RNA (and not protein), because it was rendered TCA-soluble by heating in the presence of 10% (w/v) TCA.

The next objective was to prove the causal relationship between methylation of 16S RNA and ribosomal resistance to aminoglycosides. However, these studies were hindered by several problems. In particular, both the S100 extract from *S. lividans* TSK412 and the preparation of S-adenosyl-methionine used as cofactor, appeared to contain some substance(s) which inhibited *in vitro* poly U-dependent protein synthesis. For example, 30S ribosomal subunits, from the parental strain, that had been preincubated in the presence of S-adenosyl-methionine were less active in poly U-dependent protein synthesising systems than 30S particles preincubated without added cofactor (see panels B, C and D, Fig. 6.11). Also, preincubation of 30S ribosomal particles with relatively high amounts of S100 from strain TSK412, caused a similar reduction in their protein synthetic activity (see panel A, Fig. 6.11). It appeared, therefore, that these 30S ribosomal subunits might be inhibited in protein synthesising systems owing to a direct effect of S-adenosyl-methionine and that the S100 preparation from strain TSK412 might contain trace amounts of this cofactor. Furthermore, this hypothesis also explained two other anomalous observations. First it accounted for the apparent S-adenosyl-methionine-independent resistance to kanamycin (see panel A, Fig. 6.11). Secondly, it explained the low stoichiometries of methylation observed, when 30S ribosomal subunits from the parental strain were incubated with radiolabelled cofactor and S100 from strain TSK412 (see Table 6.1).

Whatever the nature of the inhibitory substance(s), it was possible to remove it (them) by ethanol-precipitation of 30S ribosomal subunits.
Legend to Fig. 6.11
Relationship Between Cofactor-Dependent Aminoglycoside Resistance and the Input of S100 from \textit{S.lividans} TSK412.

Ribosomal 30S subunits (5 pmol) from \textit{S.lividans} TSK1 (parental strain) were pre incubated for 20 min at 35°C with various amounts of methylase extract (S100 from \textit{S.lividans} TSK412), in the presence or absence of S-adenosyl-methionine (0.5mM final concentration). The 30S subunits were then supplemented with untreated 50S ribosomal particles (5 pmol) and S100*, both from the parental strain, and their response to kanamycin (100μg ml⁻¹ final concentration) was assessed in poly U-dependent protein synthesis. (■) plus cofactor, minus kanamycin: (□) plus cofactor, plus kanamycin: (●) minus cofactor, minus kanamycin: (○) minus cofactor, plus kanamycin.
after the preincubation step, and before assaying their activity in in vitro protein synthesising systems. The results thereby obtained (see Fig. 6.10) indicated that equivalent levels of poly U-dependent synthetic activity were observed in all drug-free assays. Furthermore, there was no apparent loss in activity or change in the response to aminoglycoside antibiotics, as a result of precipitating the 30S ribosomal particles (compare panels C and D, Fig. 6.10).

Finally, attempts were made to determine the stiochiometry of methylation of 30S ribosomal particles from the parental strain and thereby estimate the number of sites of action of the 'kanamycin-apramycin' resistance methylase from S. lividans TSK412. Previous data had been obtained using relatively high inputs of methylase extract from strain TSK412 (see Table 6.1) and therefore, in view of the results discussed above, it was possible that trace amounts of cofactor in this extract might have led to an underestimation of the stiochiometries of methylation. Consequently, the levels of incorporation of radiolabelled methyl groups into 30S particles from the parental strain, were reassessed by incubation with various inputs of methylase extract from S. lividans TSK412, together with [3H]-S-adenosyl-methionine. The results showed that an optimal stiochiometry of methylation of 16S rRNA of about 0.7 could be obtained (see Fig. 6.12). Although these data do not prove that only a single methyl group is introduced into 16S RNA by the aminoglycoside-resistance enzyme in strain TSK412, they are entirely compatible with such a model. However, since the 30S ribosomal subunits in any given preparation are undoubtedly heterogeneous, a higher level of methylation of a specific sub-population of 30S particles might well have been masked. Also, trace amounts of S-adenosyl-methionine in the
Legend to Fig. 6.12

Methylation of 30S Ribosomal Subunits from \textit{S. lividans} TSK1 by S100 from \textit{S. lividans} TSK412.

Assays contained 30S ribosomal subunits (20 pmol) from \textit{S. lividans} TSK1, S-adenosyl-([\text{H}]\text{-methyl})-methionine as cofactor and methylase extract from strain TSK412 as follows: (●) 2.5% (v/v): (○) 5% (v/v): (□) 10% (v/v): (■) 20% (v/v). Time-independent background 'incorporation' into 30S particles from strain TSK41 has been subtracted.
Fig 6.12

Methyl groups incorporated (pmol/pmol 16S RNA)

Time (min)

[\text{\textsuperscript{3}}H]
S100 preparation from *S. lividans* TSK412 (suggested by the low levels of acquired resistance seen in panel B as compared with panel C, Fig. 6.10) might have led to an underestimation of the stiochiometry of methylation.

Collectively, these data prove that methylation of 16S rRNA is the cause of resistance to kanamycin, apramycin and sisomicin in *S. lividans* TSK412, and by inference, in *S. tenjimariensis*. 
3. DISCUSSION.

This is the first demonstration that specific methylation of 16S rRNA can render ribosomes resistant to antibiotics. On the other hand, it has been known for some time that failure to methylate 16S rRNA causes resistance to kasugamycin in \textit{ksg A} mutants of \textit{E.coli} (Helser \textit{et al.}, 1972). Also, changes in the nucleotide sequence of the small ribosomal subunit RNA have been shown to be responsible for antibiotic-resistance in several mutants. Such point mutations have been shown to confer resistance to paromomycin in yeast mitochondria (Li \textit{et al.}, 1982) and in \textit{Tetrahymena thermophila} (Spangler and Blackburn, 1985), resistance to streptomycin in \textit{Euglena gracilis} chloroplasts (Montandon \textit{et al.}, 1985), resistance to hygromycin in \textit{T. thermophila} (Spangler and Blackburn, 1985) and resistance to spectinomycin in \textit{E.coli} (Mark \textit{et al.}, 1983). However, resistance is purely a relative term, and the distinguishing feature of the 16S rRNA methylation discussed here, is the very high level of ribosomal resistance which is produced, in comparison with that observed in the mutants mentioned above.

Aminoglycoside antibiotics containing 2-deoxystreptamine have been reported to exert multiple effects on bacterial protein synthesis and have been shown to bind to multiple sites on the ribosome (see Chapter 2, page 22 \textit{et seq.}) Although attempts have been made to determine the primary mode of action of aminoglycosides, and to relate a given aspect of a drug’s action to a particular target site on the ribosome, no definitive conclusions could be drawn from those investigations. However, the present data and those reported previously (Piendl \textit{et al.}, 1984), strongly imply that kanamycin, apramycin and sisomicin, exert
their primary inhibitory effects as a result of interaction with the 30S ribosomal subunit. The results presented here on the stoichiometry of methylation of 16S rRNA by the aminoglycoside-resistance methylase strongly suggest, (even if they do not prove) that modification of a single residue of 16S RNA can render ribosomes resistant to kanamycin, apramycin and sisomicin. On this basis, these three aminoglycosides might be expected to possess a common target site on the ribosome. On the other hand, whatever the number of sites of methylation, such modification clearly interferes with the functional binding of these aminoglycosides to ribosomes. Therefore, it is possible that such binding normally occurs directly on 16S rRNA. Indeed, since intact 30S ribosomal subunits act as substrates for the kanamycin-apramycin resistance methylase, the site of modification of 16S rRNA is presumably exposed on these particles and, therefore, is available for interaction with aminoglycosides.

The substrate specificity of the kanamycin-apramycin resistance methylase is also relevant when considering the regulation of expression of the gene encoding this enzyme in S.tenjimariensis. At present, there is no unequivocal evidence to indicate whether this resistance determinant is expressed constitutively in the producer, or whether expression is inducible. However, given the substrate specificity of the resistance methylase, it is clear that the enzyme can modify ribosomal subunits that have already been assembled and, therefore, the gene could, but need not necessarily, be inducible. In this context it is worth noting that the determinant responsible for ribosomal resistance to kanamycin in S.kanamyceticus, is only expressed during kanamycin production (Nakano et al., 1984). Although it is not clear what causes induction of the S.kanamyceticus resistance gene, a
similar mechanism might be operating in *S. tenjimariensis*. If this is true, the aminoglycoside resistance determinant in *S. lividans* TSK412 presumably has been cloned away from the DNA sequence present in *S. tenjimariensis*, which is responsible for regulating its expression.

When the site(s) of action of the kanamycin-resistance methylase in *S. lividans* TSK412 have been determined, it should be possible to localise the primary target site(s) of these aminoglycosides to within a particular domain of the ribosome. In order to relate this ribosomal domain to a specific function, it will be important to ascertain whether *in vitro* protein synthesising systems containing such methylated ribosomes, are resistant to some or all of the effects that have been reported to be induced by aminoglycosides. In this context, it is important to emphasise that all the present data were collected in a cell-free protein synthesising system directed by poly U. On the one hand, such systems only monitor the elongation phase of protein synthesis. Therefore, although ribosomes from a given organism might be resistant to aminoglycosides when assayed in the cell-free system described here, it is not known whether such ribosomes would be resistant when introduced into a more authentic assay system. On the other hand, it is worth noting that the relative levels of resistance observed here, mimic qualitatively the response of the intact organisms. Therefore, there is currently no evidence to suggest that the putative patterns of ribosomal resistance to aminoglycosides that might be observed in other, more physiological, systems, would be any different from that seen here.

A comparison of the effects of aminoglycosides on *in vitro* protein synthesis presented here, with those reported previously using similar systems (e.g. Piendl et al., 1984), revealed several interesting
observations. When the amounts of drug present in such assays were compared on a molar basis with the respective input of ribosomes, similar levels of resistance to a given aminoglycoside were observed with ribosomes from *S. lividans* TSK41 (or *S. tenjimariensis*, see Fig. 6.1) and those from *S. tenjimariensis* (Piendl et al., 1984). Therefore, in this respect, the data were compatible. However, it was surprising to find that aminoglycoside-sensitive ribosomes showed marked differences in their overall response to antibiotics. Thus, previously, several groups had shown that such ribosomes (including those from *S. lividans* wild type, Piendl et al., 1984) undergo a triphasic response *in vitro*, in the presence of aminoglycosides of the kanamycin, gentamicin and neomycin families (Davies and Davis, 1968: Tai and Davis, 1979). This multiphasic effect was suggested to result from a stimulation of amino acid incorporation at intermediate concentrations, which obscured the inhibition of protein synthesis at low or high drug concentrations (Tai and Davis, 1979). However, no such effect was observed here with ribosomes from *S. lividans* TSK1 (parental strain), or any other aminoglycoside-sensitive 70S particle (see Figs. 6.1, 6.2, 6.3, 6.4). On that basis, it appeared that the *in vitro* poly U-directed system used here, was not stimulated by intermediate concentrations of antibiotic (i.e. did not misread) to the same extent as similar cell-free systems employed by other groups. In this context, it is important to note that the latter systems generally contain unfractionated *E. coli* tRNA, so that non-cognate aminoacids could be incorporated into the growing polyphenylalanine chain, thus representing mistranslation of the synthetic messenger. However, the cell-free system used here, is supplemented only with phenylalanine-specific tRNA, thus reducing the chances of incorporating non-cognate
aminoacids, and thereby restricting the misreading effects induced by aminoglycosides.

Several other parameters have also been suggested to influence the extent of misreading induced by aminoglycosides in vitro, including the concentration of cations (Davies et al., 1964: Tai and Davis, 1979) and the tRNA concentration (Davies et al., 1965: Tai and Davis, 1979). In particular, the stimulation of aminoacid incorporation observed in the presence of gentamicin, and associated with misreading, was shown to decrease when the magnesium concentration was raised from 8mM to 12 mM (Piendl and Böck, 1982). Although it is difficult to compare the effects of absolute concentrations of magnesium and tRNA present in various in vitro systems, the concentration of 15mM magnesium employed in the system described here, might contribute to a further restriction of aminoglycoside-induced misreading.

Whether or not these explanations account for the apparent absence of the multiphasic response from the present data, it is clear that aminoglycoside antibiotics act as potent inhibitors of the poly U-directed system employed here. Indeed, this response can only serve to simplify the interpretation of the data presented here.

In conclusion, whilst it appears that ribosomes from S. lividans TSK412 can perform the elongation phase of protein synthesis in the presence of specific aminoglycosides, it is not yet certain whether such ribosomes are also resistant to the misreading effects that can be induced by these antibiotics. In particular, it will be interesting to examine the response of ribosomes from strain TSK412 to kanamycin, in partial reactions of protein synthesis, especially those which assay translational accuracy. In E. coli, kanamycin has two distinct effects on the translational fidelity of ribosomes programmed with poly U: it
increases the error frequency of the initial selection of aminoacyl-tRNA and also decreases the efficiency with which ribosomes proof read their choice of aminoacyl-tRNA, prior to peptide bond formation (Jelenc and Kurland, 1984). It is not yet certain whether either, or both, of those effects contribute to the bactericidal action of kanamycin and, therefore, it would be interesting to know whether they can be blocked by specific methylation of 16S rRNA. If so, this would suggest that the site(s) of action of the kanamycin-apramycin resistance methylase might lie within the ribosomal domain concerned with the control of translational fidelity. On that basis, ribosomal RNA might be seen to play an important role in this crucial step of protein synthesis.
CHAPTER 7

AMINOGLYCOSIDE RESISTANCE IN CLONES OF
STREPTOAXCES LIVIDANS CONTAINING DNA
FROM MIEROMONOSPORE PURPUREA
CHAPTER 7

1. INTRODUCTION.

The experiments described in this chapter were performed in this laboratory by Dr. Jill Thompson to whom I am extremely grateful for agreeing to their presentation here. These studies concerned the biochemical analysis of the resistance mechanism(s) operating in the aminoglycoside-resistant clone, *S. lividans* JR14. (Strain JR14 was generated as described in Chapter 5, by a series of cloning experiments carried out by Dr. J. Thompson). This clone contains plasmid pLST14, a derivative of pIJ702, which includes an aminoglycoside-resistance determinant(s) from *Micromonospora purpurea*, the producer of the gentamicin C complex. As mentioned previously, this recombinant plasmid renders *S. lividans* resistant *in vivo* to several aminoglycosides, including kanamycin and gentamicin. Therefore, although strain JR14 resembles *S. lividans* TSK412 in its response to kanamycin, the two organisms clearly differ in their relative sensitivities to a variety of other aminoglycosides (see Table 5.1, page 79, for details). On that basis, these strains must contain different mechanisms of aminoglycoside resistance. Therefore, it seemed relevant to analyse the resistance mechanism(s) present in *S. lividans* JR14 and to compare the results with those obtained with *S. lividans* TSK412, as presented in the previous chapter. (The analysis of strain JR14 was carried out using essentially identical methods and conceptually similar experiments to those described in Chapter 6).
2. RESULTS.

2.1. Ribosomal Resistance to Aminoglycosides.

Previously, it had been reported that 70S ribosomes from *M. purpurea* could mimic *in vitro* the gentamicin and kanamycin resistances observed in the intact organism (Piendl and Böck, 1982). When ribosomes from the clone, *S. lividans* JR14, were supplemented with S100* from the parental strain, *S. lividans* TSK1, and assayed in cell-free protein synthesising systems, it was not surprising to find that they were indeed resistant to both these antibiotics (see Fig. 7.1).

Subsequently, 50S and 30S ribosomal subunits, prepared from *S. lividans* JR14 and the parental strain, were reformed in all four possible combinations to generate 70S particles. When the latter were assayed as before in poly U-dependent protein synthesising systems, resistance to kanamycin and gentamicin was shown to be associated with the 30S ribosomal subunit from strain JR14. These data also agreed with earlier results, which had implied that ribosomes from *M. purpurea* owed their resistance to gentamicin to some property of their 30S subunits (Piendl and Böck, 1982).

Finally, using reconstituted 30S ribosomal particles, experiments were carried out to determine whether 16S rRNA or one or more of the proteins in the 30S subunit could be linked with aminoglycoside resistance in strain JR14. To do this, total proteins (TP30) and 16S RNA were prepared from 30S ribosomal subunits from *S. lividans* JR14 and the parental strain, *S. lividans* TSK1, and combined in all four possible modes to regenerate 30S particles. These reconstituted particles were then assayed together with S100* and 50S ribosomal subunits from the
Legend to Fig. 7.1.

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine by 70S ribosomes.

Assays contained S100* from *S. lividans* TSK1 together with 5 pmol ribosomes from (○) *S. lividans* parental strain TSK1 (100% activity = 32 phe per ribosome h⁻¹);

(●) *S. lividans* clone JR14 (100% activity = 37 phe per ribosome h⁻¹).

Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h⁻¹).
Fig 7.1

Kanamycin

Gentamicin

Antibiotic Concentration (µg/ml)

Activity (%)
parental strain, in poly U-directed protein synthesis. The results indicated that resistance to kanamycin and gentamicin was associated with 16S rRNA from the clone, \textit{S. lividans} JR14 (see Fig. 7.2). Again, these data agreed with earlier reports, which had suggested that 16S rRNA was responsible for ribosomal resistance to kanamycin and gentamicin in \textit{M. purpurea} (Piendl \textit{et al.}, 1984).


The data presented so far in this chapter suggested that resistance to specific aminoglycosides in \textit{S. lividans} strains TSK412 and JR14 was associated with the same ribosomal component, namely 16S RNA. Therefore, as discussed above for \textit{S. lividans} TSK412, it was possible that 16S rRNA in strain JR14 may have acquired its ability to confer resistance, either due to post-transcriptional modification or as a result of some alteration in the nucleotide sequence of the RNA, or both. Since the piece of \textit{M. purpurea} DNA in pLST14 was about 3kb in length and only about 1.5kb of DNA would have been required to encode the entire 16S rRNA molecule, it was possible that a rRNA gene might have been cloned in \textit{S. lividans} JR14. However, a series of Northern and Southern hybridisation experiments, similar to those performed with pLST41, proved that pLST14 did not contain a ribosomal RNA gene. Therefore, it appeared that the 16S rRNA in \textit{S. lividans} JR14 must have been altered by post-transcriptional modification.

Previous studies had demonstrated that methylation of 23S rRNA was responsible for ribosomal resistance to thiostrepton and erythromycin, as discussed in the previous chapter. Also, since methylation of 16S rRNA had already been shown to confer ribosomal resistance to kanamycin
Legend to Fig. 7.2

Effect of Aminoglycosides on Poly U-directed Polyphenylalanine Synthesis by Homologously and Heterologously Reconstituted 30S Particles.

Assays contained 5 pmol 50S subunits and S100* from \textit{S. lividans} TSK1 (parental strain) together with 5 pmol reconstituted 30S subunits: (●) 16S rRNA and TP30 both from \textit{S. lividans} JR14 (100% activity = 11 phe per ribosome h$^{-1}$); (○) 16S rRNA from \textit{S. lividans} JR14 and TP30 from \textit{S. lividans} TSK1 (100% activity = 10 phe per ribosome h$^{-1}$); (■) 16S rRNA and TP30 both from \textit{S. lividans} TSK1 (100% activity = 7 phe per ribosome h$^{-1}$); (□) 16S rRNA from \textit{S. lividans} TSK1 and TP30 from \textit{S. lividans} JR14 (100% activity = 8 phe per ribosome h$^{-1}$). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h$^{-1}$).
and apramycin in S. lividans TSK412, it seemed logical to attempt to isolate methylease activity from S. lividans JR14, which would confer resistance to kanamycin and gentamicin. Unfortunately, these experiments were complicated by the observation that neither 30S ribosomal subunits nor 16S rRNA prepared from strain TSK1 (parental) were substrates for methylation by an S100\(^*\) preparation from strain JR14 (used as the source of methylase). However, when 30S ribosomal subunits from E. coli were used as substrate together with \(^{[1]}\)H-S-adenosyl-methionine as cofactor, and S100\(^*\) from strain JR14, radiolabelled methyl groups were incorporated into 16S rRNA. In control incubations, S100\(^*\) from the parental strain was used to screen for the possible presence of rRNA methylases present in S. lividans, but not in E. coli and not involved in resistance. Although \(^{[1]}\)H-methyl groups were incorporated under these conditions, the levels of incorporation were much higher when extracts from strain JR14 were used. The difference in incorporation was equivalent to a stoichiometry of about 0.5 methyl groups introduced into each molecule of 16S rRNA.

In order to establish a causal connection between methylase activity from S. lividans JR14 and ribosomal resistance to aminoglycosides, E. coli 30S ribosomal subunits were preincubated with S100\(^*\) from strain JR14 or from the parental strain, in the presence or absence of unlabelled S-adenosyl-methionine. Following supplementation with S100\(^*\) and untreated 50S subunits from the parental strain, the response of these particles to aminoglycosides was assessed in cell-free, protein synthesising systems (see Fig. 7.3). Resistance was observed only when 30S particles had been pre-incubated with S-adenosyl-methionine together with S100\(^*\) from S. lividans JR14.
Legend to Fig. 7.3.

Effect of Methylation of *E. coli* 30S Ribosomal Subunits on their Ability to Support Poly U-directed Polyphenylalanine Synthesis in the Presence or Absence of Aminoglycosides.

Ribosomal 30S subunits (5 pmol) were preincubated with S100* either from *S. lividans* clone JR14 or *S. lividans* parental strain TSK1 in the presence or absence of S-adenosyl methionine. Then they were supplemented with 5 pmol *E. coli* 50S subunits and S100* from *S. lividans* TSK1 and assayed in cell-free protein synthesis in the absence of drugs (○), or in the presence either of gentamicin (●) (1μg ml⁻¹ final concentration) or kanamycin (■) (100μg ml⁻¹ final concentration).
Fig 7:3

[\text{Phenylalanine incorporated (cpm x 10^{-3})}]$

- JR14 extract
  - plus SAM
  - minus SAM

- control extract
  - plus SAM
  - minus SAM

Time (min)
In conclusion, S. lividans JR14 was shown to possess methylase activity (possibly a single enzyme) which was capable of rendering E. coli ribosomes resistant to gentamicin and kanamycin. It is, therefore, reasonable to assume that a similar mechanism is responsible for ribosomal resistance to these aminoglycosides in M. purpurea.
The results presented here suggest that ribosomal resistance to aminoglycosides in \textit{M. purpurea} depends upon the state of methylation of 16S rRNA. Furthermore, since similar levels and patterns of aminoglycoside resistance are observed in \textit{S. lividans} JR14 and in \textit{M. purpurea}, it is possible that such methylation may be sufficient to account entirely for the resistance phenotype of this producing organism. On the other hand, previous studies had suggested that although the 30S ribosomal subunits from \textit{M. purpurea} played a major role in determining the response of ribosomes to aminoglycosides, the 50S subunits might also contribute to the resistance of these ribosomes (Piendl and Böck, 1982; Piendl \textit{et al.}, 1984). In those experiments, hybrid Micromonospora ribosomes were constructed and those containing 50S subunits from \textit{M. purpurea} were observed to respond differently to gentamicin when compared with 70S particles including 50S subunits from an aminoglycoside-sensitive strain, \textit{Micromonospora melanosporea}. Accordingly, the 50S particle from \textit{M. purpurea} could not be excluded completely from considerations of resistance to gentamicin. However, the present data do not require any such interpretation. Indeed, when the molar ratios of gentamicin to ribosomes used here were compared with those employed previously (Piendl \textit{et al.}, 1984), there was no obvious difference between the resistance levels of 70S particles from \textit{S. lividans} JR14 and those from \textit{M. purpurea}. On that basis, it appears that the resistance properties of ribosomes from \textit{M. purpurea} may result solely from the methylation of 16S rRNA.

At present, it is not known how many methyl groups are incorporated into 16S rRNA by the aminoglycoside-resistance methylase(s) in
S.lividans JR14. On the one hand, the data presented here suggest that since the stoichiometry of methylation of 16S rRNA is less than unity after subtraction of the 'background' incorporation, only a single methyl group might be required to render ribosomes resistant to both kanamycin and gentamicin. On the other hand, it is possible that only a subpopulation of the 30S ribosomal subunits were suitable substrates for methylation and that the actual stoichiometry of modification might have been higher than that observed. In this context, it is also worth noting that the cloned insert in pLST14 is about 3kb in length and, therefore, S.lividans JR14 might have acquired more than one aminoglycoside-resistance determinant (and perhaps more than one resistance methylase) from M.purpurea. These problems hopefully will be resolved by subcloning the insert in pLST14, to determine the minimal amount of DNA required for resistance to individual or multiple aminoglycosides.

When the data presented here from investigations on S.lividans JR14 are compared with those from equivalent studies on S.lividans TSK412, it is interesting that the basic mechanisms of aminoglycoside resistance present in both strains, are associated with methylation of 16S rRNA. However, in order to account for the different patterns of aminoglycoside resistance displayed by these two clones, presumably there is a difference in the number and/or location of the methylated residues within 16S rRNA. Indirect evidence in support of this hypothesis is provided when one considers that the aminoglycoside-resistance methylases derived from S.lividans strains JR14 and TSK412 have different substrate specificities: the former requires 30S ribosomal subunits from E.coli, whilst the latter acts on 30S particles from S.lividans TSK1. (Further experimental evidence to suggest that
CHAPTER 7

these resistance enzymes have different sites of action within 16S rRNA prepared from *S. lividans* TSK1, is presented in the following chapter).

The fact that at least two aminoglycoside-resistance methylases act on 16S rRNA is important when considering the primary target sites of aminoglycosides, especially as these antibiotics have been reported to bind to both ribosomal subunits (for a review, see Gale *et al.*, 1981). When the sites of action of these resistance methylases (derived from strains JR14 and TSK412), have been located within 16S rRNA, the target sites for aminoglycosides hopefully will be more clearly defined. It will also be interesting to examine the effects of specific methylation of 16S rRNA on the ribosomal response to aminoglycosides, in partial reactions of protein synthesis, as discussed in the previous chapter. On the basis of such results, it might be possible to relate a defined region of 16S rRNA to a specific functional domain of the ribosome.
CHAPTER 8

AMINOGLYCOSIDE RESISTANCE IN CLONES OF STREPTOMYCES LIVIDANS CONTAINING DNA FROM STREPTOMYCES TENERARIUS
1. INTRODUCTION.

This chapter describes investigations concerning the mechanism(s) of resistance to aminoglycosides that exist in the nebramycin producer, *S. tenebrarius*. The nebramycin antibiotic complex contains at least seven 'factors' which include kanamycin B and tobramycin (see Fig. 2.1) and their 6'-O-carbamoyl derivatives, as well as the novel aminoglycoside, apramycin (see Fig. 2.4). In addition to being resistant to its endogenous products, *S. tenebrarius* is also refractory to a wide range of other aminoglycosides (Yamamoto et al., 1982). At least two of the genes involved in resistance to these antibiotics have been cloned separately in *S. lividans* (see Chapter 5 for details). First, the determinant(s) responsible for resistance to kanamycin and gentamicin and secondly the gene(s) involved in kanamycin and apramycin resistance, have been isolated in *S. lividans* strains TSK31 and TSK51 respectively. (For full details on MIC values, refer to Table 5.1, page 79). The biochemical basis of aminoglycoside resistance in these two clones has been investigated, although the mechanisms involved have not been characterised as fully as those present in *S. lividans* strains TSK412 and JR14, as described in the preceding two chapters.
2. RESULTS.


Results from previous studies had suggested that ribosomes from _S. tenebrarius_ were resistant _in vitro_ to a variety of aminoglycosides, including kanamycin and gentamicin (Yamamoto _et al._, 1982). Therefore, as _S. lividans_ TSK31 was refractory to these and other aminoglycosides _in vivo_, initial experiments were carried out to ascertain whether the ribosomes from this organism were responsible for its resistance phenotype. In these investigations, 70S ribosomes prepared from _S. lividans_ TSK31, _S. lividans_ TSK1 (parental strain) and _S. tenebrarius_, were each supplemented with S100 from the parental strain and assayed for their response to a variety of aminoglycosides in a cell-free, protein synthesising system (see Figs. 8.1 and 8.2). Ribosomes from _S. lividans_ TSK31 were highly resistant to kanamycin, tobramycin and gentamicin, but sensitive to apramycin and neomycin.

Thus, the profile of ribosomal resistances observed _in vitro_ for strain TSK31, closely mirrored the _in vivo_ resistance pattern (see Table 5.1, page 79, for MIC data), and suggested that this strain owed its multiple resistance phenotype to some property of its 70S particles. In addition, these results showed that ribosomes from _S. tenebrarius_ were resistant to all the aminoglycosides tested (albeit to only low levels of neomycin), as was the intact organism (refer to Table 5.1). These data were compatible with previous reports which had demonstrated that ribosomes from this producer were resistant to kanamycin and gentamicin (Yamamoto _et al._, 1982) and also to apramycin and low levels of neomycin (Piendl _et al._, 1984).
Legend to Fig. 8.1.

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine.

Assays contained S100* from *S. lividans* TSK1 together with 5pmol 70S ribosomes from (■) *S. lividans* TSK31 (100% activity = 21 phe per ribosome h⁻¹): (○) *S. tenebrarius* (100% activity = 15 phe per ribosome h⁻¹): (●) *S. lividans* TSK1, parental strain (100% activity = 15 phe per ribosome h⁻¹). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h⁻¹).
Legend to Fig. 8.2

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine.

Assays contained S100* from *S. lividans* TSK1 together with 5 pmol 70S ribosomes from: (■) *S. lividans* TSK31 (100% activity = 21 phe per ribosome h⁻¹): (○) *S. tenebrarius* (100% activity = 15 phe per ribosome h⁻¹): (●) *S. lividans* TSK1, parental strain (100% activity = 15 phe per ribosome h⁻¹). Activities in drug-free controls are given as pmol phenylalanine incorporated per pmol ribosomes per hour (phe per ribosome h⁻¹).
At this stage in the investigations, the mechanism(s) of resistance to kanamycin and gentamicin in *S. lividans* JR14 had been determined, as described in the preceding chapter. When those results were compared with the data presented so far on *S. lividans* TSK31, it was obvious that the two strains were not only resistant to the same aminoglycosides in vivo (see Table 5.1 for MIC data), but also that the ribosomes from both clones showed similar patterns and equivalent levels of aminoglycoside resistance in vitro. On that basis, it was tempting to suggest that similar mechanisms of resistance might be operating in *S. lividans* strains JR14 and TSK31. If so, ribosomes from strain TSK31 might be rendered resistant to aminoglycosides by specific methylation of 16S rRNA. Evidence in support of this hypothesis was obtained when 30S ribosomal subunits from *S. lividans* TSK31 were shown to confer resistance to kanamycin. In these investigations, hybrid 70S ribosomes were generated using 30S and 50S subunits derived from *S. lividans* TSK31 and the parental strain (*S. lividans* TSK1). All four combinations of particles were constructed and after supplementation with S100* prepared from the parental strain, their responses to kanamycin were assessed in poly U-directed protein synthesis (see Fig. 8.3). Resistance to kanamycin was clearly associated with ribosomes in which the 30S subunit was derived from the clone. These results were compatible with previous reports, which suggested that 30S ribosomal subunits from *S. tenebrarius* were responsible for resistance to kanamycin and gentamicin (Piendl *et al.*, 1984). Further indirect evidence that ribosomes from strain TSK31 might owe their gentamicin resistance to some property of their 16S rRNA, also came from studies on *S. tenebrarius* ribosomes (Piendl *et al.*, 1984). Those investigations revealed that 16S rRNA from this producing organism was capable of
Legend to Fig. 8.3

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine by Homologous or Heterologous Combinations of Ribosomal Subunits.

Assays contained S100* from *S. lividans* TSK1 (parental strain) together with 5pmol 30S and 5pmol 50S ribosomal subunits, as indicated below. Assays were performed in the absence (●) or presence (○) of kanamycin, 100 μg ml⁻¹ final concentration.

Panel A. 30S and 50S particles both from *S. lividans* TSK1.
Panel B. 30S particles from *S. lividans* TSK1 and 50S particles from *S. lividans* TSK31.
Panel C. 30S and 50S particles from *S. lividans* TSK31.
Panel D. 30S particles from *S. lividans* TSK31 and 50S particles from *S. lividans* TSK1.
Fig 8.3

(A) Phenylalanine incorporated (cpm x 10^-3)

(B) Phenylalanine incorporated (cpm x 10^-3)

(C) Phenylalanine incorporated (cpm x 10^-3)

(D) Phenylalanine incorporated (cpm x 10^-3)

Time (min)
CHAPTER 8

conferring gentamicin resistance on reconstituted 30S subunits which contained ribosomal proteins from \textit{S. lividans} (wild type).

Taken collectively, these results strongly suggested (but did not prove) that the mechanisms of resistance to kanamycin and gentamicin in \textit{S. lividans} strains TSK31 and JR14 were similar, if not identical. (Further evidence in support of this hypothesis is discussed in a later section of this chapter).

2.2. Aminoglycoside Resistance in \textit{S. lividans} TSK51.

The aminoglycoside-resistance phenotype of \textit{S. lividans} TSK51 (see Table 5.1, page 79), revealed that, unlike strain TSK31, this clone was resistant to apramycin. In addition, these results demonstrated that the resistance patterns of \textit{S. lividans} strains TSK51 and TSK412 were indistinguishable. Therefore, preliminary investigations were carried out to determine whether the resistance profile of strain TSK51 could be attributed to some property of its ribosomes. To do this, ribosomes derived from this clone were supplemented with S100* prepared from the parental strain and assayed in poly U-dependent protein synthesising systems, in the presence and absence of various aminoglycosides. (The concentrations of antibiotics included in these assays were chosen on the basis of previous results obtained with ribosomes from \textit{S. lividans} TSK412 and the parental strain, see Fig. 6.7). The results (shown in Fig. 8.4), clearly demonstrated that 70S particles from \textit{S. lividans} TSK51 were resistant to kanamycin, apramycin, sisomicin and gentamicin (albeit only low-level resistance in the latter case). That being so, it appeared that ribosomes from strain TSK51 not only mimicked the resistance character of the intact organism, but also displayed a
CHAPTER 8

Legend to Fig. 8.4

Effect of Aminoglycosides on Poly U-directed Synthesis of Polyphenylalanine.

Assays contained S100* from S. lividans TSK1 together with 5pmol 70S ribosomes from S. lividans TSK51, and were performed in the absence (●) or presence (○) of aminoglycosides, as indicated, each at 10μg ml⁻¹ final concentration.
pattern of aminoglycoside resistance similar to that seen with 70S particles from *S. lividans* TSK412. Therefore, it was tempting to speculate that *S. lividans* strains TSK51 and TSK412 might possess similar mechanisms of aminoglycoside resistance. If so, ribosomes from strain TSK51 would owe their resistance to kanamycin and apramycin to specific methylation of 16S rRNA. Evidence compatible with this hypothesis was provided by studies on aminoglycoside resistance in *S. tenebrarius*. The results suggested that the 30S ribosomal subunits from this organism were responsible for resistance to kanamycin and apramycin (Piendl et al., 1984). (Further evidence in support of this hypothesis is presented below).

2.3. Aminoglycoside-Resistance Methytransferases and their Sites of Action.

At this stage in the investigations, it was clear that the aminoglycoside-resistant clones of *S. lividans*, discussed above, could be divided into two groups, according to the patterns of antibiotic resistance displayed in vitro by their ribosomes. The first group included *S. lividans* strains TSK412 and TSK51, whose ribosomes were resistant to kanamycin and apramycin, and the second group contained *S. lividans* strains TSK31 and JR14, which possessed kanamycin and gentamicin-resistant ribosomes. It has been suggested that members of both groups owe their resistance properties to methylation of their 16S rRNA. However, assuming that this is the sole mechanism of aminoglycoside resistance present in these strains, one would need to predict (in order to account for the different resistance profiles) that the sites of modification of 16S rRNA cannot be identical in both of these groups. The aim of the experiments described below was to
test this hypothesis.

According to this idea, members of a given group should possess similar aminoglycoside-resistance methylases, perhaps with identical sites of action within 16S rRNA. On that basis, one might expect that the methylase responsible for kanamycin and apramycin resistance in \textit{S. lividans} TSK412 would be incapable of introducing methyl groups into 16S rRNA in 30S ribosomal subunits prepared from strain TSK51. This was confirmed when 30S ribosomal subunits derived from \textit{S. lividans} TSK51 showed no detectable methylation during incubation with the S100 prepared from strain TSK412 in the presence of \( ^{3}H \)-S-adenosyl-methionine as cofactor (see Fig. 8.5). In contrast, in control incubations, 30S particles from the parental strain (\textit{S. lividans} TSK1), were methylated to the expected levels by the extract from strain TSK412. The simplest explanation of these data is that strain TSK51 already contains an aminoglycoside-resistance methylase, which acts at the same site(s) as the equivalent enzyme present in TSK412. However, these results do not exclude the possibility that the putative enzyme(s) responsible for aminoglycoside resistance in \textit{S. lividans} TSK51 might also act at additional sites, or that the ribosomes from strain TSK51 are rendered resistant to aminoglycosides due to an alteration in the nucleotide sequence of the 16S rRNA.

Unfortunately, analogous experiments could not be carried out with \textit{S. lividans} strains JR14 and TSK31, because the kanamycin-gentamicin resistance methylase(s) present in strain JR14 did not act on \textit{Streptomyces} ribosomal particles \textit{in vitro}, instead 30S subunits derived from \textit{E. coli} ribosomes were usually employed as substrate. Nevertheless, other investigations, described in the next section, provided data which were compatible with the hypothesis that these two
Legend to Fig. 8.5

Methylation of 30S Ribosomal Subunits by Methylase Extract from *S. lividans* TSK412.

Assays contained S100 from *S. lividans* TSK412 together with [methyl-\(^{3}H\)]-S-adenosyl-methionine, as cofactor, plus 30S ribosomal subunits prepared from various strains of *S. lividans*: (●) strain TSK51; (□) strain TSK1; (○) strain TSK31; (■) strain JR14. Time-independent background 'incorporation' into 30S particles from strain TSK41 has been subtracted.
Fig 8.5

$[\text{H}^3] \text{Methyl groups incorporated}$

(pmol/pmol 16S RNA)

Time (min)
clones possess similar aminoglycoside-resistance methylases.

In order to examine whether strains with different aminoglycoside-resistance phenotypes possess resistance methylases with different sites of action upon 16S rRNA, another series of methylation assays was carried out. The rationale was as follows. The kanamycin-apramycin resistance enzyme present in \textit{S. lividans} TSK412 should be capable of modifying 30S ribosomal subunits from \textit{S. lividans} JR14 (and also from strain TSK31), thereby rendering them resistant to apramycin. This was confirmed when 30S ribosomal subunits from \textit{S. lividans} strain JR14 and TSK31 were incubated with extracts prepared from strain TSK412 with \[^{3}H\]-S-adenosyl-methionine as cofactor. Under such conditions, radiolabelled methyl groups were incorporated into the 16S rRNA of these particles (see Fig. 8.5). As predicted, when these methylation assays were repeated using unlabelled cofactor, the resulting modified particles proved to be apramycin resistant when supplemented with untreated 50S subunits and S100*, (both from the parental strain) and assayed in a cell-free protein synthesising system (see Fig. 8.6).

These results clearly demonstrate that the kanamycin-apramycin resistance methylases in \textit{S. lividans} TSK412 and, by inference, in strain TSK51, act differently from the methylase(s) responsible for kanamycin and gentamicin resistance in \textit{S. lividans} JR14 and, by inference, in strain TSK31. On the basis of these results, it is clear that there is more than one way of methylating 16S rRNA to give resistance to kanamycin.
Legend to Fig. 8.6 A-D

Correlation Between Methylation of 30S Ribosomal Subunits and Resistance to Apramycin.

Ribosomal 30S subunits (5pmol) from *S. lividans* JR14 (panel A) or *S. lividans* TSK31 (panel C), were preincubated in the presence of S-adenosyl-methionine and 2.5% (v/v) S100 from *S. lividans* TSK412 (as the source of the kanamycin-apramycin resistance methylase) for 20 min at 35°C. Ribosomal particles were then ethanol-precipitated before being introduced into a cell-free protein synthesising system directed by poly U. Ribosomal 30S subunits (5pmol) from *S. lividans* JR14 (panel B) or *S. lividans* TSK31 (panel D), were introduced directly into protein synthesising systems, without preincubation or ethanol-precipitation. All protein synthesis assays also contained 5pmol 50S subunits and S100*, both from the parental strain, and were performed in the absence (●), or presence (○), of apramycin, 10 μg ml⁻¹ final concentration.
2.4. Homology Between Aminoglycoside-Determinants Cloned from Different Producing Organisms.

Strains of \textit{S. lividans} that are presumed to possess similar aminoglycoside-resistance methylases would be expected to contain related resistance determinants. Accordingly, from the results presented so far, one would predict that the genes responsible for resistance to kanamycin and apramycin in \textit{S. lividans} strains TSK412 and TSK51, might possess considerable sequence homology. Similarly, the DNA sequences of the genes conferring resistance to kanamycin and gentamicin in \textit{S. lividans} strains JR14 and TSK31 might be related.

In the experiments described below, hybridisation techniques have been employed to investigate whether there is any evidence to support these hypotheses. Plasmid DNA isolated from the aminoglycoside-resistant strains of \textit{S. lividans}: TSK31, TSK51, TSK412 and JR14, together with vector pIJ702, were digested to completion with Bcl I, and the restriction fragments were separated by electrophoresis in an agarose gel. These fragments were then transferred onto a nylon membrane filter, (see Chapter 3, page 55 et seq., for full details of Southern blotting and hybridisation techniques). Two identical filters were prepared, and one was probed with the kanamycin-apramycin resistance determinant present in \textit{S. lividans} TSK412, and the other with the kanamycin-gentamicin resistance determinant(s) present in \textit{S. lividans} JR14. The fragments of DNA used as the radioactive probes in these experiments were prepared as follows: the gene conferring aminoglycoside resistance in pLST412 was isolated as a \textit{Pst I} - \textit{Sph I} restriction fragment, about 0.8kb in length (see Fig. 6.5, for a restriction map of pLST412), and the determinant(s) responsible for
aminoglycoside resistance in plST14 was prepared as a Bgl II - Sph I restriction fragment, about 1.9kb in length. (see Fig. 5.5, for restriction map of plST14). Clearly, the latter fragment did not encompass all the U. purpurea DNA isolated in plST14, however, a subcloning experiment carried out by David Holmes in this laboratory had demonstrated that this piece of U. purpurea DNA contained the kanamycin-gentamicin resistance determinant(s). Both of these fragments were radiolabelled using random hexanucleotide primers and DNA polymerase I, to direct the incorporation of $^{32}$P-AdCTP. 

As expected, when the kanamycin-apramycin resistance determinant from plST412 was used as a probe, hybridisation with Bcl I restriction fragments originating from plST412 and plST51 was observed, even at the highest level of stringency (see Fig. 8.7). These complementary fragments contained the tyrosinase gene plus the determinant(s) conferring aminoglycoside resistance. Since no hybridisation was observed with the tyrosinase gene in the gel-track containing plJ702, this implied that the kanamycin-apramycin resistance genes in plST412 and plST51 (and, by inference, in S. tenjimariensis and S. tenebrarius), possessed at least 85% homology with respect to their DNA sequences. (A faint band of radioactive material was also detectable in most gel-tracks and represented hybridisation of probe DNA with the 1.7kb Bcl I fragment common to all these plasmids, as indicated in Fig. 6.6.

The simplest explanation of this observation was that the probe was contaminated with small amounts of this plJ702-derived fragment.

Hybridisation experiments involving the kanamycin-gentamicin resistance determinant(s) from plST14 as the radioactive probe, also generated the expected results (see Fig. 8.8). Hybridisation with fragments derived from plST14 and plST31 was observed, even at the
Legend to Fig. 8.7.

Hybridisation of the 'Kanamycin-Apramycin' Resistance Gene from pLST412 With Other Recombinant Plasmids.

Plasmids pIJ702, pLST412, pLST31, pLST51 and pLST14 were digested with Bcl I and the DNA fragments were separated by agarose gel electrophoresis (as indicated: tracks a-e). After transfer to a nylon membrane, the fragments were hybridised with $^{32}$P-radiolabelled Sph I - Pst I restriction fragment from pLST412 which includes the kanamycin-apramycin resistance gene from S.tenjimariensis (see Fig. 6.6). The membrane was washed at 'high stringency' (0.1 x SSC at 68°C), dried and subjected to autoradiography using Fuji RX film (see Chapter 3, page 53 et seq., for further details). The autoradiogram showed that the probe hybridised with the 4.5kb Bcl I fragment from pLST51, which includes the kanamycin-apramycin resistance determinant(s) from S.tenebrarius.
Fig 8.7

-4.5kb
-2.4kb
-1.7kb

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<th>Sample</th>
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<th>pLST412</th>
<th>pLST31</th>
<th>pLST51</th>
<th>pLST14</th>
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Legend to Fig. 8.8.

Hybridisation of the 'Kanamycin-Gentamicin' Resistance Determinant(s) from pLST14 With Other Recombinant Plasmids.

Plasmids pIJ702, pLST412, pLST31, pLST51 and pLST14 were digested with Bcl I and the DNA fragments were separated by agarose gel electrophoresis (as indicated: tracks a-e). After transfer to a nylon membrane, the fragments were hybridised with \(^{32}\)P-radiolabelled Sph I - Bgl II restriction fragment from pLST14 which includes the kanamycin-gentamicin resistance gene(s) from M. purpurea (see Fig. 5.5). The membrane was washed at 'high stringency' (0.1 x SSC at 65°C), dried and subjected to autoradiography using Fuji RX film (see Chapter 3, page 53 et seq.) for further details). Autoradiography revealed that the probe hybridised with the 4.5kb Bcl I fragment from pLST31 which includes the kanamycin-gentamicin resistance determinant(s) cloned from S. tenebrarius.
<table>
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-4.5kb
highest level of stringency employed. These complementary fragments consisted of the tyrosinase gene, in addition to the aminoglycoside-resistance determinant(s). As no hybridisation was observed with the tyrosinase gene in the track containing pIJ702, this implied that the DNA sequence of the aminoglycoside-resistance determinant(s) in pLST14 and pLST31 (and, by inference, *M. purpurea* and *S. tenebrarius*) had at least 85% homology.

In conclusion, the data from these hybridisation experiments provided further evidence that *S. lividans* strains TSK412 and TSK51 possessed similar aminoglycoside-resistance genes and that these were different from the determinants conferring resistance to kanamycin and gentamicin common to *S. lividans* strains JR14 and TSK31.
3. DISCUSSION.

At least two aminoglycoside-resistance determinants have been isolated from the nebramycin producer, *S.tenebrarius*, and together these genes can account for most of the multiple aminoglycoside-resistance phenotype of this organism. Furthermore, since the ribosomes from the two aminoglycoside-resistant clones, *S.lividans* strains TSK31 and TSK51, mimic *in vitro* the resistance character of their respective intact organisms, neither strain need necessarily contain enzymes capable of inactivating the aminoglycosides studied here. However, neither of these clones resembles *S.tenebrarius* in its response to neomycin and related antibiotics, such as lividomycin and paromomycin. (*S.tenebrarius* is highly resistant to these compounds *in vivo*). Although ribosomes from *S.tenebrarius* are resistant to low levels of neomycin *in vitro* (see Fig. 8.2), previous reports had suggested that this organism also contained an acetyltransferase enzyme (AAC 2') that was capable of inactivating neomycin and related antibiotics *in vitro* (Yamamoto *et al.*, 1982). On that basis, it is possible that *S.tenebrarius* contains two resistance mechanisms which complement one another to confer high-level resistance to neomycin. However, it is important to emphasise that it is currently not clear whether the AAC enzyme does indeed contribute to the observed resistance phenotype of *S.tenebrarius*. As an alternative possibility, this producer might possess a partially effective permeability barrier to neomycin-like antibiotics, which operates in conjunction with the ribosomal resistance, thereby rendering *S.tenebrarius* highly resistant to these aminoglycosides *in vivo*. Resolution of this matter must await further investigation.
Although the mechanisms of ribosomal resistance that exist in \textit{S. lividans} strains TSK31 and TSK51 have not been characterised directly, the data presented here strongly suggest, but do not prove, that both of these strains owe their aminoglycoside-resistance properties to methylation of their 16S rRNA. Furthermore, since \textit{S. lividans} strains TSK51 and TSK412 are thought to contain homologous resistance determinants and similarly, strains TSK31 and JR14 might possess related resistance genes, it appears that \textit{S. tenebrarius} might contain aminoglycoside-resistance determinants that resemble those present in \textit{S. tenjimariensis} and \textit{M. purpurea}. Further evidence that these genes are homologous could be obtained by DNA sequencing and detailed restriction mapping of the cloned determinants. Also, using a given aminoglycoside-resistance gene as the radioactive probe, it would be interesting to carry out hybridisation experiments, to identify whether other aminoglycoside-producing organisms contain similar resistance determinants.

This leads on to the broader question of how many different aminoglycoside-resistance methylases exist in the various organisms which produce these antibiotics. In this context, it is worth noting that when compared with \textit{M. purpurea}, other producers of gentamicin-like compounds share strikingly similar phenotypic responses to aminoglycosides (Matkovic \textit{et al.}, 1984). Moreover, in each of these organisms, the 30S ribosomal subunit is thought to be responsible for the resistance properties. Accordingly, it is possible that all producers of gentamicin-like antibiotics might share with \textit{M. purpurea}, a common mode of ribosomal resistance involving specific methylation of 16S rRNA. If so, these organisms might all possess similar aminoglycoside-resistance determinants.
CHAPTER 8

Recent studies on the kanamycin producer, *S. kanamyceticus* have also provided some interesting results relating to the mechanism(s) of aminoglycoside resistance that exist in this organism (Murakami et al., 1983; Nakano et al., 1984). In those investigations, the determinant(s) conferring resistance to kanamycin, gentamicin and sisomicin were transferred from *S. kanamyceticus* into *S. lividans* using vector pIJ702. One of the resultant clones appeared to possess a similar pattern of aminoglycoside resistance *in vivo* to that observed with *S. lividans* strains JR14 and TSK31 and also with *M. purpurea*, although the response of the clone to apramycin was not determined. It is not yet clear whether aminoglycoside resistance in *S. kanamyceticus* is caused by methylation of rRNA, although in view the results presented here, one is tempted to hypothesise that this will prove to be the case. In support of this idea, biochemical analysis of the *S. lividans* clone containing the resistance gene(s) from *S. kanamyceticus*, revealed that the ribosomes were responsible for resistance to kanamycin. Thus, it is possible that the similar aminoglycoside-resistance phenotypes of *S. kanamyceticus* and *M. purpurea* are determined by homologous resistance genes.

It may be that specific methylation of 16S rRNA is a mechanism of aminoglycoside resistance that is prevalent among organisms which produce these antibiotics. However, it is interesting that the methylases characterised here confer resistance to antibiotics belonging to the kanamycin and gentamicin families (as well as the novel aminoglycoside, apramycin), but not to neomycin-like antibiotics. A possible explanation for this phenomenon is based on the chemical structures of these drugs (refer to Figs. 2.1, 2.2, 2.3.). Although the kanamycins and gentamicins have very different
substituents on their aminosugar moieties, both families contain a 2-deoxystreptamine nucleus which is disubstituted at positions 4 and 6, whereas in neomycin-like antibiotics, the 2-deoxystreptamine moiety is 4,5-disubstituted. Consequently, these two classes of aminoglycosides will possess radically different stereochemical structures and from the results presented here, presumably bind to separate sites on the ribosome. Evidence in support of this hypothesis was provided by studies on the binding of radiolabelled paromomycin to 70S ribosomes from E. coli (Lando et al., 1976). The results implied that paromomycin could bind specifically to a single site on these ribosomes and that related compounds (e.g. neomycin B, neomycin C and lividomycin A), were effective competitors for this ribosomal binding site. On the other hand, gentamicin C was a poor competitor and kanamycin A did not compete at all with paromomycin, suggesting that these two aminoglycosides probably bind to other sites within the ribosome.

In conclusion, ribosomes can be rendered resistant to different aminoglycosides by post-transcriptional methylation of separate sites within 16S rRNA. Thus, resistance to gentamicin is conferred by modification of 16S rRNA at a different location to that which renders ribosomes resistant to apramycin. This implies that these two aminoglycosides possess different ribosomal binding sites and, therefore, they presumably do not share a common mode of action. On the other hand, it is interesting that there are two ways of methylating 16S rRNA to confer ribosomal resistance to kanamycin. It is possible that this antibiotic has two, or more, functional target sites on the ribosome and, consequently, it has multiple effects on protein synthesis, as suggested previously (Jelenc and Kurland, 1984). An alternative possibility is that the kanamycin-apramycin resistance
methylase and the kanamycin-gentamicin resistance enzyme(s) act at adjacent sites within 16S rRNA, as it exists in the intact ribosome and, therefore, that modification by either enzyme can prevent the binding of kanamycin. Hopefully, the aminoglycoside-resistance determinants which have been isolated during the course of this work, will provide useful tools for further investigation of some of these hypotheses, particularly with respect to the analysis of the mode of action of specific aminoglycosides.
CHAPTER 9

GENERAL DISCUSSION
CHAPTER 9


Early hypotheses on ribosomal function were based on the idea that the protein components of the ribosome performed functional roles, whereas the rRNA merely provided a scaffold for the assembly of the various proteins (Fellner, 1974). However, recent studies have revealed that rRNAs also participate directly in the translational process. For example, affinity labelling experiments have suggested that peptidyl transferase activity is associated with 23S rRNA in E.coli (Breitmeyer and Noller, 1976; Greenwall et al., 1974; Barta et al., 1984). Similarly, 16S rRNA has been strongly implicated in ribosomal binding of both mRNA (Fiser et al., 1977; Towbin and Elson, 1978) and tRNA (e.g. Prince et al., 1982, as discussed below).

Genetic studies have also provided evidence that rRNA plays a major role in the translational functions of the ribosome. Many of these investigations have involved mitochondria, which usually have only a single set of rRNA genes, in contrast to most eubacteria. This has facilitated the isolation of mutants containing rRNA genes with point mutations. These single base changes in mitochondrial rRNA sequences have been shown to confer dramatic functional alterations of ribosomes, particularly with respect to their sensitivity to specific antibiotics (Sor and Fukuhara, 1982; Li et al., 1982; Kearsey and Craig, 1981, see below for further details). Recently, however, using a multicopy plasmid containing a rRNA operon (rrnH) from E.coli, it has been possible to isolate antibiotic-resistant mutants of this organism, with altered 16S and 23S rRNA sequences (Sigmund et al., 1984; Ettayebi et al., 1985, as discussed below). Other minor alterations in rRNA (but not proteins) have also been shown to cause drastic effects on
translation. For example, the toxic proteins colicin E3 and alpha sarcin, completely inactivate protein synthesis by cleavage, in each case, of single phosphodiester bonds in rRNA (Senior and Holland, 1971; Chan et al., 1983).

Taken collectively, the above data provide strong support for the idea that rRNA plays a direct role in translation. Indeed, given that rRNA comprises about two thirds the mass of the bacterial ribosome, it is, perhaps, not surprising that it is directly involved in the functioning of this organelle. In this context, it is worth considering the possible functional roles of rRNA from an evolutionary point of view. On the one hand, if ribosomal proteins were originally important in defining the translational interactions that occur during protein synthesis, it is difficult to imagine how these proteins would have been synthesised. On the other hand, if the primitive translational machinery was defined by nucleic acids, it is possible, at least in principle, that ribosomal proteins evolved later to modulate the translational activity of rRNA and, thereby, improve the speed and fidelity of protein synthesis. In agreement with the latter idea, rRNAs from various organisms have been shown to possess highly conserved sequences and secondary structural elements (Woese et al., 1983). Thus, it appears that a knowledge of rRNA structure is of fundamental importance to increasing our understanding of the overall topology, as well as the functioning of the ribosome.

Although the sequence and secondary structure of 23S, 16S and 5S rRNAs from E.coli are known in considerable detail (Noller, 1984; Brimacombe et al., 1983), the three-dimensional packing of these rRNAs into a 220Å diameter ribosomal particle is only poorly understood. Similarly, very little is known about the functional roles of rRNA.
However, results from several experimental approaches have recently provided evidence that specific regions of the rRNA are involved in particular functions (for a review, see Noller, 1984). These data are outlined briefly below.

2. Structure-Function Relationships in 23S Ribosomal RNA.

Early studies, using affinity probes of the peptidyl transferase site of the ribosome had suggested that 23S rRNA was directly involved in this step of protein synthesis (Breitmeyer and Noller, 1976; Greenwall et al., 1974), although the precise position of the reaction with rRNA has only recently been established (Barta et al., 1984). The main site of modification was shown to be U-2584, within 23S rRNA. Interestingly, this residue is close in the secondary structure of 23S RNA to several rRNA mutations which have been shown to confer resistance to antibiotics that impair peptide bond formation. For example, chloramphenicol-resistance mutations mapping in 23S-like RNA genes have been found in yeast, mouse and human mitochondria, at positions corresponding to residues 2447, 2451, 2503 and 2504 in E.coli 23S rRNA (Dujon, 1980; Kearsey and Craig, 1981; Blanc et al., 1981a, b). Also, an erythromycin-resistance mutation in yeast mitochondria has been shown to result from a base substitution at a position corresponding to residue 2058 in E.coli 23S rRNA (Sor and Fukuara, 1982), which lies in the same domain of the RNA as the chloramphenicol-resistance mutations discussed above. Further evidence that residue 2058 in E.coli 23S rRNA is involved with the binding of erythromycin to the bacterial ribosome, was provided by studies on S. erythraeus, the producer of erythromycin. This antibiotic-producing
organism contains a methylase, which introduces two methyl groups into 23S rRNA from *B. stearothermophilus*, thereby rendering ribosomes containing such modified RNA resistant to specific MLS-type antibiotics (Skinner and Cundliffe, 1982). The site of action of the 'erythromycin-resistance methylase' from *S. erythraeus* within *B. stearothermophilus* 23S RNA was determined and was shown to correspond to residue 2058 in *E. coli* 23S rRNA (Skinner et al., 1983: see also Chapter 6, page 103 et seq.) Since chloramphenicol and some of the macrolides impair peptide bond formation, (for a review see Gale et al., 1981), the region of 23S rRNA containing the corresponding sites of mutation which confer resistance to these antibiotics, may well be present in the peptidyl transferase centre of the prokaryotic ribosome.

Recent evidence in support of this hypothesis was provided by the isolation of chloramphenicol and erythromycin-resistant mutants of *E. coli*, which contained a rRNA operon (rrnH), on a multicopy plasmid. The sites of mutation were localised to residues 2057 and 2058 within the 23S rRNA (Ettayebi et al., 1985: Sigmund et al., 1984).

The base changes in 23S-like rRNAs, discussed above, are clustered around a highly conserved region of rRNA (Noller, 1984). These extensive sequence homologies not only allow correlation of specific bases in the primary sequence between rRNAs from a wide variety of sources, but also, perhaps even more importantly, indicate that this region should play a crucial functional role in the ribosome.

The GTPase centre of the ribosome, which is the target site for thiostrepton (Gale et al., 1981), has also been localised within 23S rRNA. Methylation of A-1067 in the sequence of 23S RNA has been shown to confer ribosomal resistance to thiostrepton (Thompson et al., 1982d),
CHAPTER 9

indicating that this region of 23S RNA is involved in ribosomal GTPase functions (for further details, see Chapter 1, page 12 et seq.)

Finally, the region of 23S rRNA concerned with elongation factor binding of tRNA has been localised, since this interaction is blocked by the activity of a cytotoxin, alpha sarcin (Hobden and Cundliffe, 1978), which introduces a single cut after G-2661 of 23S rRNA (Chan et al., 1983).

Collectively, these data help us to build up a preliminary picture of the regions of 23S rRNA that are concerned with particular ribosomal functions, although it is obvious that a great deal of research remains to be done in this field. It is worth emphasising again, the value of antibiotics in elucidating such ribosomal structure-function relationships. Indeed, it is striking that this type of experimental approach has generated most of the evidence that is currently available on this subject.

3. Structure-Function Relationships in 16S rRNA.

In the context of the work presented in this dissertation, it is particularly relevant to consider the experimental evidence for relationships between ribosomal structure and function in 16S rRNA (for a review, see Thompson and Hearst, 1983).

3.1. Initiation of Protein Synthesis and mRNA Binding.

The role of 16S rRNA in recognising and binding mRNA in the initiation complex, is well established (Shine and Dalgarno, 1974; Steitz and Jakes, 1972). A comparative analysis of a large number of
mRNA sequences showed that there was a varying degree of complementarity between mRNA at a position about 10 nucleotides to the 5' side of the initiator codon and the conserved CCUCC sequence at position 1535-1539 of 16S rRNA, close to the 3' terminus. Furthermore, initiation factor IF-3, which is involved in promoting the dissociation of vacant 70S ribosomes and the binding of mRNA during the initiation of translation (for a review, see Grunberg-Manago, 1980), has been cross-linked to 16S rRNA (Cooperman et al., 1977). At least one of the binding sites of IF-3 has been located towards the 3' terminus of 16S rRNA (Wickstrom, 1983). In addition, this region of 16S RNA contains two dimethylated adenine residues, which are responsible for determining the sensitivity of the ribosome to kasugamycin (Helser et al., 1972). In this context, it is interesting that this antibiotic has been claimed to interfere with the initiation of protein synthesis (Okuyama et al., 1971). Therefore, three independent lines of evidence imply that the 3'-terminal domain of 16S rRNA is involved in the initiation phase of protein synthesis.

3.2. Binding of tRNA and Decoding.

Several groups have suggested that 16S rRNA is directly involved in the binding of tRNA and the decoding of mRNA. For example, when N-acetylvalyl-tRNA was bound to the ribosomal P site (peptidyl-tRNA site), a modified base in the wobble position of the codon loop of tRNA was shown to cross-link to C-1400 in *E. coli* 16S rRNA (Prince et al., 1982; Ehresmann et al., 1984). Furthermore, analogous cross-linking has been found in yeast (Ofengand et al., 1982; Ehresmann et al., 1984) and *Artemia salina* (Ciesolka et al., 1985a). Interestingly, when
photoaffinity probes were attached to the 5' anticodon site of valyl-tRNA bound to the ribosomal A site (aminoacyl-tRNA site), the major cross-linked product again involved C-1400 of 16S rRNA (Ciesolka et al., 1985b). The significance of these data is that they place the ribosomal site of codon-anticodon interaction in the middle of one of the most highly conserved sequences known, the 1390-1410 region of 16S rRNA (Noller, 1984). The function of this interaction between 16S rRNA and the tRNA anticodon is not yet clear. However, the fact that both the sequence and the proximity of 16S RNA to the anticodon is conserved in many species is a strong indication that this region of 16S rRNA plays an important role in the tRNA binding and decoding function of the ribosome.

4. Aminoglycoside-Resistance Methyldases and Predicted Sites of Action Within 16S rRNA.

Aminoglycosides have been shown to interfere with the translocation step of protein synthesis and to cause misreading of RNA transcripts (Davies and Davis, 1968; Davies et al., 1965). On this basis, it is possible that the sites of action of the aminoglycoside-resistance methylases studied here, might lie within the ribosomal domain(s) concerned with decoding the mRNA. It has been suggested that C-1400 in 16S rRNA might lie within such a domain of the ribosome, as discussed above and, therefore, it is tempting to speculate that the aminoglycoside-resistance methylases might modify 16S RNA within the vicinity of this residue. Further evidence to support this hypothesis is discussed below.

The results on the substrate specificities of these resistance
methylases, revealed that 30S ribosomal subunits, but not 70S particles, could be modified. This suggested that these enzymes might be acting upon a region of 16S rRNA which is situated at the subunit interface of 70S particles, or on a sequence which is exposed on 30S subunits, but unavailable on 70S monomers, perhaps due to a conformational change associated with the interaction of 30S and 50S subparticles. In this context, it is worth mentioning previous studies, which involved the use of single strand and double strand-specific probes, to assign particular residues of 16S rRNA to the region of the subunit interface of 70S ribosomes. These residues were rendered resistant to such probes, when 30S and 50S ribosomal subunits associated to form 70S particles (Chapman and Noller, 1977; Vassilenko et al., 1981). Several of these protected sites, that were crucial for intermolecular interaction between the large and small ribosomal subunits, were located towards the 3' terminus of 16S rRNA (Herr et al., 1979). Therefore, it appears that this region of rRNA is located at the subunit interface of 70S ribosomes, and might be available for modification by the aminoglycoside-resistance methylases in 30S subparticles.

Previous studies on various aminoglycoside-resistant mutants have provided further evidence that the 3' terminal sequence of 16S rRNA might contain the sites of action of the aminoglycoside-resistance methylases. Resistance to hygromycin and to paromomycin in Tetrahymena, has been associated with point mutations towards the 3' end of small subunit (17S) ribosomal RNA (Spangler and Blackburn, 1985). The mutation to hygromycin resistance was shown to be a U to C mutation at position 1711 of 17S rRNA (corresponding to position 1496 in E.coli 16S rRNA). Interestingly, this residue lies within a
sequence of 14 nucleotides which is essentially unchanged throughout evolution, implying that it is functionally important in the translation process. In each of the three paromomycin-resistant mutants of *Tetrahymena* that were isolated, a G to A transition was found at position 1707 in 17S rRNA (equivalent to position 1492 in *E. coli* 16S rRNA). This point mutation disrupts the first base pair of a large hairpin loop, which is a highly conserved secondary structure located near the 3' terminus of rRNA. It is striking that the paromomycin-resistance mutation which has been located in yeast mitochondrial 15S rRNA, affects the opposite member of that base pair, since it is a C to G transition at the residue corresponding to position 1409 in *E. coli* 16S rRNA (Li *et al.*, 1982). Therefore, both paromomycin-resistance mutations might be expected to have the same effect on rRNA secondary structure, and presumably, the resultant change in conformation impairs the binding of paromomycin to the ribosome. Since paromomycin induces extensive misreading and hygromycin impairs the translocation step of protein synthesis in *Tetrahymena* (Eustice and Wilhelm, 1984a:b), it is interesting that the point mutations in rRNA that are associated with resistance to these aminoglycosides, have been located close to the region of 16S rRNA which has been cross-linked to the anticodons of several tRNAs.

Previous studies concerning the bacteriotoxin, colicin E3, also suggested that aminoglycosides interact with the ribosomal domain containing the 3' terminal region of 16S rRNA. Normally, this toxin inactivates intact 70S ribosomes from *E. coli*, by cleaving an oligonucleotide ('E-3 fragment') 49 residues long, from the 3' end of 16S RNA (Bowman *et al.*, 1971: Senior and Holland, 1971). However, in the presence of certain antibiotics, this cleavage was prevented.
(Dahlberg et al., 1973). In particular, ribosomes that had been pretreated with tetracycline, which blocks the binding of aminoacyl-tRNA to the ribosomal A site (for a review, see Gale et al., 1981) or with gentamicin, were protected from subsequent inactivation by colicin E3. Of course, it is possible that these antibiotics cause conformational changes in the ribosome, so that the site of action of the toxin is no longer available. However, in view of the evidence discussed above, it is likely that gentamicin, at least, might prevent cleavage of 16S rRNA by colicin E3, as a result of direct steric hinderance. It is interesting that although the E-3 fragment contains the geminal pair of dimethylated adenine residues, which determine the response of the ribosome to kasugamycin, this antibiotic did not protect ribosomes from the lethal action of colicin E3. On the basis of these results, it appears that kasugamycin (which inhibits the initiation of protein synthesis) and gentamicin possess separate binding sites on the ribosome and consequently these two antibiotics might have different modes of action.

In conclusion, the 3' terminal region of 16S rRNA clearly plays a functionally important role in the translation process, particularly in the initiation and decoding phases of protein synthesis. The evidence discussed above, strongly implies that aminoglycoside antibiotics interact with this domain of 16S rRNA. It is, therefore, tempting and plausible to speculate that the sites of action of the aminoglycoside-resistance methylases characterised here, might lie close to residue C-1400 in the 3' terminal domain of 16S rRNA.
Concluding Remarks.

The data presented in this dissertation clearly substantiate the hypothesis that ribosomal RNA plays a crucial role in the functioning of ribosomes. Moreover, the aminoglycoside-resistance genes that have been isolated, have provided novel approaches to studying the primary mode(s) of action of aminoglycosides and will hopefully enable specific regions of 16S rRNA to be assigned to particular functions in the translation process. It is hoped that such investigations will eventually answer many, if not all, the questions that have arisen during two decades of research on these antibiotics. In addition, these antibiotic-resistance determinants have obvious potential as selectable markers, in the development of new cloning vectors, particularly (but not exclusively) for use in *Streptomyces*. 
REFERENCES

Antimicrob. Ag. Chemother. **19** 798-806

BARTA, A., STEINER, G., BROSIUS, I., NOLLER, H.F.
Proc. Natl. Acad. Sci. USA. **81** 3607-3611

BENIGNI, R., ANTONOV, R.P. and CARERE, A. (1975)
Appl. Microbiol. **30**, 324-326

BENVENISTE, R. and DAVIES, J. (1973a)
Proc. Natl. Acad. Sci. USA. **70**, 2276-2280

BENVENISTE, R. and DAVIES, J. (1973b)


BIBB, M.J., SCHOTTEL, J.L. and COHEN, S.N. (1980b)
Nature **284**, 526-531

Dev. Ind. Microbiol. **21**, 55-64

BIBB, M.J., WARD, J.M., KIESER T., COHEN, S.N. and

BIBB, M.J., WARD, J.M., KIESER, T., JANSSEN, G.R., and
BLANC, H., ADAMS, C.W. and WALLACE, D.C. (1981a)
Nucleic Acids Res. 9, 5785-5795

BLANC, H., WRIGHT, C.T., BIBB, M.J., WALLACE, D.C. and
CLAYTON, D.A. (1981b)
Proc. Natl. Acad. Sci. USA. 78, 3789-3793

Biotech. Bioeng. 13, 843-864

J. Gen. Microbiol. 130, 2883-2891

BOLLEN, A., CABEZÓN, T., DEWILDE, M., VILLARROEL, R. and
HERZOG, A. (1975)
J. Mol. Biol. 99, 795-806

BOWMAN, C.M., DAHLBERG, J.E., IKEMURA, T., KONISKY, J and
NOMURA, M. (1971)
Proc. Natl. Acad. Sci. USA. 68, 964-968

BREITMEYER, J.B. and NOLLER, H.F. (1976)
J. Mol. Biol. 101, 297-306

Prog. Nucl. Acids Res. Mol. Biol. 28, 1-48

In Plasmids and Transposons: Environmental Effects and
Maintenance Mechanisms (Eds. C. Stuttard and K.R. Rozee)

BRYAN, L.E. and VAN DEN ELZEN, H.M. (1977)
Antimicrob. Ag. Chemother. 12, 163-177

Mol. Gen. Genet. 158, 47-54

CABAÑAS, M.J. VÁZQUEZ, D. and MODOLELL, J. (1978a)
CAMPUZANO, S., VÁZQUEZ, D. and MODOLELL, J. (1979)


CELLA, R. and VINING, L.C. (1975)
Canad. J. Microbiol. 21, 463-472

CHAMBLISS, G., CRAVEN, G.R., DAVIES, J., KAHAN, L.
and NOMURA, M. (Eds.) (1980)
Ribosomes - Structure, Function and Genetics
University Park Press, Baltimore.

J. Biol. Chem. 258, 12768-12770

CHAPMAN, N.M. and NOLLER, H.F. (1977)
J. Mol. Biol. 109, 131-149


CIESOLKA, J., NURSE, K., KLEIN, J. and OFENGAND, J. (1985a)
Biochemistry 24, 3233-3239

CIESOLKA, J., GORNICKI, P. and OFENGAND, J. (1985b)
Biochemistry 24, 4931-4938

COOPERMANN, B.S., DONDON, J., FINELLI, J., GRUNBERG-MANAGO, M.
and MICHELSON, A.M. (1977)
FEBS. Letts. 76, 59-63

COX, D.A., RICHARDSON, K. and ROSS, B.C. (1977)
In Topics in Antibiotic Chemistry (Ed. Sammes, P.)
Vol. 1 pp. 5-90, John Willey and Sons, Inc.
CUNDLIFE, E. (1978)

Nature 272, 792-795

CUNDLIFE, E. (1983)


J. Mol. Biol. 132, 235-252

CUNDLIFE, E. and THOMPSON, J. (1979)

Nature 278, 859-861


Biochemistry 12, 948-950


J. Virol. 33 390-400

DAVIES, J. (1980)


DAVIES, J., CHOUK, M., YAGISAWA, and WHITE, T.J. (1979)

In Genetics of Industrial Microorganisms (Eds. O.K. Sebek and A.J. Laskin) pp. 166-169 (Ash:) Washington D.C.

DAVIES, J. and DAVIS, B. (1968)

J. Biol. Chem. 243, 3312-3316

DAVIES, J., GILBERT, W. and GORINI, L. (1964)

DAVIES, J. Gorini, L. and Davies, B. (1965)

Mol. Pharmacol. 1, 93-106


In New Trends in Antibiotics: Research and Therapy
Elsevier/North-Holland Biomedical Press.


Ann. Rev. Microbiol. 32, 469-518

DEWILDE, K., CABEZÓN, T., VILLARROEL, R., HERZOG, A. and
BOLLEN, A. (1975)

Mol. Gen. Genet. 142, 19-33

DUJON, B. (1980)

Cell 20, 185-197

EHRESMANN, C., EHRESMANN, B., MILLON, R., EBEL, J.-P.,

Biochemistry 23, 429-437

ENQUIST, L. W. and BRADLEY, S. G. (1971)

Dev. Ind. Microbiol. 12, 225-236


J. Bacteriol. 162, 551-557

EUSTICE, D. C. and WILHELM, J. M. (1984a)

Biochemistry 23, 1462-1467

EUSTICE, D. C. and WILHELM, J. A. (1984b)

Antimicrob. Ag. Chemother. 26, 53-60


Mol. Gen. Genetic. 190, 394-398

FELLNER, P. (1974)

In Ribosomes (Eds. M. Nomura, A. Tissières and P. Lengyel)

154
FISER, I., SCHEIT, K.H. and KUECHLER, E. (1977)
Eur. J. Biochem. 74, 447-456

GALE, E.F., CUNDLIFFE, E., REYNOLDS, P.E., RICHMOND, M.H.
and WARING, M.J. (1981)
The Molecular Basis of Antibiotic Action.
J. Wiley and Sons, London

Gene 25, 119-132

GORINI, L. (1974)
In Ribosomes (Eds. M. Nomura, A. Tissières and P. Lengyel)

GRAHAM, M.Y. and WEISBLUM, B. (1979)
J. Bacteriol. 137, 1404-1467

GREENWALL, P., HARRIS, R.J. and SYMONS, R.H. (1974)
Eur. J. Biochem. 49, 539-554

In Ribosomes - Structure, Function and Genetics
Eds. G. Chambliss, G.R. Craven, J. Davies, L. Kahan and

J. Antimicrob. Chemother. 8, 429-445

Gene 22, 167-174

HELSER, T.L., DAVIES, J.E. and DAHLBERG, J.E. (1971)
Nature New Biol. 233, 12-14

HELSER, T.L., DAVIES, J.E. and DAHLBERG, J.E. (1972)
Nature New Biol. 235, 6-9

HERR, W., CHAPMAN, N.M. and NOLLER, H.F. (1979)
J. Mol. Biol. 130, 433-439
HIGGINS, C.E. and KASTNER, R.E. (1968)

HIGHLAND, J.H., HOWARD G.A. and GORDON, J. (1975a)
Eur. J. Biochem. 53, 313-318

HIGHLAND J.H., HOWARD, G.A., and OCHSNER, E., STÖFFLER, G.,
HASSENBANK, R. and GORDON, J. (1975b)
J. Biol. Chem. 250, 1141-1145

HOBDEN, A.N. and CUNLiffe, E. (1978)
Biochem. J. 170, 57-61

Analit. Biochem. 114, 193-197

J. Bacteriol. 162, 406-412

In Genetic Engineering Vol. 4
(Eds. Setlow and Hollaender) pp. 119-145
Plenum Press, London

Plasmid 11, 1-16

J. Gen. Microbiol. 129, 2257-2269

HOPWOOD, D.A., LYDIATE, D.J. MALPARTIDA, F.
and WRIGHT H.M. (1985)
In Plasmids in Bacteria (Eds. D.R. Helsinki, S.N. Cohen
D.B. Clewell, D.A. Jackson, A. Hollaender). pp. 615-634

J. Antibiot. 34, 1175-1182

J. Antibiot. 36, 1789-1791


J. Biol. Chem. 259, 14151-14157


Plasmid 3, 312-318


Bacterial Revs. 41, 449-474


J. Gen. Microbiol. 129, 2703-2714


Nature 290, 607-608


Mol. Gen. Genet. 185, 223-238

Kirby, R. and Hopwood, D.A. (1977)

J. Gen. Microbiol. 98, 239-252


In Microbiology - 1981 (Ed. D. Schlessinger)


Kühberger, R., Piepersberg, W., Petzet, A., Bückel, P.

and Böck, A. (1979)

Biochemistry 18, 187-193


Nature 227, 680-685

Lai, C-J, and Weisblum, B. (1971)

Proc. Natl. Acad. Sci. USA. 68, 856-860
Eur. J. Biochem. 66, 597-606

Biochemistry 16, 4743-4751

J. Biol. Chem. 257, 5921-5928


Gene 35, 223-235

MALIK, V.S., (1972)

Canad. J. Microbiol. 16, 173-179

MALIK, V.S. and VINING, L.C. (1972)
Canad. J. Microbiol. 18, 583-590

Nature 309, 462-464

J. Bacteriol. 155, 989-994

Microbiol. Revs. 44, 230-251

FEMS. Microbiol. Letts. 24, 273-276

J. Bacteriol. 99, 401-405

J. Bacteriol. 104, 8-12
MISUMI, M., NISHIMURA, T., KOMAI, T. and TANAKA, N. (1978)


MONTANDON, P-E, NICOLAS, P., SCHURMANN, P. and STUTZ, E. (1985)

Nucleic Acids Res. 13, 4299-4310

MURAKAMI, T., NOJIRI, C., TOYAMA, H., HAYASHI, E., KATUMATA, K.,
ANZAI, H., KATUHASHI, Y., YAMADA, Y.,
and NAGAOKA, K. (1983)

J. Antibiot. 36, 1305-1311


J. Bacteriol. 157, 79-83


Proc. Natl. Acad. Sci. USA. 79, 2817-2821

OKAMI, Y., HORTA, K., YOSHIDA, M., IKEDA, D., KONDO, S.
and UMEZAWA, H. (1979)

J. Antibiot. 32, 964-966


OKUYAMA, A. and TANAKA, N. (1972)


J. Gen. Microbiol. 129, 529-537

PALMER, E., WILHELM, J.M., and SHERMAN, F. (1979)

Nature 277, 148-149


PERZYNSKI, S., CANNON, M., CUNDLIFFE, E., CHAHWALA, S.
   and DAVIES, J. (1979)

   Lancet Vol. II, 311-315


   Doktorarbeit, Universitat Munchen.

   Antimicrob. Ag. Chemother. 22, 231-236

   Mol. Gen. Genet. 197, 24-29

PIRT, S.J. and RIGHELATO, R.C. (1967)
   Appl. Microbiol. 15, 1284-1290

POLDERMANS, B., ROZA, L. and VAN KNIPPERBERG, P.H. (1979)
   J. Biol. Chem 254, 9094-9100

PRINCE, J.B., TAYLOR, B.H., THURLOW, D.L., OFFENGAND, J

   Biochem. Soc. Trans. 586-587

RINEHART, K.L. and STROSHANE, R.M. (1976)
   J. Antibiot. 29, 319-353

RODICO, M.R., KING, A.A., BRUTON, C.J. SINCLAIR, R.B.,
   In John Innes Report, 1983-84 pp. 111-112

SCHMIDT, F.J., THOMPSON, J., LEE, K., DIJK, J.
and CUNDLIFFE, E. (1981)
J. Biol. Chem. 256, 12301-12305

SEMEKOV, Y.P., KATUNIN, V.I., MAKAROV, E.M.
and KIRILLOV, S.V. (1982)
FEBS. Lett. 144, 121-124

SENIOR, B.W., and HOLLAND, I.B. (1971)
Proc. Natl. Acad. Sci. USA. 68, 964-968

Proc. Natl. Acad. Sci. USA. 71, 1342-1346


Nucleic Acids Res. 12, 4653-4663

SINGH, A., URSIC, D. and DAVIES, J. (1979)
Nature 277, 146-148

SKINNER, R.H. and CUNDLIFFE, E. (1980)
J. Gen. Microbiol. 120, 95-104

J. Gen. Microbiol. 128, 2411-2416

SKINNER, R., CUNDLIFFE, E. and SCHMIDT, F.J. (1983)
J. Biol. Chem. 258, 12702-12706

Nucleic Acids Res. 10, 6571-6577

J. Biol. Chem. 260, 6334-6340

STEITZ, J.A. and JAKES, K. (1972)
Proc. Natl. Acad. Sci. USA. 72, 4734-4738

161
TAI, P-C. and DAVIS, B.D. (1979)
Biochemistry 18, 193-198

In Handbook Experimental Pharmacology Vol. 62
(Eds. Umezawa, H. and Hooper, I.R) pp. 221-266

Biochem. Biophys. Res. Commun. 72, 522-529

TANGY, F., MOUKKADEM, M., VIRIDIMIAN, E., CAPMUA, M.L.
and LEGOFFIC, F. (1985)
Eur. J. Biochem. 147, 381-386

J. Bacteriol. 120, 316-321

THOMPSON, C.J. and GRAY, G.S. (1983)
Proc. Natl. Acad. Sci. USA. 80, 5190-5194

Gene 20, 51-62

THOMPSON, C.J., SKINNER, R.H., THOMPSON, J., WARD, J.M.,
HOPWOOD, D.A. and CUNDLIFFE, E. (1982b)
J. Bacteriol. 151, 678-685

Nature 286, 525-527

J. Bacteriol. 151, 668-677

J. Gen. Microbiol. 124, 291-297

THOMPSON, J., CUNDLIFFE, E. and STARK, M. (1979)
Eur. J. Biochem. 98, 261-265
THOMPSON, J. and HEARST, J. (1983)
   Cell 33, 19-24


THOMPSON, J., SCHMIDT, F.J. and CUNDLIFFE, E. (1982d)
   J. Biol. Chem. 257, 7915-7917


THORBJARNARDOTTIR, S.H., MAGNUSDOTTIR, R.A., EGGERTSON, G.,
   KAGAN, S.A. and ANDRESSON, O.S. (1978)

TOWBIN, H. and ELSON, D. (1978)
   Nucleic Acids Res. 5, 3389-3407

TRAUB, P., MIZUSHIMA, S., LOWRY, C.V. and NOMURA, M. (1971)
   Methods in Enzymol. 20, 391-407

   J. Gen. Microbiol. 131, 1657-1669

   J. Mol. Biol. 152, 699-721

VINING, L.C. (1979)

WALKER, M.S., and WALKER, J.B. (1970)
   J. Biol. Chem. 245, 6683-6689

WALKER, M.S. and WALKER, J.B. (1971)
   J. Biol. Chem. 245, 7034-7040

WATANABE, S. and TANAKA, K. (1976)
   Biochem. Biophys. Res. Commun. 72, 522-529
WEINEN, B., EHRlich, R., STÖFFLER-MEILICKE, M., STÖFFLER, G.,
SMITH, I., WEISS, D., VINCE, R. and PESTKA, S. (1979)
J. Biol. Chem. 254, 8031-8041

WEISBLUM, B. (1975)
In Microbiology - 1974 (Ed. D. Schlessinger) pp.199-206
American Society for Microbiology, Washington DC.

WHITE, J.R. and WHITE, H.L. (1964)
Science 146, 772-774

WICKSTROM, E. (1983)
Nucleic Acids Res. 11, 2035-2052

Microbiol. Rev. 47, 621-669

YAMAKI, H. and TANAKA, N. (1963)
J. Antibiot. (Ser.A) 16, 222-226

YAMAMOTO, H., HOTTA, K., OKAMI, Y. and UMEZAWA, H. (1981a)
J. Antibiot. 34, 824-829

Biochem. Biophys. Res. Commun. 100, 1396-1401

J. Antibiot. 35, 1020-1025

ZIERHUT, G., PIEPERSBURG, W. and BÖCK, A. (1979)
Eur. J. Biochem. 98, 577-583