

**INDUCIBLE MACROLIDE AND LINCOSAMIDE  
RESISTANCE IN  
STREPTOMYCES LIVIDANS**

A dissertation submitted for the degree of  
Doctor of Philosophy  
at the  
University of Leicester

by

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**To my parents**

## Abstract

### INDUCIBLE MLS RESISTANCE IN *STREPTOMYCES LIVIDANS*

Gail Jenkins

Resistance to macrolide, lincosamide, and streptomycin B type antibiotics, is widespread amongst Gram positive bacteria and is particularly prevalent in the genus *Streptomyces*. The classical MLS resistance phenotype has, in a number of cases, been associated with N<sup>6</sup>,N<sup>6</sup> dimethylation of 23S rRNA, and more specifically to methylation of the adenine residue at a position equivalent to 2058 in *E.coli* 23S rRNA. However, phenotypes differing from that associated with classical MLS resistance have also been reported, and this study describes the analysis of one such inducible resistance phenotype found in the non-producing organism *Streptomyces lividans* TK21.

Two resistance genes have been isolated from *S.lividans*, with the aid of shotgun cloning techniques, and both have been analysed at the nucleotide level. The first, *lrm*, encodes a ribosomal RNA methylase of 36 KDa, that monomethylates the N<sup>6</sup> amino group of an adenosine residue at a position equivalent to 2058 in 23S rRNA. The second resistance gene codes for a protein of 46 KDa, and though the precise function of this protein is as yet undetermined, a distant similarity does exist between this gene product and various eukaryotic UDP-glucuronosyl transferases.

Transcription of *lrm* initiates from two promoters that lie some 253 and 377 nucleotides upstream from the translational initiation codon. The leader sequence is capable of forming a series of stable hairpin loops, and an area exists within this region that could encode a short leader peptide of 38 amino acids. It is therefore suggested that induction of *lrm* may be regulated by translational attenuation.

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## ABBREVIATIONS

|       |   |
|-------|---|
| ATP   | Adenosine 5'-triphosphate   |
| bp    | Base pairs  |
| BPB   | Bromophenol blue  |
| BSA   | Bovine serum albumin  |
| CIAP  | Calf intestinal alkaline phosphatase                                      |
| cpm   | Counts per minute   |
| CTAB  | Hexadecyltrimethylammonium bromide  |
| CTP   | Cytosine 5'-triphosphate  |
| dATP  | 2'-deoxyadenosine 5'-triphosphate   |
| dCTP  | 2'-deoxycytosine 5'-triphosphate  |
| dGTP  | 2'-deoxyguanosine 5'-triphosphate   |
| ddATP | 2',3'-dideoxyadenosine 5'-triphosphate                                    |
| ddCTP | 2',3'-dideoxycytosine 5'-triphosphate                                     |
| ddGTP | 2',3'-dideoxyguanosine 5'-triphosphate                                    |
| ddTTP | 2',3'-dideoxythymidine 5'-triphosphate                                    |
| DNA   | Deoxyribonucleic acid   |
| dNTP  | deoxynucleoside 5'-triphosphate   |
| DMSO  | Dimethyl sulphoxide   |
| DNase | Deoxyribonuclease   |
| DDT   | Dithiothreitol  |
| EDTA  | Diaminoethane tetra-acetic acid   |
| EGTA  | Ethylene glycol-bis (b-aminoethyl ether) N,N,N',N', -tetra<br>acetic acid |
| GTP   | Guanosine 5'-triphosphate   |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid                     |

|                       |   |
|-----------------------|---|
| IMS                   | Industrial Methylated Spirits                                 |
| IPTG                  | Isopropyl $\beta$ -D-thiogalactopyranoside                    |
| kb                    | Kilobase pairs  |
| kDa                   | Kilodaltons   |
| mRNA                  | Messenger ribonucleic acid                                    |
| N.C.I.B               | National Collection of Industrial Bacteria                    |
| O.D.                  | Optical density   |
| PEG                   | Polyethylene glycol   |
| PIPES                 | Piperazine-N,N'-bis [2-ethanesulphonic acid]                  |
| psi                   | pounds per square inch  |
| rev min <sup>-1</sup> | Revolutions per minute  |
| RNA                   | Ribonucleic acid  |
| rRNA                  | Ribosomal ribonucleic acid                                    |
| tRNA                  | Transfer ribonucleic acid                                     |
| S                     | Svedberg unit ( $10^{-3}$ seconds)                            |
| SDS                   | Sodium dodecylsulphate  |
| TCA                   | Trichloroacetic acid  |
| TES                   | N-tris (hydroxymethyl) methyl-2-aminoethane<br>sulphonic acid |
| TEMED                 | N,N,N',N',-tetramethylethylenediamine                         |
| Tris                  | Tris(hydroxymethyl) aminomethane                              |
| TTP                   | Thymidine 5'-triphosphate                                     |
| UTP                   | Uridine 5'-triphosphate                                       |
| UWGCG                 | University of Wisconsin Genetics Computer Group               |
| (v/v)                 | Ratio of volume to volume                                     |
| (w/v)                 | Ratio of weight to volume                                     |
| X-gal                 | 5-bromo-4-chloro-3-indolyl $\beta$ -D-galactopyranoside       |

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# **CHAPTER 1**

## **INTRODUCTION**



## Introduction

*Actinomycetes* include more than thirty genera of gram-positive bacteria that show branching filamentous, or irregular rod-shaped cell morphology. The genus *Streptomyces* is an important member of this order and has been particularly useful to both the agricultural and medical professions over the years. Biodegradation by these organisms is extremely beneficial in waste removal and plays an integral part in the recycling of materials in nature; a number of mesophilic *Streptomyces* are able to decompose lignocellulose from plant residues, by producing a variety of cellulases, xylanases, amylases, and ligninases. Other beneficial activities relating to agriculture include the production of fungicides, herbicides and antiparasitic agents with bialophos, an antibiotic initially isolated from *Streptomyces viridochromogenes*, and subsequently found as a product of *Streptomyces hygroscopicus* by Meiji (Kondo, et al., 1973), being one example of a streptomycete product with antibacterial, antifungal, and herbicidal activities. Avermectin, produced by *Streptomyces avermitilis*, was first isolated by Merck, Sharpe, and Dohme (Campbell, et al., 1983), and is an example of an antiparasitic drug which has exceptional activity against nematode and arthropod parasites but lacks antibiotic activity against bacteria and fungi.

The greatest contribution made by *Streptomyces* to the medical world is the production of antibiotics, but other useful secondary metabolites being discovered include immunodepressants, immunostimulants, and enzyme inhibitors with examples of the latter including inhibitors of angiotensin converting enzyme, glycosidases, and proteases which may prove useful in the treatment of hypertension, diabetes, and cancer respectively.

### 1.1. Control of Differentiation and Secondary Metabolism in *Streptomyces*.

In addition to their commercial value, scientific interest has focused on *Streptomyces* as interesting organisms in their own right. They are morphologically complex prokaryotes, undergoing a process of colony development that depends on differential gene expression. During their life cycle, a branching network of substrate mycelium gives rise to aerial hyphae that subsequently develop into chains of mature spores. The biosynthesis of many antibiotics occurs after the most active period of vegetative growth and is intrinsically linked with the onset of aerial mycelium and spore development.

In *Streptomyces*, as in the majority of unicellular bacteria, there is a tendency for genes concerned with the same biochemical pathway, including those for antibiotic biosynthesis, to be grouped together in clusters, though evidence so far suggests that such clusters will rarely be found to represent single transcriptional units. In contrast to this physical linkage of genes for biochemical pathways, the genes involved in morphological development are somewhat scattered. In recent years considerable progress has been made in understanding the complex network of processes involved in the switch from primary to secondary metabolism, and the concurrence of morphological and biochemical differentiation indicates the existence of common mechanisms for the regulation of genes involved in both of these processes.

An important regulatory factor that controls secondary metabolism and cell differentiation in a number of actinomycetes is the compound A-factor, which is encoded by the gene *afsA* in *S.coelicolor* and is required for the

initiation of sporulation and antibiotic production in that strain [Horinouchi, et al., 1983] (Hara, et al., 1983). It is however thought that the *afsA* gene product may condense two pre-existing primary metabolites to give active A-factor. In addition, two further regulatory genes, *afsB* and *afsC*, have been isolated from *S.coelicolor*, and both appear to be needed for production or enhancement of production of A-factor and the four antibiotics produced by *S.coelicolor*, namely undecylprodigiosin (Rudd and Hopwood, 1980), methylenomycin (Wright and Hopwood, 1976), actinorhodin (Wright and Hopwood, 1976), and a calcium dependent antibiotic (Hopwood and Wright, 1983).

The *afsB* product appears to be a DNA binding protein (Horinouchi, et al., 1986) and has been shown to have a positive effect on actinorhodin production by stimulating the *act* genes (Horinouchi, et al., 1989). The second gene *afsC* was found as an open reading frame contiguous with *afsB* following DNA sequencing and appears to act by enhancing the effect of the *afsB* gene product.

A-factor has also been found to be essential for streptomycin production and sporulation in *S.griseus*. Preliminary data have suggested that A-factor switches on transcription of the streptomycin-6-phosphotransferase gene which, along with the adjacent *strR* gene is the key gene for both streptomycin resistance and biosynthesis in the strain (Hara and Beppu, 1982) (Distler, et al., 1987). The product of the *strR* gene is important in regulating the transcription of other genes in the streptomycin biosynthetic pathway; for example *strB1* (a putative amidinotransferase) is only expressed in the presence of a functional *strR* gene (Distler, et al., 1987). However, transcription from the *strB1* promoter can occur in the absence of the *strR* protein implying that *strR* does not encode a sigma factor, and it has

therefore been proposed that the *strR* product might act as an antitermination factor. The transcriptional activities in the central segment of the *str* gene cluster are shown in Fig1.1, and from this it has been postulated that full length transcripts initiating from *strB1* are only produced if the *strR* protein binds to a sequence immediately upstream of the *strB1* coding sequence, thereby preventing premature termination of transcription.

Specific binding proteins for A-factor have recently been identified (Miyake, et al., 1989) and a simple model has been proposed for regulation of gene expression by A-factor. In the absence of A-factor, these binding proteins are thought to attach to some specific region of DNA, presumably the promoter regions, and repress expression of specific genes. However, when A-factor is present it binds to the latter proteins, and results in their release from DNA thereby enabling transcription of the previously repressed genes. The timing of expression of the genes in question may therefore be determined by the concentration of A-factor in the cell, in that transcription of certain genes may be repressed by these binding proteins until A-factor is produced within the cell, immediately prior to the onset of sporulation and antibiotic production (Miyake, et al., 1990). Support for this hypothesis has been provided by the observation that mutants of *S.griseus* capable of streptomycin production and sporulation in the absence of A-factor are also found to be defective in A-factor binding protein, and reversal of this defect in the latter protein results in simultaneous loss of streptomycin production and sporulation.

A number of other genes have also been isolated that are involved in morphological development, these are the so called *bld* and *whi* genes. Mutations within the *bld* genes (except in *bldC*) are pleotropic, in that they not only abolish aerial mycelium formation, but also prevent production of

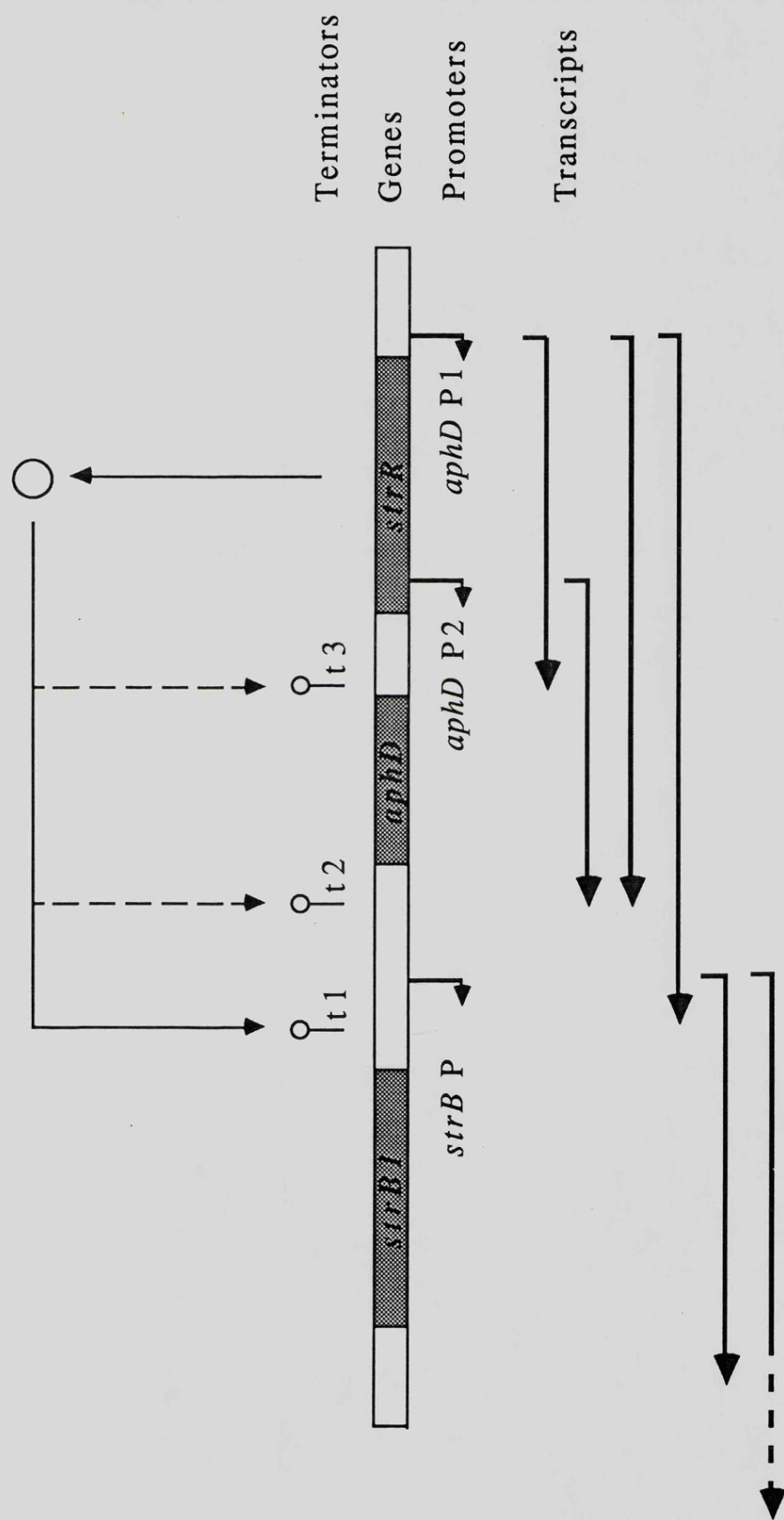


Fig. 1.1. Predicted model for the regulation of *strB1* expression, in the streptomycin biosynthetic pathway of *S. griseus*. The promoters were identified by S1 nuclease mapping, with *t1* to *t3* indicating the putative terminators. Activation of *strB1* is thought to occur by antitermination via the *strR* gene product

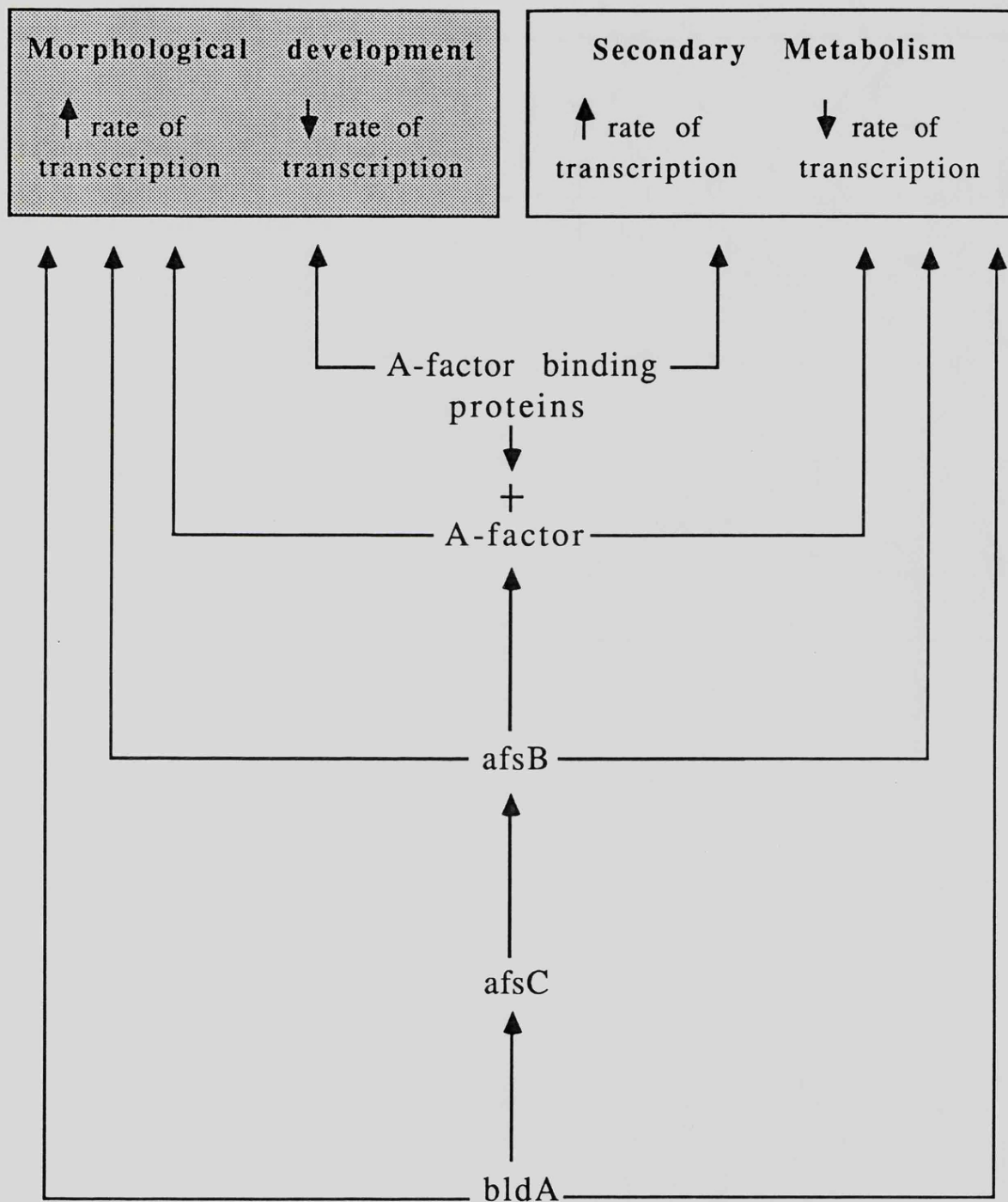
the four antibiotics found in *S.coelicolor*, and given that the typical white mycelium does not form in these mutated strains, they are referred to as being 'bald'. The link between this *bld* phenotype and antibiotic production has been broken by mutagenesis of a *bldA* strain where mutants have been isolated that are capable of undecylprodigiosin biosynthesis but remain unable to sporulate, and the involvement of at least one locus (*pwb*-pigmented while bald) has been demonstrated. It seems likely that the influence of *bldA* on undecylprodigiosin production is *via* a regulatory gene and further information concerning this may lead to identification of gene(s) involved in a complex network that links secondary metabolism and differentiation.

The *bldA* gene is also present in *S.lividans*, a close relative of *S.coelicolor*, and by sequence comparison has been shown to encode a t-RNA whose anticodon would base pair with the UUA codon for leucine (Lawlor, et al., 1987). However, since the G+C content of *Streptomyces* DNA is extremely high (70-75%), UUA codons would not be expected to occur very often in *Streptomyces* genes. Three genes containing TTA codons, (*lacZ* from *E.coli*, a spectinomycin resistance gene, and a macrolide/lincosamide resistance gene *carB* from *S.thermotolerans*) were introduced into *bldA* mutants of *S.lividans* and *S.coelicolor*, and were shown to be expressed only in *bldA*<sup>+</sup> background, which strongly suggests that the *bldA* product is the principal tRNA by which TTA codons can be translated in these two strains. Since *bldA* mutants grow well, it seems likely that genes involved in vegetative growth contain few TTA codons. As expected such codons are extremely rare in the *Streptomyces* genes that have been sequenced and have only been found in genes relating to antibiotic synthesis and resistance. This would provide an obvious mechanism by which *bldA* could control physiological and morphological differentiation. If TTA codons are confined to some or all of the genes

involved in such processes and *bldA* is only expressed in the late stages of growth, then production of aerial mycelium and antibiotic production would be confined to such a time as when *bldA* is expressed. Indeed, RNA transcripts from *bldA* are much more abundant in old cultures than in young (Lawlor, et al., 1987). The above hypothesis appears to be ruled out however, in the case of morphological development, since genes for aerial mycelium production and sporulation are all expressed when *bldA* mutants are grown on mannose as a carbon source, as opposed to glucose; in addition, four of the *whi* genes referred to below are expressed in *bldA* mutants. In the case of morphological development, *bldA* may simply divert specific nutrients into production of aerial mycelium by regulating the expression of genes involved in such pathways. Conversely, in the absence of *bldA*, alternative biochemical pathways may be followed which utilise different carbon sources.

Possibly *bldA* expression is closely connected with that of *afsB*, although the sequence of this latter gene has been determined and does not contain any TTA codons. however, given that its expression is apparently influenced by *afsC*, the presence of any TTA codons within the latter gene sequence could provide a simple pathway by which secondary metabolism and cell differentiation may be linked (Fig. 1.2).

A further set of genes involved in sporulation are the *whi* genes, so called because mutations within them appear to block subsequent sporulation and result in the colonies retaining the immature white colour of aerial mycelium. The 9 *whi* genes so far identified have been found scattered around the chromosome; three have been cloned, and one, *whiG*, is thought to encode a putative sigma factor (Gribskov and Burgess, 1986). Since *whiG* mRNA is more abundant during aerial mycelium formation than in earlier vegetative growth (Chater, et al., 1988) it has been suggested that the



**Fig 1.2. Suggested pathway for the control of differentiation in *Streptomyces*.** The genes involved in morphological and physiological development may be controlled by a series of regulatory proteins that interact with each other



increased availability of the *whiG* gene product during the late growth phase leads to subsequent sporulation. Presumably, the sigma factor encoded by *whiG* is necessary for the transcription of genes involved in sporulation. The molecular role of other *whi* genes is unknown at present but it is likely that they are structural genes for the components of aerial hyphae or mature spores.

The production of alternative sigma factors is now thought to be a further important mechanism in the co-ordinate regulation of gene expression in *Streptomyces*, in that altering the specificity of the RNA polymerase holoenzyme may permit the selective expression of discrete gene sets. Multiple forms of RNA polymerase were initially detected in *E.coli* and *B.subtilis*. The major sigma factor in *E.coli* ( $\sigma^{70}$ ), encoded by the *rpoD* gene is approximately 70 000 daltons in size (Burgess, et al., 1969), and holoenzymes containing this sigma factor are responsible for the initiation of transcription from the majority of *E.coli* promoters, which exhibit two hexameric conserved nucleotide sequences, centred approximately 35 and 10 base pairs upstream of the transcript start point and separated from each other by about 16-18 bp (Hawley and McClure, 1983). The characterisation of a large number of *E.coli* promoters has allowed a consensus sequence of TTGACA for the -35 region and TATAAT for the -10 region to be obtained (Pribnow, 1975), although no natural promoter has yet been found to exhibit both a perfect -35 and -10 sequence.

Alternative sigma factors were first demonstrated in *E.coli* following studies on heat shock proteins, when synthesis of these proteins was found to increase if *E.coli* was shifted to higher temperatures. This response was subsequently attributed to a novel sigma factor ( $\sigma^{32}$ ) encoded by *rpoH*,

whose levels were increased following heat shock and which was responsible for increased initiation of transcription from heat shock promoters (Grossman, et al., 1984). Presumably as a result of the temperature upshift, the increased levels of  $\sigma^{32}$  directly affect transcription of the genes encoding heat shock proteins; alternatively the increase in temperature may affect transcription of other factors such as activators or repressors which themselves regulate expression from heat shock promoters. Following the isolation of  $\sigma^{32}$ , a number of other alternative sigma factors have now been described in *E.coli*.

To date, nine holoenzyme forms have been isolated from *B.subtilis*, with at least three being involved in the transcription of sporulation (*spo*) genes (Losick and Youngman, 1984). The principal sigma factor, encoded by *rpoD*, is  $\sigma^{43}$  (formerly  $\sigma^{55}$ ). The principle holoenzyme  $E\sigma^{43}$  is equivalent to  $E\sigma^{70}$  of *E.coli*, in its ability to direct transcription initiation at promoters with the canonical sequences TTGACA and TATAAT centred approximately 35 and 10 bp preceding the transcription start. These are the so called *veg* promoters (Moran, et al., 1982). Unlike *E.coli*, *B.subtilis* exhibits programmed gene expression changing in response to nitrogen, phosphorus, and carbon, in a transition from vegetative growth to stationary phase. It is therefore not surprising that a large number of sigma factors conferring a variety of promoter recognition specificities have been detected in *B.subtilis*. Vegetative cells of *B.subtilis* contain at least three minor species of sigma factor, two of which,  $\sigma^{37}$  and  $\sigma^{32}$ , are responsible for transcription initiation of genes switched on after the exponential phase of growth (Ollington, et al., 1981, Wang and Doi, 1984). In particular two sporulation genes, *spoVG* and *spoVC*, are recognised by these latter two holoenzymes, but not by  $E\sigma^{43}$ , the major polymerase associated with vegetative growth. Presumably the

delayed production of  $\sigma^{37}$  and  $\sigma^{32}$  prevents transcription of sporulation genes during early vegetative growth, until the sigma factors are expressed at the onset of sporulation, under the control of a regulatory gene *spoOH*. In addition a third gene *ctc*, expressed after the exponential phase of growth and shown to be discrete from but adjacent to *spoVC*, is transcribed by  $E\sigma^{37}$  and  $E\sigma^{32}$ . The promoter region of *ctc* has been well characterised and shown to differ somewhat from the consensus sequence described for the *veg* promoter (Igo and Losick, 1986).

The third sigma factor present in vegetative cells of *B.subtilis* is  $\sigma^{28}$ , which has been found to initiate transcription from *E.coli* heat shock promoters *in vitro*, suggesting its possible analogy to *E.coli*  $\sigma^{32}$  (Briat, et al., 1985). Sporulating cells of *B. subtilis* contain a number of sigma factors whose synthesis is specifically associated with the course of spore formation, and in particular,  $\sigma^{29}$  continues to direct transcription from *spoVG* and *spoVC* as well as a further sporulation gene *spoIIG*, which is not recognised by  $\sigma^{43}$  or  $\sigma^{37}$ .

It would therefore appear that an important feature of differential gene expression in *Bacillus* is the existence of multiple forms of RNA polymerase, each with the ability to utilise a characteristic set of promoters, and it was this information that led to the suggestion that differential gene expression may be regulated in a similar way in *Streptomyces*. When RNA polymerase from *Streptomyces* was initially analysed it was shown to resemble those of other prokaryotes, in having a core structure  $\alpha_2\beta\beta'$  (Chater and Cooper, 1975), and several promoters from *E.coli* and *Bacillus*, closely resembling the consensus prokaryotic sequence have been shown to function in *S.lividans* (Bibb and Cohen, 1982). This latter streptomycete must therefore possess an

RNA polymerase that recognises and uses *E.coli* transcriptional signals, despite the high G+C content of *Streptomyces* DNA.

Furthermore, when the *ampC*  $\beta$ -lactamase gene of *E.coli*, whose promoter region has close similarity to the consensus sequence of *E.coli* vegetative promoters, was cloned in *S.lividans*, mutations in the -10, -35, and intervening spacer regions of the promoter that alter the level of transcription in *E. coli* had comparable effects in *S.lividans* (Jaurin and Cohen, 1984). When up-promoter mutations were made in these areas creating sequences which more closely resembled the 'consensus' promoter, those which led to an increase in promoter efficiency in *E.coli* also led to an increase in transcription of *ampC* mRNA in *S.lividans*; however the base substitutions involved did not lead to the same degree of change in both organisms. This was perhaps to be anticipated since *S.lividans* DNA has a high G+C content typical of *Streptomyces*, and the RNA polymerase of *S.lividans* recognising the *ampC* promoter has probably evolved in such a way as to yield optimal promoter activity with 'consensus' sequences that differ slightly from those of *E.coli*. Consistent with this idea is the observation that *ampC* mRNA levels were 5-fold higher when wild type *ampC* was present in *E.coli* than when it was present in *S.lividans*, although a difference in gene copy number or in the rate of mRNA degradation between the two species could also explain these data. Promoters have since been isolated from *Streptomyces* that resemble typical *E. coli* promoters in base composition and structural organisation and have been termed SEP's (*Streptomyces-E.coli* like promoters) (Jaurin and Cohen, 1985); they do, however, display a pattern of sequence conservation and multiple direct repeats that appears to be unique to *Streptomyces* genes. Such promoters are expressed in *E.coli*, and the level of transcription is somewhat higher than when the same promoter is present in *S.lividans*. The possibility exists

therefore that these promoters may be subject to some form of regulation in *Streptomyces*, possibly mediated *via* the direct repeat sequences, whilst existing in a derepressed state in *E.coli*.

It is believed that SEP's constitute a relatively minor fraction of promoters in *Streptomyces* (~2.5%), since a large number of *Streptomyces* DNA fragments with promoter activity in *S.lividans* failed to be transcribed in *E.coli* (Bibb and Cohen, 1982). A number of *Streptomyces* promoters have now been characterised (Table 1.1) which appear to have regions similar to the prokaryotic consensus sequence for vegetative promoters, and are presumably recognised by the *veg* polymerase of *S. lividans* (some differences do exist and a *Streptomyces* consensus sequence is identified in Table 1.1). However, despite the apparent similarity in sequence, these *Streptomyces* promoters failed to be expressed in *E.coli*, the reason for which is unknown. Possibly a distinguishing feature of promoters outside the -10 and -35 regions, such as overall A+T content may play a part in the ability of promoters to be used by heterologous hosts. The promoters of *E.coli* and *Bacillus* are exceptionally high in A+T content (Banner, et al., 1983), whilst *Streptomyces* promoters on the other hand are remarkably deficient in A-T base pairs. It is possible therefore that the high G+C content of *Streptomyces* DNA, particularly in and around its promoter sequences, may be irrelevant to promoter use in that genus, but presents a significant barrier to transcription initiation in other hosts. Alternatively the high G+C content of *Streptomyces* promoters may prevent strand separation by *E.coli* or *Bacillus* RNA polymerase, hence preventing transcriptional initiation.

Some *Streptomyces* promoters have also been identified that show either resemblance to the prokaryotic consensus in the -10 region only or show no resemblance at all to the canonical sequences (See Table 1.1). The

Table 1.1 Promoter regions of some streptomycete genes

| Promoter                         | -45 region to -35 region | -10 region   | mRNA start |
|----------------------------------|--------------------------|--------------|------------|
| <i>E.coli</i> <sup>a</sup>       | TTGACa                   | TAtAaT       |            |
| <i>gyI</i> P1                    | TTGACG                   | GAGACT       | -8 bp- U   |
| <i>gyI</i> P2                    | TCGAAC                   | TAGAGT       | -6 bp- A   |
| <i>ermE</i> P1                   | TGGACA                   | TAGGAT       | -6 bp- G   |
| <i>ermE</i> P2                   | TTGACG                   | GAGGAT       | -6 bp- C   |
| <i>Streptomyces</i> <sup>a</sup> | TTGaca                   | tAGgaT       |            |
| <i>aph</i> P1                    | CGAAAGCGCGGAACGGGT       | CATGAT       | -8 bp- A   |
| <i>aph</i> P2                    | CGGTGGGGGATTCCGGCCGA     | CCATGT       | -8 bp- U   |
| <i>tsr</i> P1                    | TTGCCGGTCAGGGCAGCCAT     | TAGGGT       | -6 bp- A   |
| <i>tsr</i> P2                    | GCTCGACGCAGCCAGAAAT      | AATACT       | -6 bp- U   |
| <i>endoH</i>                     | ATTGCATGATTGA            | CCGGCGGCAGGG | -7 bp- G   |

<sup>a</sup> consensus sequence

significance of these variations seems to reflect transcription by alternative forms of RNA polymerase, and distinct forms of the holoenzyme have indeed been detected in *S.coelicolor*. During heterologous complementation experiments, RNA polymerase associated polypeptides from *S.coelicolor* were tested in the presence of core RNA polymerase from *B.subtilis* for their ability to stimulate transcription from the *ctc* and *veg* promoters (Westpheling, *et al.*, 1985). Two proteins of molecular weight 49 000 Da and 37 000 Da (subsequently referred to as  $\sigma^{49}$  and  $\sigma^{37}$ ) were isolated that strongly stimulated transcription of *ctc* DNA by core RNA polymerase, and a 35 000 Da protein ( $\sigma^{35}$ ) was detected that strongly enhanced *veg* RNA synthesis by the core enzyme.

The *S. coelicolor* enzyme ( $E\sigma^{35}$ ) appeared to be similar in its specificity to the major holoenzymes  $E\sigma^{70}$  and  $E\sigma^{43}$  of *E. coli* and *Bacillus* respectively, in that it efficiently used a promoter strongly conforming to the consensus sequence of the principal class of bacterial promoters, but in contrast the enzyme containing  $\sigma^{49}$  (or  $\sigma^{37}$ ) resembled in specificity the holoenzyme  $E\sigma^{37}$  found in *B.subtilis*, where it is involved in the transcription of developmentally regulated genes.

During these studies a *Streptomyces* promoter was also identified that could be used by the *ctc* transcribing RNA polymerase. The promoter was that of the *endoH* gene from *S.plicatus* (Robbins, *et al.*, 1984), whose product is a secreted endo-N acetylglucosaminidase that hydrolyses the glycosidic bonds between N-acetylglucosamine residues of 'high mannose' oligosaccharides (Tarantino and Maley, 1974). Whilst transcripts could be generated from this promoter using *ctc* transcribing RNA polymerase from *S.coelicolor*, the *veg* transcribing form of RNA polymerase was totally

inactive with *endoH*-containing DNA as template. When the *endoH* promoter was compared with that of *ctc* there was no overall similarity; they did however partially conform to each other at positions functionally important in *ctc* promoter use (Fig1.3). Therefore, *endoH* may be one example of a *ctc* like promoter in *Streptomyces* which probably directs transcription of genes responsible for morphological development and production of secondary metabolites. The utilisation of the *Streptomyces endoH* promoter by *ctc* transcribing RNA polymerase was the first demonstration of the physiological significance of different holoenzyme forms to transcription of *Streptomyces* genes.

A further  $\sigma$  factor was subsequently isolated from *S.coelicolor*, during studies of the agarase gene, *dagA* (Buttner, et al., 1988), which enables the organism to utilise agar as the sole carbon source. Not surprisingly, expression of *dagA* is subject to induction by agar degradation products and repression by glucose and a number of other sugars (Hodgson and Chater, 1981). Transcription of *dagA* was found to occur from four promoters, whose sequences are markedly heterogeneous (Fig 1.4), and by separating RNA polymerase from *S.coelicolor* into fractions that differed in their ability to recognise the four *dagA* promoters, three different holoenzymes were isolated, each of which transcribed from only one of the *dagAp2*, *dagAp3*, and *dagAp4* promoters. The holoenzymes which initiated transcription from *dagAp3* and *dagAp4* were shown to direct transcription of *ctc* and *veg* promoters respectively, suggesting that the  $\sigma$  factors responsible corresponded to the previously isolated  $\sigma^{49}$  (for transcription of *dagAp3*) and  $\sigma^{35}$  (for transcription of *dagAp4*). Indeed, *dagAp4* closely resembles the consensus sequence for the major class of eubacterial promoters (Fig1.4). A novel sigma factor of approximately 28 KDa ( $\sigma^{28}$ ) was found to direct



|                                  |                           |          |              |
|----------------------------------|---------------------------|----------|--------------|
| TTTCGAGGTTTAAATCCTTATCGTTATGGGTA | TTGTTT                    | GTAATAGG | <i>ctc</i>   |
|                                  | *                         | **       |              |
| ATTGACTGATTGACGCGC               | TTCCGGCGGGCAGGGAGGCACGGTG |          | <i>endoH</i> |

Fig. 1.3. Nucleotide sequence of the *ctc* and *endoH* promoter regions. Positions in the *ctc* promoter thought to be important for polymerase recognition are indicated ( • ). There is little overall homology between the two transcription initiation regions, but the promoters do partially conform to each other at positions functionally important in *ctc* promoter use ( \* ).



transcription from *dagAp2* by core RNA polymerase and in addition failed to initiate transcription from either the *veg* or *ctc* promoters.

At least three different forms of RNA polymerase holoenzyme containing different sigma factors ( $\sigma^{49}$ ,  $\sigma^{35}$ ,  $\sigma^{28}$ ) have now been detected in *S.coelicolor*, and it seems likely that a further unique sigma factor, yet to be isolated, directs transcription of *dagAp1* by core RNA polymerase. In addition, *whiG* of *S.coelicolor* is thought to encode a further novel  $\sigma$  factor, since cell free extracts of *whiG* mutants contain all 4 *dagA* transcribing activities. This would indicate that *S.coelicolor* contains at least five different sigma factors, which are probably members of a larger family, that determine the differential expression of different gene sets during the organisms life cycle. These sets probably include those involved in differentiation and secondary metabolism, although other gene classes may also be regulated in this way, an idea which is consistent with the marked sequence heterogeneity of *Streptomyces* promoters.

Interestingly, a further example of temporal regulation exerted at the level of transcription has been described for the *gal* operon of *S.lividans*. The operon contains three genes *galT*- encoding galactose-1-phosphate uridyl transferase, *galE*- encoding UDP glucose-4-epimerase and a galactokinase gene-*galK*, as shown in Fig.1.5 (Fornwald, *et al.*, 1987). Two promoters were found to be responsible for transcription of the operon, one (*galP1*) is located upstream of *galT* whilst the other (*galP2*) is found within the intergenic space between *galT* and *galE*, and whilst *galP2* is constitutively expressed *galP1* is repressed by glucose and induced in the presence of galactose. The apparently constitutive expression of *galE* and *galK* reflects a need for these enzymes under non-inducing conditions; in *E.coli* at least, UDP



glucose-4-epimerase (encoded by *galE*) is needed for the conversion of UDP-glucose to UDP galactose, in the absence of galactose, for use in cell wall biosynthesis. A second role for galactokinase (encoded by *galK*) is unknown, but it is possible that the inducer of *galP1* is galactose-1-phosphate rather than galactose itself and expression of *galK* would therefore be a necessary pre-requisite for expression of *galT*; in fact, transcription from *galP1* is virtually undetectable in *galK* mutants (Brawner, *et al.*, 1988).

During inspection of *galP1*, regions typical of the -10 and -35 consensus sequences of the major class of eubacterial promoters were identified; they were however separated by 21 bp instead of the usual 17 bp spacing. This spacing is reminiscent of the *spoIIIE* and *spoIIG* promoters of *Bacillus subtilis* (Guzman, *et al.*, 1988) (Kenney and Moran, 1987), both of which have sequences nearly identical to the consensus -35 and -10 regions but have an uncharacteristic spacing of 21 bp. and 22 bp. respectively. It is at present unclear which holoenzyme may recognize *galP1*, although *galP1* transcribing activity could not be clearly separated from  $E\sigma^{35}$  holoenzyme activity. Studies of *galP2* transcription showed that it was recognised by the previously identified holoenzyme  $E\sigma^{28}$  (Westpheling and Brawner, 1989).

Recently multiple *rpoD* genes have been detected in *S. coelicolor*, a unique phenomenon in prokaryotes, since species examined so far have only been found to possess one copy of such a gene. When the *rpoD* gene of *Myxococcus xanthus*, originally isolated using the *rpoD* gene of *E. coli*, was used to probe the genome of *S.coelicolor*, four homologues of the *rpoD* gene were found (*hrdA*→*hrdD*)(Tanaka, *et al.*, 1988)(Buttner, *et al.*, 1990). In addition, of the four other *Streptomyces* strains examined, *S.lividans*, *S.parvulus*, *S.griseus*, and *S.griseosporus* were found to possess 4,4,3, and 3

*rpoD* homologues respectively, indicating that multiple *rpoD* signals are common in *Streptomyces*. To investigate the roles of these *rpoD* genes in *S.coelicolor*, they were disrupted *in vivo* by mutational cloning, and whilst disruption of *hrdA*, *hrdC* and *hrdD* gave rise to mutants not visibly affected in colony morphology, differentiation or production of the pigmented antibiotics (i.e. undecylprodigiosin and actinorhodin), disruption of *hrdB* proved to be a lethal event. Given the close similarity of each of the four *hrd* gene products to  $\sigma^{70}$  of *E.coli*, it seems likely that they direct core RNA polymerase to transcribe from promoters with strong similarity to the *E. coli* consensus sequence. Such a sigma factor,  $\sigma^{35}$  has already been described in *S. coelicolor*, and it seems likely that  $\sigma^{35}$  is encoded by one of these *hrd* genes. Given the apparent essentiality of *hrdB* it would seem the most likely candidate.

It is possible that the multiple *rpoD* genes encode sigma factors which have a similar function, i.e. the transcription of *veg*-like promoters, with the presence of multiple genes being necessary to maintain sufficient levels of sigma factors in the cell. Alternatively and probably most likely, the *rpoD* gene products may have a similar but nevertheless distinct functional specificity, with each product recognising a slightly different kind of promoter. In accordance with this idea, two different RNA polymerase holoenzymes have been detected in *S.coelicolor* that direct transcription from two *E.coli* consensus like promoters. One directs transcription from the *veg* promoter of *B.subtilis* whilst the other is responsible for transcription of XP55, which is a major secreted protein of *S.lividans* (Burnett, et al., 1987). These two promoters differ from each other by only one base pair in the -10 region and one in the -35 region, both having a spacing of 17 bp, however there is no evidence as yet linking these two transcribing activities with

holoenzymes containing *hrd*-encoded  $\sigma$  factors. The previously described *galP1* was shown to have -10 and -35 sequences similar to the *E. coli* vegetative promoter with a unique spacing of 21 bp. Perhaps a sigma factor encoded by one of the *hrd* genes recognises -10 and -35 consensus sequences with this latter spacing and is responsible for transcription of *galP1*.

Since all the *hrd* gene products should resemble in structure and function the principle sigma factors of *E.coli* and *B.subtilis*, it is possible that *Streptomyces* cells have multiple physiological states during the vegetative stage of growth, indeed a cell having the capacity for such complex and distinct physiological changes can be expected to be different from simple unicellular bacteria like *E.coli* and *B.subtilis*. Possibly, even during the vegetative stage of growth, different sigma factors are required for the transcription of all essential genes in *Streptomyces*.

The identification of multiple forms of RNA polymerase, and a number of regulatory factors in *S.coelicolor* has begun to provide some explanations for the observed coupling of antibiotic production with morphological differentiation, although the way in which these processes are initiated is still undetermined. Recently a causal connection has been made between the stringent response, resulting from nutrient limitation, and the onset of morphological and physiological differentiation, since these latter two events usually occur when nutrients become scarce. Studies on *S.griseoflavus*, which produces the antibiotic bicozamycin, have shown that the cellular ppGpp (guanosine-5' diphosphate-3' diphosphate) pool size is undetectable at the early growth phase, but during mid-growth phase ppGpp accumulates accompanied by a drop in the GTP pool size, and this accumulation coincides exactly with the onset of aerial mycelium formation and bicozamycin

production in the cell (Ochi, 1988). It has been suggested that these changes may be stimulated more specifically by limitation of N source, and indeed if levels of aspartate are increased in the growth medium the onset of aerial mycelium formation is delayed. Further evidence in favour of the above hypothesis was provided by the observation that a relaxed (*rel*) mutant, which was severely reduced in its capacity to accumulate ppGpp was found to produce substantially less bicozamycin and its ability to form aerial mycelium was somewhat reduced. In contrast, high bicozamycin producing strains accumulated levels of ppGpp 2-3 fold greater than the wild type strain.

Exactly how levels of GTP and ppGpp influence the initiation of morphological and physiological differentiation is unknown, but possibly the nutrient limitation (particularly amino acid starvation) which occurs around this time results in the formation of ppGpp which then directly affects polymerase function. Perhaps it acts by loosening protein-protein interactions between the vegetative sigma factor and core RNA polymerase, thereby altering the relative affinity of the various sigma factors for the core polymerase and facilitating the replacement of one sigma factor with another.

## **1.2. Resistance Mechanisms in Antibiotic-Producing *Streptomyces*.**

Antibiotics produced by *Streptomyces* comprise a large class of chemically diverse compounds, which arise from the modification of intermediates in primary metabolism and from the assembly of these primary metabolites into complex structures *via* the action of specific enzymes. The biosynthetic genes encoding these enzymes are, as indicated previously, usually located in clusters and are only expressed under certain



physiological conditions, generally involving nutrient limitation. Obviously any organism producing a potentially lethal substance needs to afford some kind of protection towards itself, and the genes encoding such resistance determinants have consistently been located in or around the respective biosynthetic gene cluster. This relationship has been of great value in studying antibiotic biosynthesis, since a number of biosynthetic gene clusters have now been isolated using the corresponding resistance gene to probe cosmid libraries. Prior isolation of *ermE*, the erythromycin resistance gene from *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*), allowed for the identification of a number of erythromycin biosynthetic genes clustered around the resistance determinant (Stanzak, et al., 1986). Interestingly, *ermE* is located approximately in the centre of the gene cluster and is transcribed divergently from the closely located transcripts of *eryC1* and *eryD* (Bibb, et al., 1985), the products of which are involved in the attachment of a deoxysugar unit to erythronolide B resulting in the formation of the first antibiotically active compound, erythromycin D. The possible significance of this arrangement is that it might ensure that expression of the resistance gene would be a pre-requisite for the subsequent transcription of these late biosynthetic genes. A similar feature is seen for several other resistance genes; the methylenomycin resistance gene of *S.coelicolor* is transcribed divergently from a presumed biosynthetic pathway (Neal and Chater, 1987) and similarly for the neomycin resistance gene of *S.fradiae* (Bibb and Janssen, 1987) and streptomycin resistance in *S.griseus* (Distler, et al., 1987).

In general there are three broad mechanisms by which an antibiotic-producing streptomycete can protect itself against autotoxicity. Firstly, the production of antibiotic may be spatially compartmentalised until such time that it can be excluded from the cell, with a permeability barrier

existing to prevent re-entry of exogenous drug. Alternatively, inactivation of the antibiotic may occur by substitution or hydrolysis, and finally the target site for the antibiotic may be modified rendering it unavailable for drug binding and action (Cundliffe, 1984; Cundliffe, 1989). It has been speculated that these resistance determinants, particularly modifying enzymes, have a physiological role in the cell other than conferring resistance. In particular, aminoglycoside phosphotransferases which phosphorylate drug molecules have been closely associated with native protein kinases, suggesting that they are possibly evolutionarily related.

Due to the enormity of the subject, the examples of resistance mechanisms described below all concern antibiotics which inhibit protein synthesis with particular emphasis being placed on the latter of these three mechanisms.

#### 1.2.i Antibiotic Exclusion Systems.

Any antibiotic-producing strain must possess a mechanism by which the newly synthesised drug can be secreted from the cell. In addition, this mechanism may also double as a device for expelling any previously exported drug that has found its way back into the cell, and is particularly important in producing strains where the targets for the autogenous drug remain sensitive. An example of such an exclusion system is found in *S.rimosus*, the producer of tetracycline. This drug is thought to prevent protein synthesis by inhibiting the binding of the [aminoacyl-tRNA:EFTu:GTP] ternary complex to the A-site of the ribosome. However, when tested the producing strain was found to possess tetracycline sensitive ribosomes. Of the tetracycline resistance genes cloned from *S.rimosus*, one (*tetB*) appears to encode a membrane associated protein that promotes energy dependent

efflux of the drug; the second resistance gene has not been characterised fully but is thought to encode a cytoplasmic protein that might sequester the antibiotic (Ohnuki, et al., 1985).

Resistance to methylenomycin in the producing strain, *S.coelicolor* is also thought to occur by a similar mechanism. As yet the mode of action of methylenomycin is not known, but the resistance determinant *mmr*, has been sequenced and the putative product resembles known membrane proteins (Neal and Chater, 1987). It would therefore appear that membrane exclusion, or possibly an efflux system operates, which protects the cell against the action of methylenomycin

In *S.griseus*, a phosphatase enzyme exists that is associated with streptomycin export (Walker and Walker, 1970). Streptomycin-phosphate is the penultimate compound in the biosynthetic pathway, and it appears that this latter substance is de-phosphorylated at the cell membrane to produce antibiotically-active streptomycin, which is immediately exported from the cell. A permeability barrier is also thought to exist against the uptake of streptomycin, since influx is markedly reduced in cells during the period of antibiotic biosynthesis (Cella and Vining, 1975). An additional mechanism also exists in *S. griseus* (see below) whereby any drug entering the cell is immediately inactivated and expelled as described above.

#### 2.1.ii. Antibiotic Modification Systems.

The ability to inactivate their antibiotic products is crucial to the survival of a number of producing organisms. Despite the apparent chemical diversity of these antibiotics, only two modes of modification have been described, namely N-acetylation of amino groups and O-phosphorylation of

hydroxyls, using as the cofactors acetyl coenzyme A (acetyl CoA) and ATP respectively. Acetyltransferase activity has been detected in *S.kanamyceticus*, the producer of kanamycin (Benveniste and Davies, 1973), but whilst the gene encoding this enzyme (*aac*) functions well as a resistance determinant when cloned into *S.lividans* (Matsuhashi, et al., 1985), it is not thought to have a major role in resistance of the producing strain. Acetyl transferase activity [AAC (6')] was only detected in *S.kanamyceticus* during early growth phase and declined prior to the onset of drug production (Sato, et al., 1975), it would therefore appear that the organism employs ribosomal modification as its major mechanism of defence towards autogeneously produced drug (see later). Possibly, the *aac* product has a biosynthetic role in *S.kanamyceticus* since it apparently acetylates intermediates of the kanamycin biosynthetic pathway, and if extra copies of *aac* are introduced into the latter strain the yield of kanamycin is significantly increased (Cramer and Davies, 1986).

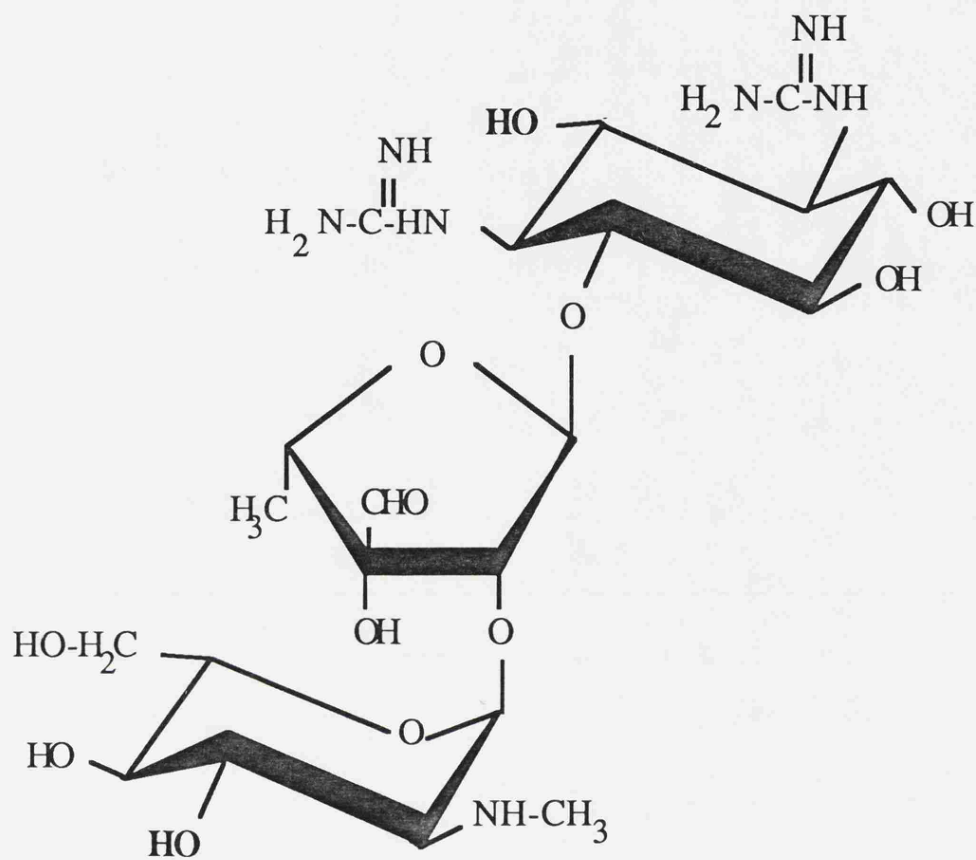
Two acetyl transferases AAC(6') and AAC(2') have also been detected in the nebramycin producer *S.tenebrarius*. Again, the precise function of these enzymes is unknown, but since the strain possesses two different rRNA methylase genes whose products render ribosomes resistant to the nebramycin complex, they seem unlikely to function as resistance determinants.

In contrast, phosphorylating enzymes have a more important role to play as determinants of resistance. In cases where such enzymes have been detected in producing strains, they usually constitute the primary resistance mechanism employed by that organism. Two different enzymes that phosphorylate streptomycin have been detected in *S.griseus* (Nimi, et al., 1971). The first phosphotransferase characterised, and the principal

streptomycin modifying enzyme in *S.griseus*, is SPH (6), whose production appears to be inducible in producing strains and is present at high levels during streptomycin production. Streptomycin is produced from myo-inositol *via* streptidine, and this latter compound and all subsequent intermediates are substrates for SPH (6'). Thus, following streptidine, the pathway of streptomycin production proceeds along a series of 6-phosphorylated intermediates that are devoid of biological activity. Possibly, the first antibiotically active compound in this pathway would be streptidine and by inactivating this intermediate the organism protects itself against potential suicide. Active drug is subsequently generated in the final reaction during transport through the cell membrane, where the penultimate compound in the pathway is dephosphorylated, possibly through the phosphatase activity of APH (6), and active streptomycin is excreted.

A second streptomycin phosphotransferase exists in *S.griseus* with specificity for the 3"-OH group (Fig.1.6) and is encoded by *aphE* (Heizel, et al., 1988). However the precise function of this enzyme with regards to streptomycin metabolism is unknown, since the APH (6) enzyme is the more strongly expressed resistance determinant during the productive phase, and expression of *aphE* in *S.griseus* has not yet been demonstrated. Similar enzyme activities have been detected in the hydroxy-streptomycin producer *S.glaucescens* (Ono, et al., 1983), with further phosphotransferases being detected in *S. hygroscopicus* and *S. vinaceus*, where they inactivate hygromycin and viomycin respectively (Pardo, et al., 1985).

Interestingly, despite the inhibitory effect of the latter three antibiotics on protein synthesis, ribosomes from the respective producing strains remain sensitive, indicating that these phosphorylating enzymes are consistently more important as resistance determinants than the previously



**Fig. 1.6** The structure of streptomycin. The hydroxyl groups modified by the two phosphotransferases, APH(6) and APH(3'') are indicated in bold.

described acetyl transferases. A further type of modification exists towards antibiotics produced by *Streptomyces*, namely O-adenylation, and whilst this type of modification has not yet been detected among antibiotic producers, such activity is not uncommon among clinical isolates. A close relationship has been described between phosphotransferase proteins and eukaryotic protein kinases (Distler, et al., 1987) with a similar situation thought to exist for acetyl transferases and methyl transferases (described in the next section), and has led to the suggestion that resistance genes coding for phosphotransferases, acetyl transferase and methylases could all be derived from cellular control genes.

### 1.2.iii. Target Site Modification.

Resistance to antibiotics will obviously occur if the target to which they bind becomes modified or replaced in such a way as to weaken or prevent interaction of the drug. Clearly modification of ribosomes rendering them resistant to inhibitors of protein synthesis provides a valuable means of defence in organisms producing such antibiotics. However, target site modification is not restricted to producers of ribosome inhibitors, similar systems exist in *Ammycolatopsis mediterranei* (formerly *Nocardia mediterranei*), *S.lydicus*, and *S.spectabilis* all of which produce RNA polymerase inhibitors (Blanco, et al., 1984).

Resistance to novobiocin in the producer, *S.sphaeroides* is due to an altered gyrase B subunit (encoded by *gyrB*) which presumably fails to bind the antibiotic (Thiara and Cundliffe, 1988). In fact, *S.sphaeroides* possesses two *gyrB* genes, one which encodes a novobiocin sensitive product that is expressed constitutively, and a second which is inducible by novobiocin and encodes the resistant B subunit. However, since the interest here lies

primarily in protein synthesis inhibitors, this section will deal exclusively hereafter with the modification of ribosomal targets.

The ribosomal component responsible for resistance in producing strains has generally been identified by a series of cross-over reconstitution experiments involving the construction *in vitro* of chimeric ribosomes using rRNA and proteins from either sensitive or resistant strains. In all cases examined so far, resistance in producing strains has been attributed to some property of the ribosomal RNA, be it 23S rRNA of the 50S subunit or 16S rRNA of the 30S subunit, as opposed to the ribosomal proteins. Such observations lend favour to the argument that the catalytic functions of the ribosome are associated with the RNA, and that the main function of ribosomal proteins is to stabilize the active conformations of the ribosome.

Resistance in the thiostrepton producer, *S. azureus* was the first ribosome modification system to be characterised. Initial 'cross-over reconstitution' analysis involved the dissociation of 50S ribosomal subunits from *S. azureus* and from a sensitive control strain to yield proteins and 23S rRNA. Following their re-association *in vitro* in the various possible combinations, hybrid ribosomal particles containing 23S rRNA from *S. azureus* were shown not to bind thiostrepton. Subsequently, resistance was shown to be due to the action of a specific RNA methylase (Cundliffe, 1978), which was isolated from *S. azureus* and shown to act upon free 23S rRNA, into which introduces a single methyl group onto the ribose sugar of an adenosine moiety at a position equivalent to A-1067 in *E. coli* 23S rRNA (Thompson, et al., 1982). These data were supported when DNA fragments from *S. azureus* were ligated into plasmids and introduced into *S. lividans* (Thompson, et al., 1982). The thiostrepton resistant transformants generated in these studies were shown to possess the resistance methylase and contained 23S rRNA which



could not be methylated by the enzyme purified from *S.aureus*. The 2'-O-methyl adenosine produced by this reaction resides in the 61 base oligonucleotide protected by ribosomal protein L11 from ribonuclease T<sub>1</sub> digestion (Schmidt, et al., 1981). This region of the 23S RNA molecule had previously been associated with GTP hydrolysis, and thiostrepton has come to be known as an inhibitor of the GTPase centre of the bacterial ribosome, providing an excellent example of how the study of ribosomal modification sites can help us to understand antibiotic action and ribosome function.

Thiostrepton has been shown to bind directly to rRNA, with binding of the drug being strongly promoted by L11; presumably this protein promotes thiostrepton binding by stabilizing a particular folded conformation of 23S rRNA. More specifically the drug has now been shown to bind to the 60 base oligonucleotide protected from nuclease digestion by L11, which includes A-1067 (J. Thompson; unpublished data), and it therefore seems feasible that residue 1067 might lie within the drug binding site and that methylation of this residue directly blocks thiostrepton binding to it. Supporting this statement, was the observation that an A→U or A→C substitution at position 1067 within 23S rRNA of *E. coli* reduced binding of thiostrepton, whilst an A→G substitution had only a marginal effect (Thompson, et al., 1988). Thiostrepton is the only example so far of a ribosomally directed antibiotic for which the target site is known, other ribosomal modification sites leading to antibiotic resistance have been located (see below), but as yet no direct link has been provided between these sites and the actual binding sites of the respective antibiotics.

Another group of antibiotics which inhibit protein synthesis by binding to the 50S subunit of the ribosome are the MLS antibiotics, but since this

group are of particular interest here they will be dealt with in a separate section (see section 1.3).

Target site modifications involving the small subunit have also been described. During a series of 'cross-over' experiments using 50S and 30S subunits derived from sensitive and resistant ribosomes, it was ascertained that the smaller (30S) subunit was the source of resistance to aminoglycosides in *Micromonospora purpurea* (Piendl and Böck, 1982), *S.tenjimariensis* and *S.tenebrarius*, with the state of 16S rRNA subsequently being shown to be responsible for resistance in each case (Piendl, et al., 1984).

In studying aminoglycoside resistance in *S.tenebrarius*, DNA fragments were introduced into *S.lividans* and transformants resistant to either gentamicin or apramycin were selected. Two distinct groups of clones were obtained, one being highly resistant to gentamicin with the second being more highly resistant to apramycin, whereas both were highly resistant to kanamycin. The genes responsible for these resistance phenotypes were subsequently referred to as *kgmB* and *kamB* respectively (Skeggs, et al., 1987) and were both shown to encode rRNA methylases which acted upon residues G-1405 and A-1408 in 16S rRNA respectively (A.A.D. Beauclerk; unpublished data). Presumably, 16S rRNA in *S.tenebrarius* is doubly modified to give broad aminoglycoside resistance observed in this strain, it does not account however for the neomycin resistance phenotype, since ribosomes of *S.tenebrarius* are sensitive to neomycin. The existence of two acetyl transferases in the strain (described earlier) may be therefore be of greater significance with respect to the resistance phenotype than at first suspected.

A resistance determinant *grm* isolated from the gentamicin producer *Micromonospora purpurea*, has also been shown to encode a rRNA methylase which acts upon 30S subunits. Whilst the exact site of methylation has not yet been determined it is known to lie within residues 1490 and 1504 of 16S rRNA (B. Vasiljevic; unpublished data). An additional resistance gene has also been cloned from this strain though the exact function of its product is unknown. The organism *M.purpurea* could therefore become another example of an aminoglycoside producer which possesses two mechanisms of defence to autogeneious drug.

The presence of aminoglycoside-modifying activity in *S.kanamyceticus* was described earlier, and initially this was believed to be responsible for resistance in the strain, since ribosomes isolated from the producer were reported to be sensitive to kanamycin (Hotta, et al., 1981). It has subsequently been demonstrated that ribosomes of *S.kanamyceticus* do become resistant to kanamycin following growth in production medium and that the ribosomal resistance mechanism operates via the 30S subunit (Nakano, et al., 1984). The ribosomal resistance determinant has been cloned from *S.kanamyceticus*, and strains of *S.lividans* harbouring the gene (*kan*) have the same phenotype as *S.lividans* containing *kgm* (Thompson, et al., 1985). It has therefore been suggested that the ribosomal resistance mechanism is similar to that specified by the *kgm* gene of *S.tenebrarius*, and indeed when 16S rRNA from *S.lividans* containing *kan* was examined, residue G-1405 was found to be modified [Beauclerk & Cundliffe; unpublished data]. It has been established that *kan* expression is regulated at the level of transcription (Nakano and Ogawara, 1987), though induction by kanamycin itself can possibly be eliminated as growth of *S. kanamyceticus* in medium containing kanamycin does not induce expression of *kan* [M. J. Calcutt; unpublished data]; this is assuming, however, that the cell membrane is

permeable to exogenous kanamycin. Alternatively, one or more of the biosynthetic intermediates may act as inducer.

The methylation events described so far which confer aminoglycoside resistance in producing organisms occur in a region of rRNA previously associated with codon anticodon interaction (Prince, et al., 1982). Evidence for this includes the fact that when tRNA<sup>val</sup> was bound in the ribosomal P site, photoirradiation resulted in an interaction between the 3' anticodon base and residue C1400 of 16S rRNA. This residue must therefore lie close to (i.e within about 4 angstroms) the anticodon of P site bound tRNA. In addition, the role of the 3'-terminal portion of 16S rRNA in translational initiation involving Shino Dalgarno sequences in mRNA is well established (Steitz, 1980). It has therefore been inferred that the 'C1400 region' of 16S rRNA might interact with mRNA codons and/or with tRNA anticodons, and regulate the decoding process in some way.

Aminoglycosides exert various effects upon protein synthesis, including inhibition of the translocation reaction during polypeptide chain elongation and initiation of protein synthesis; in addition, at certain concentrations, they cause misreading of mRNA codons (Davies, et al., 1965). Possibly, the binding of these drugs to the region around C1400 may explain their effect on translational accuracy.

One final ribosomal modification system that deserves mention is found in *S.pactum*. This organism produces the drug pactamycin, which is active against bacteria of both Gram types and also against eukaryotes (White, 1962), and is thought to exert its effect on protein synthesis in two ways. Firstly, the binding of amino-acyl tRNA to the ribosomal A site is inhibited and in addition formation of the ribosome initiation complex is also thought

to be prevented (Kappen, *et al.*, 1973). Resistance to pactamycin was again shown to be due to modification of 16S rRNA of the 30S subunit, when the resistance determinant was cloned from *S.pactum* into *S.lividans* (Calcutt and Cundliffe, 1989) and shown to encode a methylating enzyme which modified a position equivalent to A964 in *E.coli* 16S rRNA [J.P.G. Ballesta; unpublished data].

Specific methylation of rRNA has been shown to render the ribosome highly resistant to selected antibiotics, presumably by interfering with the binding of those drugs. A direct relationship is therefore indicated between the methylation sites and the drug target sites, and by relating the effects of any given antibiotic on protein synthesis to the site of modification which leads to its resistance, we can begin to understand the specific functions of particular regions of the ribosome in protein synthesis.

### 1.3. Macrolide, Lincosamide, and Streptogramin-type B Antibiotics.

The macrolide, lincosamide and streptogramin B family (MLS group of antibiotics) are a chemically diverse group of antibiotics that are linked together by the fact that they all inhibit protein synthesis in bacteria by binding to the 50S ribosomal subunit in mutually competitive fashion. In doing so they interfere in unknown ways with the peptidyltransferase domain of the ribosome. However for the purpose of discussing the relative modes of action of these antibiotics, each group will initially be considered as a separate entity.

Macrolide antibiotics possess a lactone ring comprising of 12-16 atoms, which contains few double bonds and no nitrogen atoms. The ring is also substituted with one or more sugar residues, and it is the addition of these

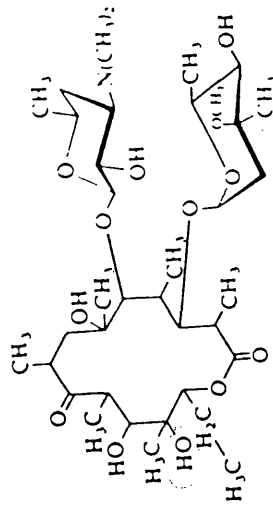
sugar moieties that is thought to result in antibiotically active compounds, with macrolides devoid of one or more of their normal sugars exhibiting altered or diminished activity (Wilhelm, et al., 1967). The nature and relative arrangement of these sugar moieties enables the group to be divided into a number of classes (Fig.1.7).

Lincomycin (Fig.1.8) is a member of the lincosamide group of antibiotics, with biological activity of the molecule depending upon alkyl substitution of both the sulphur atom and the ring nitrogen; N-demethyl-lincomycin and dethiomethyl-lincomycin are weakly or totally inactive (Mason and Lewis, 1964). The streptogramin B type antibiotics (Fig.1.9) are cyclic hexadepsipeptides containing uncommon amino acids.

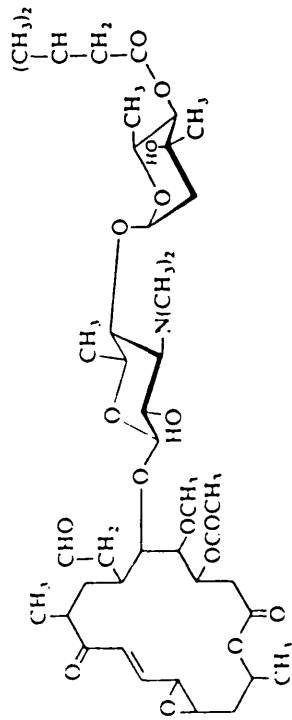
### 1.3i Mode of Action.

Despite being grouped under the same name, not all macrolide antibiotics act identically, as was demonstrated when different macrolides were compared in systems synthesising polylysine in response to polyA. In a detailed analysis, dilysine synthesis was shown to be inhibited by carbomycin, unaffected by spiramycin and tylosin, but stimulated by erythromycin. However, synthesis of trylsine was inhibited by carbomycin, tylosin and spiramycin, whilst the effects of erythromycin varied according to the experimental conditions. In subsequent studies, carbomycin was found to inhibit peptidyl transferase reactions in *in vitro* systems employing N-acylated amino acids, dipeptides or oligopeptides as donated substrates (Monroe and Vazquez, 1967; Mao and Robishaw, 1971), and inhibition remained when the donor substrate was prebound to the ribosome, eliminating any discrepancies in the assays that may have arisen due to possible inhibition of donor substrate binding to the ribosome by carbomycin

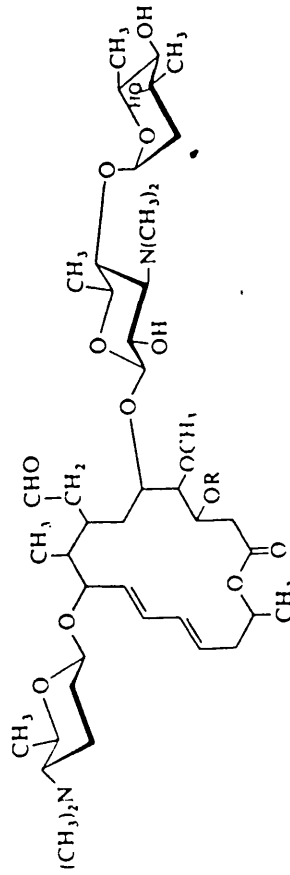
**Fig. 1.7 The macrolide antibiotics.** The macrolide ring systems have been simplified for purposes of comparison, with the nature and relative position of substitution of the sugar moieties emphasised. The detailed structure of one macrolide from each group represented is also shown



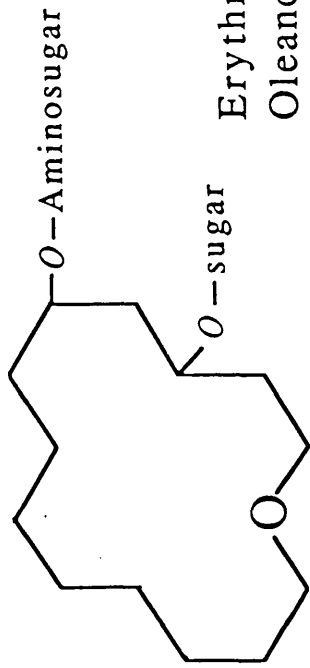
Erythromycin A



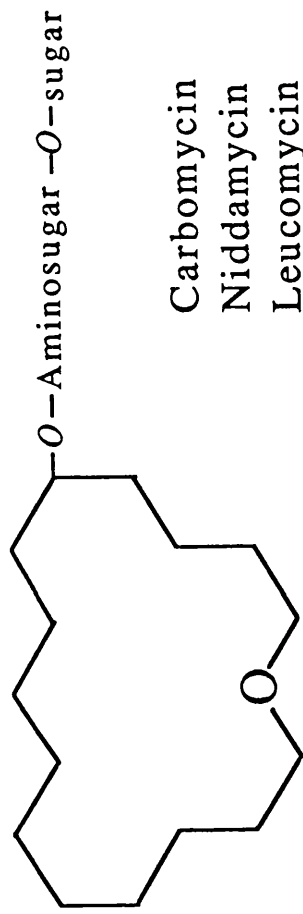
Carbomycin A



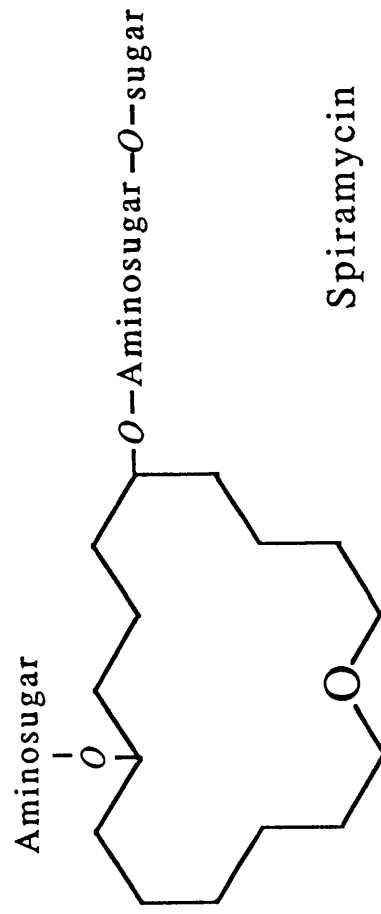
Spiramycin I R = H  
II R = COCH<sub>3</sub>  
III R = COCH<sub>2</sub>CH<sub>3</sub>



Erythromycin  
Oleandomycin

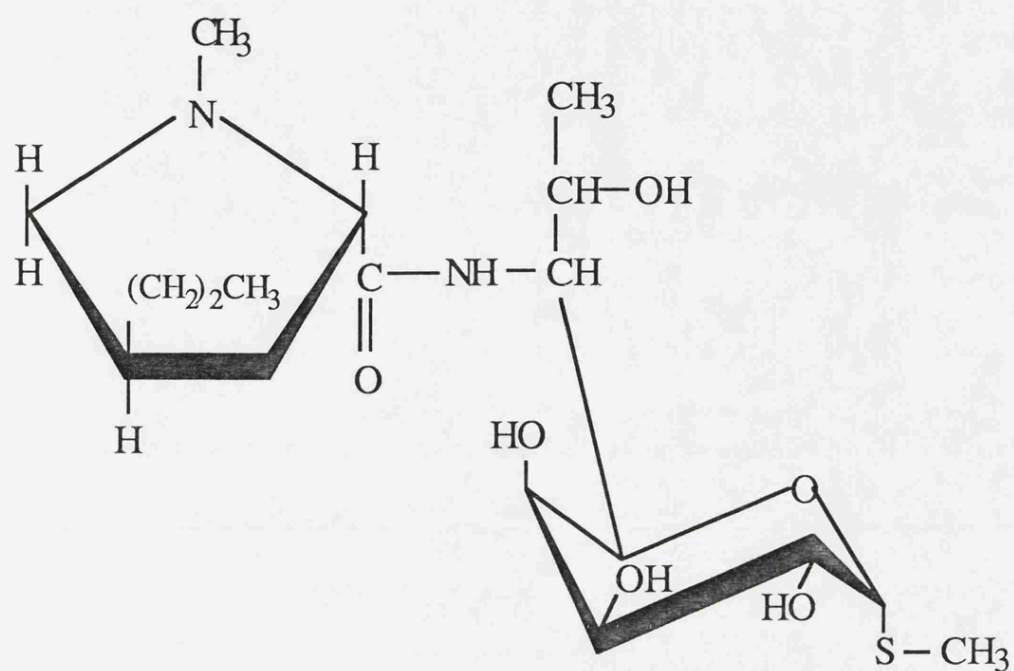


Carbomycin  
Niddamycin  
Leucomycin



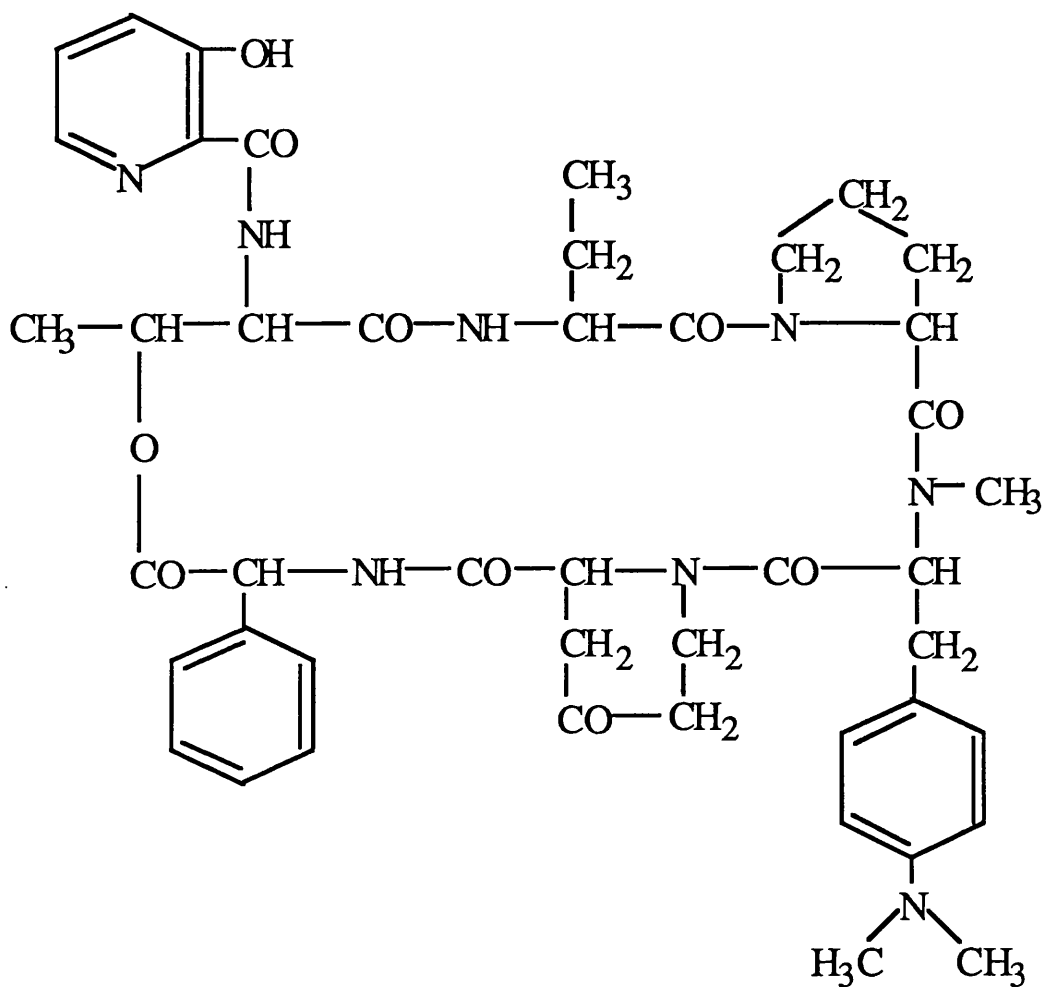
Spiramycin





Lincomycin

**Fig. 1.8. Structure of Lincomycin.** Biological activity depends upon alkyl substitution of both the sulphur atom and the ring nitrogen atom



Streptogramin B

**Fig. 1.9 The structure of Streptogramin B.** Members of the Streptogramin B group are usually cyclic heptadepsipeptides containing uncommon amino acids.

(Celma, et al., 1970). Since macrolides do not prevent the binding of aminoacyl tRNA to ribosomes nor inhibit ribosome dependent GTP hydrolysis, carbomycin may possibly inhibit the peptidyl transferase reaction by blocking recognition of acceptor substrates. Spiramycin and tylosin also inhibited peptidyl transferase assays regardless of whether donor substrate was prebound or not. Unlike carbomycin however, these drugs did not inhibit peptidyl transferase reactions when the donor substrate was fmet-tRNA (Mao and Robishaw, 1971). This result is in agreement with that obtained during studies of oligo lysine synthesis previously mentioned, and indicates that spiramycin and tylosin only inhibit peptidyl transferase when the nascent peptide is of a certain length (presumably > 2 amino acids). Again results with erythromycin were confusing, with its effect on peptidyl transferase depending upon the nature of the transferred substrate. The transfer of N-acylated amino acids was usually stimulated (Monro and Vazquez, 1967) due to an effect upon the rate of transfer, whilst transfer of dipeptidyl donor substrates was sometimes inhibited and sometimes stimulated (Mao and Robishaw, 1972); the drug did however block the transfer of oligolysyl peptides (Jayaraman and Goldberg, 1968). The mode of action of erythromycin is unclear, but given that it can stimulate the rate of peptidyl transfer, it seems unlikely that it acts primarily as an inhibitor of peptidyl transferase.

The effect of macrolides on protein synthesis has also been examined *in vivo*, and again distinct differences were observed. Using protoplasts of *Bacillus megaterium*, spiramycin was shown to cause rapid breakdown of polyribosomes (Cundliffe, 1969), such an effect was however inhibited if the protoplasts were treated with other antibiotics known to inhibit polypeptide chain elongation. It would therefore appear that the breakdown of polyribosomes was due to normal ribosomal run-off and not to dissociation

of the ribosome during protein synthesis. Similar results have been obtained with carbomycin and tylosin (Ennis, 1972) and it has therefore been suggested that these antibiotics only inhibit the elongation of short peptides and that ribosomes bearing nascent peptides longer than a certain length are unaffected. Possibly the longer nascent peptides may block access of these large drug molecules to their target site.

Erythromycin when added to protoplasts stabilizes polyribosomes which still contain their nascent peptides (Cundliffe and McQuillen, 1967), similar preservation was observed in intact cells of *E. coli* (Ennis, 1972), suggesting that binding of erythromycin, a smaller molecule than the former three, is not affected to the same extent by the length of nascent peptides. The ability of erythromycin to preserve polyribosomes intact so that their nascent peptides are unable to react with puromycin can be attributed to either direct inhibition of the peptidyl transferase reaction or inhibition of translocation- which would leave nascent peptides in the A site therefore blocking entry of puromycin. However, if protoplasts were pre-treated with chlorotetracycline, which inhibits binding of aminoacyl tRNA to the A site thereby confining nascent peptides to the P site, erythromycin no longer inhibited the reaction with puromycin. Drugs known to inhibit peptidyl transfer continued to inhibit such reactions indicating that the latter of the two possible explanations is correct. Data supporting this idea came when erythromycin was found not to inhibit the puromycin reaction directly *in vitro*, but inhibited that part of the reaction dependent upon the presence of factor EF-G and GTP, i.e. translocation of nascent peptide from the A→P site. In addition, translocation-dependent release of deacylated-tRNA from ribosomes was also inhibited (Igarashi, et al., 1969). Following the discovery that peptidyl tRNA was displaced from ribosomes in the presence of drug (Otaka and Kaji, 1975) it was proposed that translocation is prevented by

deacylated tRNA sequestered in the P site, leading to eventual loss of peptidyl-tRNA in the presence of erythromycin. Whilst the mode of action of erythromycin has not been established conclusively it seems likely that the drug inhibits translocation by sequestering deacylated tRNA in the ribosomal P site.

Stimulation of dissociation of peptidyl tRNA from ribosomes, by carbomycin, erythromycin and spiramycin has been demonstrated (Menninger and Otto, 1982) and is thought to occur during attempted translation. It has been suggested that this is the major mechanism of action of all macrolide antibiotics, with the differing effects of various macrolides on the cell-free synthesis of different peptides being explained by differential effects on the rates of dissociation of different peptidyl tRNA's from the ribosome. This argument has been used to explain some of the conflicting observations on the effects of macrolide antibiotics previously observed. It remains likely however that macrolides exert multiple effects on ribosome function which vary between the individual antibiotics.

The lincosamide, lincomycin, has been shown to compete with erythromycin and chloramphenicol for binding sites on the prokaryotic ribosome (Fernandez-Munoz, et al., 1971), indicating that these three antibiotics bind in mutually exclusive fashion to partially (or completely) overlapping sites on ribosomes. Lincomycin inhibits peptidyl transferase reactions in *in vitro* systems as demonstrated when the drug inhibited the formation of fmet-puromycin from fmet-hexanucleotide (Monro and Vazquez, 1967), however the drug also prevented binding of acetyl-leucyl-pentanucleotide and leucyl pentanucleotide which bind to P and A sites respectively of the ribosome (Celma, et al., 1971), indicating that its effect on peptide bond formation may reflect the blockade of either of

these two sites.

Lincomycin, like spiramycin and carbomycin caused rapid breakdown of polyribosomes (Cundliffe, 1969) which could be prevented by inhibitors of polypeptide chain elongation. Again this effect could be due either to inability of the ribosome to initiate peptide chain synthesis or to the ineffect of lincomycin on ribosomes bearing nascent peptides of a certain length. Inhibition of peptide chain initiation by lincomycin has not been demonstrated; in fact the drug failed to inhibit factor-mediated binding of fmet-tRNA (Reusser, 1975). It would therefore appear that the drug only acts upon ribosomes bearing short peptide chains which is supported by the fact that lincomycin will not bind to polyribosomes unless nascent peptides are first removed (Pestka, 1972). Perhaps lincomycin inhibits peptidyl transferase reactions by affecting the ability of the enzyme to recognise either its donor or acceptor substrate.

Streptogramin B antibiotics bind with 1:1 stoichiometry to 50S ribosomal particles, and like macrolide or lincosamide antibiotics, prevent the interaction of chloramphenicol with the ribosome (Chang, et al., 1969), thereby indicating possible related binding sites to those of erythromycin and lincomycin. Again, polyribosomes are extensively degraded in the presence of Streptogramin B type antibiotics (Ennis, 1970), suggesting that this drug is also unable to bind to ribosomes carrying long peptide chains. There is as yet no explanation for the inhibitory effects of streptogramin B antibiotics upon protein synthesis; they did not inhibit binding of donor or acceptor substrates to the peptidyl transferase centre (Pestka, 1969) nor did they affect peptide bond formation (Monro and Vazquez, 1967).

As previously mentioned several antibiotics of the MLS group compete

with each other and with chloramphenicol for binding sites on the ribosome, and this led to the conclusion that these three groups of antibiotics interact with overlapping binding sites, presumably close to that of chloramphenicol. The binding region was proposed to be in or around the peptidyl transferase centre, based on the ability of chloramphenicol to inhibit specifically the process of peptide bond formation (Celma, et al., 1970). Evidence which supports this idea and which implicates a particular ribosomal domain (domain V) in peptidyl transferase function has come from studies of ribosomal RNA mutations which confer resistance against these antibiotics. Two such resistant mutants of *E.coli* with altered RNA sequences have been isolated by *in vivo* chemical mutagenesis of an *E.coli* strain containing the *rrnH* operon cloned on a multi-copy plasmid (Sigmund, et al., 1984). Prior isolation of this operon was deemed necessary by the fact that mutations in the *rrnB* operon of *E.coli* only have a detectable phenotype when they are isolated in an *rrn* operon that contributes to a larger percentage of the rRNA than do any of the seven chromosomal *rrn* operons of *E.coli*.

Sequence analysis revealed that an A→U transition at 2058 in 23S rRNA conferred resistance to MLS antibiotics whilst a G→A substitution at 2057 resulted in resistance to macrolides with a 14-membered lactone ring, such as erythromycin and to chloramphenicol. It is highly probable therefore that residues 2057 and 2058 lie within the peptidyl transferase domain of the ribosome.

A number of yeast mitochondrial mutants have also been isolated which have sequence changes in the rRNA of their large subunit. An A→G change at a position equivalent to 2058 in *E.coli* 23S rRNA again led to MLS resistance (Sor and Fukuhara, 1984), whilst a C→G transversion at position 2761

conferred resistance to erythromycin and spiramycin; though resistance to other members of the MLS group were not tested in this case. Moreover, erythromycin resistance and spiramycin resistance results from a C→G or C→T substitution respectively in yeast mitochondria at a position which is homologous to C2610 of *E. coli* 23S rRNA (Bolotin- Fukuhara, et al., 1983). The only example of a ribosomal RNA mutation in *Streptomyces* leading to resistance against any of these MLS antibiotics is the transition of an A→G at a position corresponding to 2058 of *E.coli* 23S rRNA in a mutant strain of *S.ambofaciens* (Pernodet, et al., 1988). This and the other point mutations described are indicated in Fig.1.10

Further evidence implicating the latter region of 23S rRNA in peptidyl transfer is the observation that benzophenone derivatized tRNA, a peptidyl tRNA analogue, can be bound to the P site of the 70S ribosome and crosslinked to 23S rRNA near the sites affected by these mutations (Fig.1.10)(Barta, et al., 1984). In addition puromycin, which binds to the A site, can also be crosslinked nearby (Eckerman and Symons, 1978), and since this small region of 23S rRNA is intimately associated with both substrate binding sites and various inhibitors of peptidyl transferase, it is not improbable that the region is associated with the catalytic site for peptidyl transfer.

Examination of a rRNA mutation leading to erythromycin resistance in a mutant of *E. coli*, has also provided evidence for the functional interaction between domains II and V of 23S rRNA. The mutation affording low levels of erythromycin resistance was localised to a region of domain II, and sequencing showed that positions 1219-1230 of the 23S rRNA gene were deleted in the mutant. Since all previously characterised rRNA mutations





conferring resistance to erythromycin have been found exclusively in domain V it would appear that there is some form of interaction between domains II and V. These data support those of (Stiege, et al., 1983), who have demonstrated, by U.V. cross linking studies, that sequences 571-577 and 739-748 in domain II are proximal to positions 2030-2032 and 2609-2618 respectively in domain V. However the deleted section is not thought to constitute part of the erythromycin binding site since the sequence between positions 1213 and 1236 shows high phylogenetic variability and given that erythromycin inhibits protein synthesis in a wide range of bacteria its binding site would be expected to be highly conserved. In addition the levels of erythromycin resistance are substantially lower than observed with mutations in domain V. Perhaps this region of the RNA is needed for the binding of a ribosomal protein involved in quaternary interactions bridging domains II and V, thereby generating the correct ribosomal conformation required for the binding of erythromycin.

The above data provide strong evidence that antibiotics of the MLS group bind directly to 23S rRNA, in a region of the ribosome associated with peptidyl transferase activity. Further information supporting this idea includes the report that erythromycin, carbomycin and vernamycinB protect overlapping sites in the proposed peptidyl transferase region (Moazed and Noller, 1987). Using DMS and kethoxal to probe antibiotic- ribosome complexes erythromycin was found to protect residues A-2058, A-2059 and G2505 of 23S rRNA, carbomycin protected A-2058, A-2059, A-2062, A-2451, and G-2505, whilst vernamycinB showed strongest protection to A-2062 and G-2505. A number of chloramphenicol protected sites were also identified. From this study it has been proposed that peptidyl transferase is inhibited as a result of antibiotics binding to or close to A-2451, whilst binding proximal to A-2058 is thought to interfere with growth of the nascent

peptide. Interestingly, vernamycin B also protected A-753 present in domain II providing further evidence for an association between this region of 23S rRNA and domain V.

Despite the widening opinion that antibiotics bind to RNA a few mutations within ribosomal proteins, that confer resistance to erythromycin have been described. Alteration of protein L4 and L22 are both thought to be responsible for resistance to erythromycin in some *E. coli* strains, and have led to the suggestion that these altered proteins change the conformational structure of the ribosome around the proposed peptidyl transferase domain, thereby preventing recognition by erythromycin of its target site. However in view of the fact that protein L15 is able to bind erythromycin weakly in solution and that it restored drug-binding capacity to core particles, the possibility also exists that these former proteins constitute part of the erythromycin binding site.

Specific base changes within rRNA by no means constitute the major mechanism of resistance used by bacteria against antibiotics within the MLS group. In fact the use of ribosomal mutational alterations as a defence mechanism against autotoxicity has not been described in any strain which produces MLS antibiotics. The primary mechanism of resistance detected in both producing organisms and other MLS resistant strains is described in the next section.

### 1.3.ii MLS Resistance.

Cross resistance of bacterial strains to macrolides, lincosamides, and streptogramin B antibiotics was first detected in clinical isolates of *Staph. aureus* in the mid-1950's (Chabbert, 1956)(Garrod, 1957). Initial studies

indicated that ribosomes from erythromycin resistant *Staphylococcus aureus* had a lower affinity for erythromycin (Kono, et al., 1966), and furthermore the drug remained unmodified after incubation with extracts from resistant cells (Nakajima, et al., 1968) thereby suggesting that resistance was due to modification of the antibiotic target.

In further biochemical studies of resistant strains, sub-inhibitory concentrations of erythromycin were found to induce high level resistance in isolates of *Staph. aureus* to this drug and also to other macrolides, lincosamides, and streptogramin B antibiotics, hence the collective term 'MLS' was introduced to embrace the three groups of antibiotics against which cross resistance was expressed. Following induction with erythromycin, resistant cells appeared within a few minutes and within approximately one generation virtually all the population had become resistant, though induced cells reverted to sensitivity after a few generations of growth following the withdrawal of erythromycin (Weisblum, et al., 1971). Such resistance was only induced by erythromycin, its close derivative oleandomycin and the lincosamide celesticetin (Weaver and Pattee, 1964)(Allen, 1977).

Subsequent work on the mechanism of resistance, showed that no altered ribosomal proteins were detected following induction, but that rRNA from induced cells contained the modified base N<sup>6</sup>,N<sup>6</sup>, -dimethyladenine, which was absent from the 23S rRNA of sensitive or uninduced cells (Lai and Weisblum, 1971). A causal connection between such methylation and resistance to MLS antibiotics was established by means of ribosome reconstitution experiments in which 23S rRNA from uninduced and induced cells was reconstituted *in vitro* with the total ribosomal protein fraction plus 5S rRNA from sensitive cells of *Bacillus stearothermophilus*. When tested for antibiotic-resistant protein synthesis, the reconstituted 50S ribosomal

subunits were found to be resistant or sensitive according to the source of the 23S rRNA (Lai, *et al.*, 1973). The resistance determinant was found to be contained within plasmid pE194, isolated from *Staph. aureus* (Iordanescu and Surdeanu, 1980) and when introduced into *B. subtilis* (Gryczan and Dubnau, 1978) resulted in inducible MLS resistance associated with dimethyladenine in a form indistinguishable from that originally observed in *Staph. aureus* (Weisblum, *et al.*, 1979). The sequence of the resistance determinant revealed an open reading frame encoding a possible 29 KDa protein, suggested to be the putative methylase previously predicted.

MLS resistance has also been detected in certain strains of streptococci, *Strep. pyogenes* possesses an inducible MLS resistance phenotype (Sanders, *et al.*, 1968), but unlike *ermC*, all three classes of MLS antibiotics can induce resistance in this strain (Dixon and Lipinski, 1974, Malke, *et al.*, 1981). A similar situation is seen in the case of *Strep. sanguis*, where the resistance determinant is located on plasmid pAM77 and induction is associated with the appearance of a 29 KDa protein (Horinouchi, *et al.*, 1983). Constitutive MLS resistance has also been demonstrated, in *Strep. faecalis*, which in the presence of the native plasmid pAM $\beta$ 1 was found to possess dimethylated adenine in 23S rRNA, whilst strains from which pAM $\beta$ 1 had been eliminated did not (Graham and Weisblum, 1979). MLS resistance has also been detected in a number of other clinical isolates including strains of *Clostridium perfringens* (Berryman and Rood, 1989), *Bacillus subtilis* (Mahler and Halvorson, 1980), *Bacillus licheniformis* (Docherty, *et al.*, 1981), and *Bacteroides fragilis* (Privitera, *et al.*, 1979).

MLS resistance is also widely distributed amongst the genus *Streptomyces*, the producers of these antibiotics, with the first suggestion of its presence being based on the report that ribosomes from *Sacc. erythraea*

(formerly *S.erythraeus*) were resistant to both erythromycin and lincomycin (Teraoka and Tanaka, 1974). Constitutively expressed MLS resistance was subsequently observed in the producing strain and was associated with the presence of dimethylated adenine in 23S rRNA detected during a survey of six macrolide, lincosamide or streptogramin B producing streptomycetes (Graham and Weisblum, 1979). Of all the streptomycetes tested for resistance to MLS antibiotics, only *Sacc. erythraea* was found to be resistant to all three classes in a way that resembled the pattern seen in studies of *Staph. aureus*, in addition it was the only streptomycete tested found to contain dimethylated adenine in 23S rRNA:- the structural modification associated with the classical MLS resistance phenotype.

Of the other producing organisms, *S.fradiae* and *S.cirratus*, which produce tylosin and cirramycin respectively, were found to contain monomethylated adenine but not dimethyladenine whilst the remaining three streptomycetes contained neither mono- or dimethyladenine (Fujisawa and Weisblum, 1981). These results therefore suggested a possible diversity from the classical MLS resistance phenotype described previously and further studies were therefore undertaken to examine the extent of divergence and also to detect any possible inducible phenotypes in these streptomycetes (Fig. 1.11).

In the case of *S.lincolnensis*, addition of lincomycin to the growth medium resulted in the appearance of monomethyladenine in 23S rRNA, thus presenting an apparent paradox in which *S.lincolnensis* requires lincomycin to become induced, and requires induction before going into production. Possibly the strain initially makes subinhibitory inducing levels of antibiotic prior to full production late which occurs later in the growth cycle, it would however seem more likely that a different endogenous

| <i>S. streptomyces</i> spp. | Antibiotic produced | Methylated residue                                | Expression               |
|-----------------------------|---------------------|---|--------------------------|
| <i>S. fradiae</i>           | tylosin             | m <sup>6</sup> A or m <sub>2</sub> <sup>6</sup> A | constitutive & inducible |
| <i>S. cirratus</i>          | cirramycin          | m <sup>6</sup> A                                  | constitutive             |
| <i>S. lincolnensis</i>      | lincomycin          | m <sup>6</sup> A                                  | inducible                |
| <i>S. diastaticus</i>       | virginiamycin       | m <sub>2</sub> <sup>6</sup> A                     | inducible                |
| <i>S. viridochromogenes</i> | none                | m <sup>6</sup> A                                  | inducible                |
| <i>S. hygroscopicus</i>     | maridomycin         | m <sup>6</sup> A + m <sub>2</sub> <sup>6</sup> A  | constitutive             |

**Fig. 1.11 MLS resistance phenotypes of various *Streptomyces* spp.** The MLS resistance phenotype is widespread amongst the genus *Streptomyces* and is associated with the presence of monomethylated adenine (m<sup>6</sup>A), or dimethylated adenine (m<sub>2</sub><sup>6</sup>A). The phenotype can be expressed either constitutively or inducibly.

inducer- possibly one of the intermediates in the biosynthetic pathway, serves to initiate expression of MLS resistance. As observed in the previous studies, uninduced strains of *S.fradiae* contained only monomethylated adenine, however induction with erythromycin resulted in the subsequent appearance of dimethylated adenine within the 23S rRNA, since the strain has now been studied in more detail this unexpected observation can be explained (See later) and forms part of a complicated story now emerging with respect to MLS resistance in *S.fradiae*. The streptomycete *S.hygrosopicus* represents a unique case in terms of MLS resistance, in that both mono- and dimethylated adenine were detected in 23S rRNA from an uninduced culture, possibly further investigations into this strain may reveal the presence of two distinct modified residues associated with MLS resistance. Two further organisms, *S.diastaticus* and a non-producing strain *S.viridochromogenes* were found to possess di- and monomethyl adenine respectively following induction.

Despite the observation that a few macrolide producers appear to possess constitutively methylated adenine residues, the possibility exists that these producing strains may synthesise their own endogenous inducers in the form of antibiotic biosynthetic intermediates or the final antibiotic produced. In cases where an inducible phenotype was detected the specificity of induction varied between the species.

The variations in mode of expression, variety of inducers, and patterns of rRNA methylation demonstrated that MLS resistance existed in a more diverse and general form than at first anticipated, prompting the need for a redefinition of the MLS phenotype to account for the fact that distinct subsets of MLS antibiotics, not previously found to induce, effectively induce resistance and that the induced ribosomal alteration can involve



monomethylation or dimethylation (or both) of adenine.

There was at the time only a causal connection linking rRNA methylation and MLS resistance, however confirmation of this hypothesis came from studies of MLS resistance in *Sacc. erythraea*. An indication that dimethylation of 23S rRNA is necessary and sufficient to render ribosomes of this latter strain resistant to erythromycin and other MLS antibiotics was provided when a rRNA methylase gene was cloned from *Sacc. erythraea* into *S. lividans*, where it conferred the classical MLS resistance phenotype (Thompson, et al., 1982). More direct evidence was obtained when the methylase was purified (Skinner and Cundliffe, 1982) and used to modify rRNA from *B. stearoothermophilus* in the presence of unlabelled SAM as cofactor, and subsequently used in the reconstitution of 50S subunits. Reconstituted particles containing methylated RNA were found to be substantially resistant to erythromycin and lincomycin, whilst those containing unmethylated RNA were totally sensitive, moreover the exact site of methylation was subsequently determined and shown to correspond to A-2058 in *E. coli* 23S rRNA (Skinner, et al., 1983).

In addition rRNA was isolated from *B. stearoothermophilus* carrying pE194 (which includes *ermC*) after growth in the presence and absence of induction, whilst rRNA from an uninduced culture was a good substrate for the *Sacc. erythraea* methylase, that from an induced culture remained unmodified by the enzyme. Since the *S.erythraea* methylase has been shown to act at a single site within 23S rRNA, the simplest explanation for these data is that both enzymes act at the same site, they do not however exclude the possibility that the *ermC* product acts at additional sites, though there is no data as such to suggest this.

Residue A-2058 is located within the proposed peptidyl transferase centre of 23 S rRNA and as discussed previously, single base substitutions at this site have resulted in resistance to erythromycin and other members of the MLS group. Co-resistance in these two organisms can therefore be rationalised on the basis that such drugs all bind to closely related ribosomal sites centered at or around A-2058, and that methylation of this latter site therefore reduces the affinity with which the MLS antibiotics bind to the ribosome by either directly blocking their target site or by sterically inhibiting access to it.

Dimethylation at the same site has also been shown to contribute to MLS resistance in the tylosin producer *S. fradiae* (Zalacain and Cundliffe, 1989), the resistance determinant, *tlrA*, (Birmingham, *et al.*, 1986) encodes a dimethylase whose expression is induced by erythromycin, and acts upon A-2058 within 23S rRNA. The same gene has been cloned independently by Kamimiya *et al.* and designated *ermSF* (Kamimiya and Weisblum, 1988). The situation in *S. fradiae* is however complicated by the presence of other macrolide/lincosamide resistance genes, including *tlrB* and *tlrC*, and whilst both resistance determinants have been cloned (Birmingham, *et al.*, 1986) the nature of their products is as yet undetermined, though strains of *S. griseofuscus* containing *tlrC* possess ribosomes sensitive to tylosin and erythromycin indicating that the *tlrC* product does not act at the level of the ribosome (M. Zalacain; unpublished data).

### 1.3 iii Control of Inducible MLS Resistance.

The existence of MLS resistance phenotypes inducible by members of the same group raised the curious question as to how an inhibitor of protein

synthesis can switch on expression of its own resistance gene. The possibility that regulation was exerted at the level of transcription was eliminated by the demonstration that following inhibition of RNA synthesis by the prior addition of rifampicin, *ermC* expression could still be induced in the presence of erythromycin, and furthermore the rate of transcription of *ermC* was similar in induced and uninduced cells of *B. subtilis* (Shivakumar, *et al.*, 1980). These data suggested that *ermC* mRNA is produced constitutively and activated by induction which occurs post-transcriptionally. In subsequent experiments, when the ribosomal affinity for erythromycin was reduced as a consequence of a chromosomal mutation, the ability of the drug to induce *ermC* was also impaired, providing a strong indication that post-transcriptional activation of *ermC* mRNA must involve binding of erythromycin to the ribosome.

Following the localisation of the *ermC* gene and its control element within pE194, sequencing revealed the presence of two open reading frames capable of encoding a 19 amino acid peptide and a 243 amino acid protein (Gryczan, *et al.*, 1980, Horinouchi and Weisblum, 1980), with the latter protein corresponding to the predicted 29 KDa methylase produced under inducing conditions, in *B. subtilis* cells containing pE194, and subsequently purified (Shivakumar and Dubnau, 1981, Shivakumar, *et al.*, 1979). Immediately preceeding this coding sequence was a series of complementary repeat sequences and the open reading frame capable of encoding the 19 amino acid peptide (Fig1.12). These complementary inverted repeat sequences were found to be potentially capable of assuming alternative stem-and-loop structures and it was proposed that translation of *ermC* mRNA is regulated by the ability of the leader sequence to undergo conformational re-arrangement.

**Fig. 1.12. Alternative conformations for *ermC* mRNA.** The mRNA is capable of folding into two different configurations. The inactive conformation (A) shows sequestration of SD-2 in the loop formed by association of stems 3 and 4, whilst in the active conformation (B) SD-2 has been freed, allowing ribosome attachment and methylase synthesis. Sites of point mutations which result in constitutive expression are indicated with open arrows.



The model subsequently proposed (Fig.1.12) postulates that the methylase mRNA is initially synthesised in a completed but inactive form and that the process of induction subsequently converts it to an active form (Gryzcan, et al., 1980, Horinouchi and Weisblum, 1980). The 5' end of the mRNA contains a set of four inverted complementary sequences designated 1,2,3, and 4, which are capable of base pairing to give two possible conformations (Fig.1.12A and Fig.1.12B). In addition, a further set of complementary repeat sequences A and A' are also present but bear no resemblance to 1,2,3 or 4. The conformation represented in Fig.1.12A corresponds to the inactive configuration of the control region, since the ribosome binding site associated with the methylase gene (SD-2) is sequestered by base-pairing between 3 + 4. Activation of the methylase gene is achieved by hindered translation from SD-1 of the 19 amino acid 'leader' peptide encoded within stems A and 1. If ribosomes stall during translation of the leader peptide, stem 2 is freed from its base-pairing with sequence 1 thereby allowing the association of stems 2 + 3, this in turn results in the previously occluded SD-2 being unmasked. Ribosome stalling is presumably brought about by the binding of an MLS antibiotic capable of inducing the system, however the unmasking of SD-2 could also be brought about by the spontaneous dissociation of stems 3 + 4, possibly resulting in basal level expression in the wild type strain, in the absence of induction. Clearly, any mutation which de-stabilizes the association of stems 3 + 4 is likely to result in the unmasking of SD-2 with a higher degree of probability than in the wild type strain.

The proposed model resembles that of transcriptional attenuation involved in the control of amino acid biosynthesis, in that both require interference with the synthesis of a control peptide which in turn leads to modification of the secondary structure of mRNA. However this latter model

allows continued transcription whilst the effect of the former model is to allow translation, hence control is said to be by translational attenuation.

Evidence supporting the model has come from studies of *Staph. aureus* mutants, which are generated by selection on media containing inhibitory concentrations of a non-inducing antibiotic and express the MLS resistance phenotype constitutively due to alterations within the *ermC* leader sequence (Horinouchi and Weisblum, 1980). Of the constitutively resistant mutants isolated all were found to possess alterations in either sequence 1, 3, or 4, whilst none of the mutations involved sequence 2. Such results would be predicted by the model since any mutation in sequence 2 would not only weaken interaction between stems 1 + 2 but also between stems 2 + 3 thereby reducing the efficiency with which the active conformation could occur. The most frequent mutations occurred in stems 3 and 4, indicating not only the importance of the pairing stability of stems 3 + 4, but also the biological significance of these two residues in that loss of either clearly favours dissociation of this particular stem and loop structure. Single base changes were also detected in sequence 1 which presumably de-stabilized 1 + 2 base pairing thereby favouring the association of 2 + 3, in addition a further mutant was isolated in which the whole of sequence 1 was deleted and according to the model, *ermC* mRNA from this strain would immediately adopt the active 2 + 3 conformation resulting in constitutive expression of methylase activity. By restriction digests of pE194, mutants were also obtained in which sequences 1, 2 and 3 had been removed, and whilst deletion of sequences 1 and 2 yielded a partially repressed phenotype deletion of sequence 3 resulted in a constitutive phenotype.

Further support for the postulated model has been provided by ribosome protection studies which demonstrated that, in accordance with the

postulated model, SD-1 is exposed and accessible to ribosomes whilst SD-2 is unavailable- presumably the latter ribosome binding site is sequestered by base-pairing (Narayanan and Dubnau, 1985).

Regulation of MLS resistance by this mechanism is subject to a form of negative feedback, in that the translational attenuation model predicts that synthesis of methylase should eventually become self limiting. Only sensitive ribosomes will stall in the control region in the presence of inducer, indicating that when the level of resistant ribosomes (formed as a result of sufficient *ermC* expression) reaches a critical level they will no longer be able to stall in the leader sequence, thus preventing synthesis of further methylase, hence the system will become de-sensitised. Results supporting this were obtained by the demonstration that in erythromycin-sensitive, *ermC* deletion mutants which specified truncated methylase, induction lead to an overproduction of the defective product, presumably since the ribosomes were not being methylated, induction was not self limiting by a decrease in the number of sensitive ribosomes. Similarly, when a translational fusion was constructed between the attenuator plus the first 219 nucleotides of the *ermC* structural gene and the *lacZ* gene, high levels of  $\beta$ -galactosidase were produced following induction. However a similar construction containing the intact *ermC* gene produced substantially lower levels of  $\beta$ -galactosidase, suggesting that the *ermC* product exerts a negative feedback control on expression (Kirsch and Lai, 1984).

However a more direct mechanism for shutting down methylase production also appears to operate, namely translational autoregulation by the methylase protein itself. In an analysis of several mutants that specified altered *ermC* products, those producing 'inactivated' truncated methylases



were found to be deregulated in that induction led to overproduction of the defective product, a fact explained by the continued ability of sensitive ribosomes to translate the message. However, in one of the mutant strains, which possessed a point mutation in the methylase coding sequence giving rise to a full size but inactive protein, the kinetics of accumulation of that product were normal (Denoya, *et al.*, 1986). Since all the ribosomes in this latter strain remain sensitive, translation of *ermC* mRNA must be controlled by some other mechanism other than decreased availability of sensitive ribosomes. It has therefore been suggested that the *ermC* methylase can regulate its own production by binding to and preventing utilisation of its own mRNA, indeed the observation has been made that the sequence around SD-2 of *ermC* mRNA resembles that region of 23S rRNA within which the methylation site conferring MLS resistance is located (see Chapter 6). There is as yet no evidence demonstrating that the *ermC* methylase does bind to its own mRNA at the SD-2 sequence, however it has been reported that the *ermC* methylase does bind to 23S rRNA at a region in the vicinity of domain V and surrounding the site of methylation (Su and Dubnau, 1990).

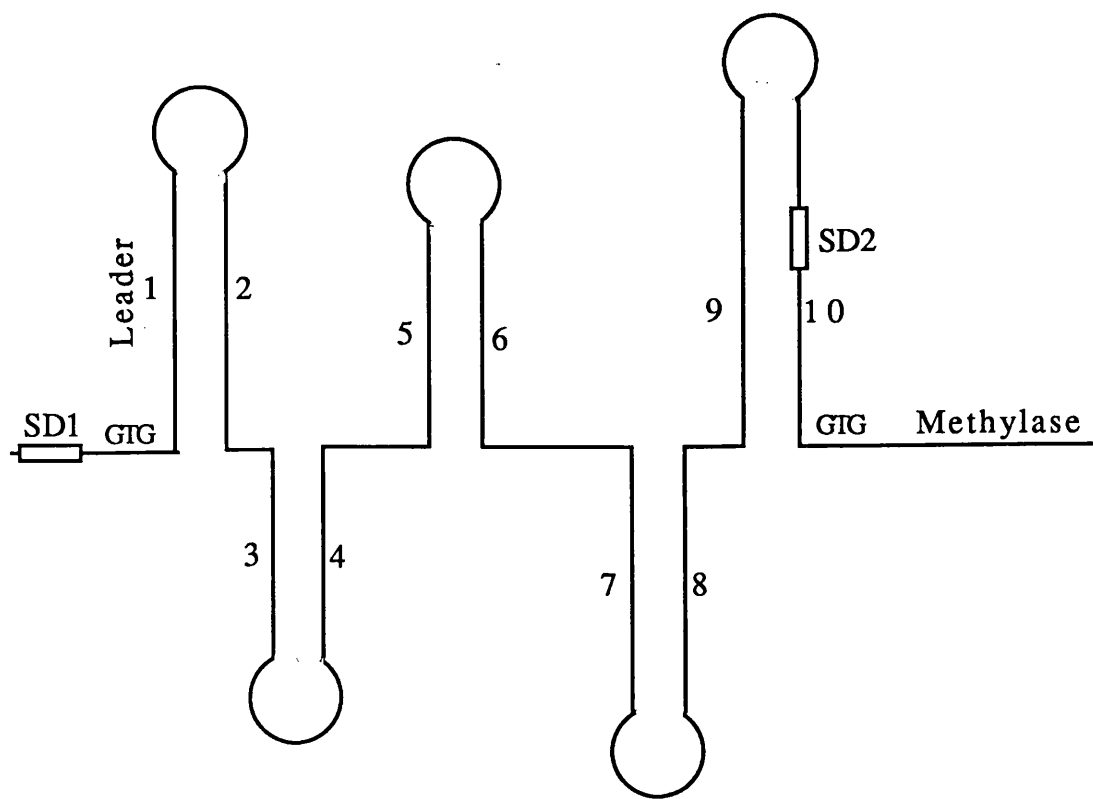
The gene, *ermC*, is the most extensively studied of all the *erm* type genes, but among the others that are inducible translational attenuation regulation has been proposed for *ermAM* of *Strep. sanguis* (Horinouchi, *et al.*, 1983), *ermD* of *B. licheniformis* (Gryczan, *et al.*, 1984) and *ermG* of *B. sphaericus* (Monod, *et al.*, 1987). A further gene that is thought to be controlled by translational attenuation and deserves particular mention here is *tlrA* (or *ermSF*) of *S. fradiae*. Sequence analysis and transcript mapping have revealed the presence of a 385 nucleotide leader sequence upstream of the methylase coding gene, which contained an open reading frame capable of encoding a 24 amino acid peptide (Kamimiya and Weisblum, 1988). This leader sequence, like that of *ermC* appears capable of pairing internally to form

stable secondary structures (Fig.1.13) and it is postulated that the leader sequence again functions as a translational attenuator whereby stalling of ribosomes during attempted translation of the leader peptide would ultimately lead to unmasking of SD-2. Antibiotic induced ribosome stall in segment 1 would free segment 2 allowing it to pair with segment 5, this then allows segment 6 to associate with segment 9 which in turn frees segment 10, containing SD-2, the putative ribosome binding site for methylase synthesis. Segments 3 + 4 and 7 + 8 would be expected to remain invariant irrespective of induction. Clearly the proposed model for regulation of *tlrA* is more complicated than that suggested for *ermC*, though it is supported by mutational analysis involving deletions in the proposed attenuator, particularly by deletion of segment 9 which results in expression of constitutive resistance.

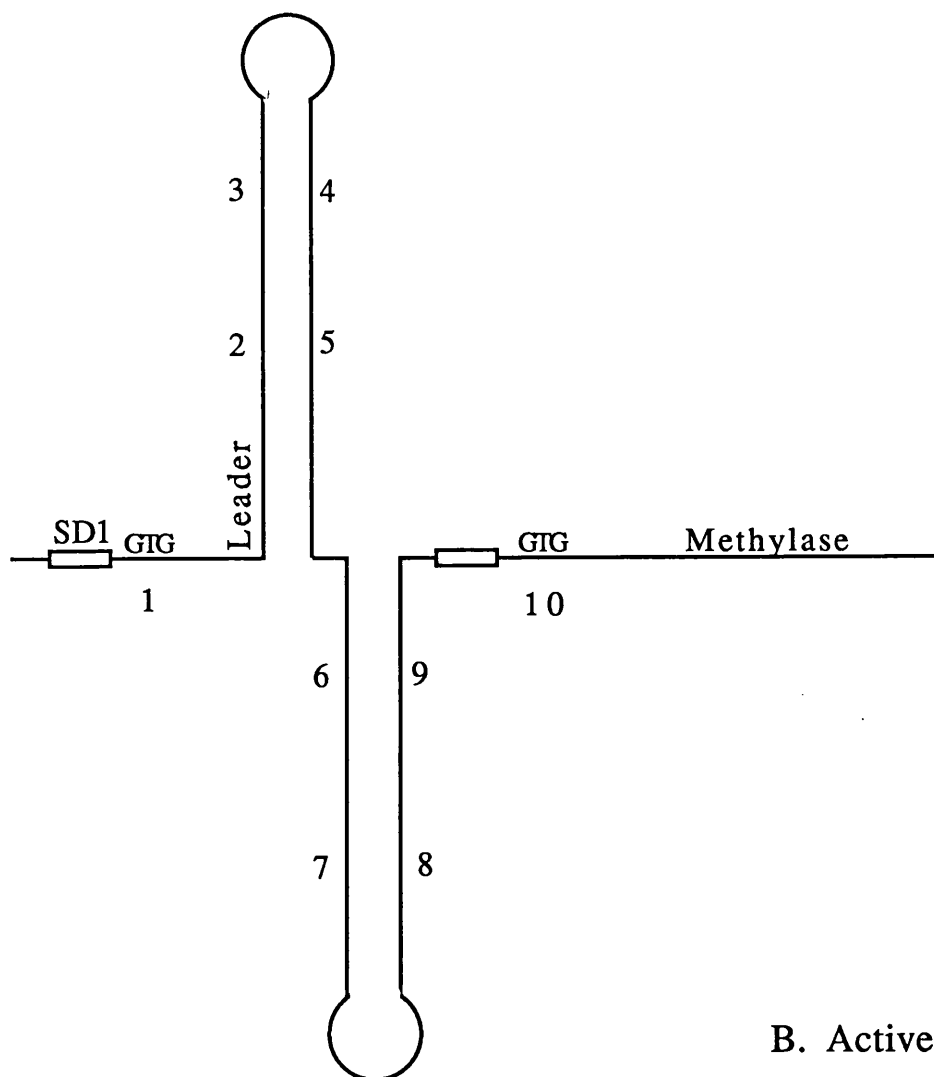
In the various attenuator systems, the length of the control peptide can vary from 14 (*ermD*) to 36 (*ermAM*) amino acid residues and the amino acid composition is also seen to be highly variable, in addition the number of conformational states available to some of the mRNA control sequences appear to be much greater than for *ermC*.

It might be thought that any inhibitor of protein synthesis should cause the ribosome to stall in these systems thereby inducing methylase production, but as already indicated the different MLS resistance genes are all induced by different sub-sets of MLS antibiotics which raises the intriguing question as to what determines the specificity of induction of any given system. The variations between leader peptides and their possible involvement with induction specificity is discussed in chapter 6. The data presented in chapters 3,4, and 5 aim to add to the current knowledge regarding MLS resistance in prokaryotes and to offer an explanation for the

**Fig 1.13. Alternative conformations for *tlrA* mRNA.** A translational attenuation model has been proposed for control of *tlrA*. In the inactive conformation (A) SD-2 is sequestered by the association of stems 9 and 10, whilst in the inactive conformation (B) SD-2 is freed allowing expression of the rRNA methylase.



A. Inactive



B. Active

Fig. 1.13

apparent diversity in phenotypes observed in *Streptomyces* species.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

## METHODS

### 1. Origin, Maintenance, and Growth of Organisms

#### 1.1 Bacterial Strains

The sources of bacteria used in this work and their relevant characteristics are indicated in Table 2.1

#### 1.2 Growth and Preservation of *Streptomyces*

Spores from *Streptomyces* strains were obtained by growth on NE agar which contained 1%(w/v) glucose, 0.2%(w/v) yeast extract, 0.1%(w/v) beef extract, 0.2%(w/v) casamino acids and 2%(w/v) agar, adjusted to pH7.0 with KOH.

All *Streptomyces* strains were preserved as spore suspensions in glycerol. These were prepared by removing spores from the surface of an NE agar plate using 5-10ml of sterile water and filtering them through a cotton wool plug to remove mycelium. Glycerol was then added to a final concentration of 20%, and the spore suspension stored at -20°C.

Confluent plates of streptomyces were prepared by spreading a loop of spore suspension over the surface of an NE agar plate, followed by incubation for 4-5 days at 30°C. For the preparation of liquid cultures, spores and aerial mycelia were removed from a confluent NE agar plate by agitation in 5ml of sterile water, and used to inoculate 2 X 1L YEME medium supplemented with 5mM MgCl<sub>2</sub> and 0.5%(w/v) polyethylene glycol 6000. YEME medium contained (per litre) 3g yeast extract, 3g malt extract, 5g peptone and 10g glucose. Cultures were grown at 30°C for 14-20 h in a New Brunswick orbital shaker at 220-250 rev min<sup>-1</sup>.

For the preparation of DNA or protoplasts from *Streptomyces lividans*

Table 2.1 Bacterial strains

| Organism              | Strain     | Relevant characteristics             | Source                                       |
|-----------------------|------------|--------------------------------------|--|
| <i>S.lividans</i>     | TK21       | plasmid free derivative of strain 66 | Prof. D.A. Hopwood, J.I. Institute, Norwich. |
| <i>S.lividans</i>     | TSK1       | strain TK21 + pIJ702                 | Dr. P. Skeggs, this laboratory.              |
| <i>S.lividans</i>     | AT391      | strain TK21 + pLST391                | Dr. A. Thiara, this laboratory.              |
| <i>S.lividans</i>     | TB700      | strain TK21 + pLST700                | Dr. M. Calcutt, this laboratory.             |
| <i>S.griseofuscus</i> |            | No MLS resistance phenotype          |  |
| <i>S.vellosus</i>     | NCIB 11180 | lincomycin producer                  | NCIB   |
| <i>S.caelestis</i>    | NCIB 9751  | celesticetin producer                | NCIB   |
| <i>S.fradiae</i>      | NCIB 10812 | tylosin producer                     | NCIB   |
| <i>S.lincolnensis</i> | UC 2376    | lincomycin producer                  | Dr. A. Dietz, Upjohn Co., Kalamazoo.         |
| <i>S.spectabilis</i>  |            | streptovaricin producer              | Dr. J. Salas, University of Oviedo, Spain.   |
| <i>S.ambofaciens</i>  | NRRL 2420  | spiramycin producer                  | NRRL   |
| <i>Sacc.erythraea</i> | NRRL 2338  | erythromycin producer                | Prof. J. Davies, Pasteur Institute, Paris.   |
| <i>E.coli</i>         | NM522      |                                      | Dr. M.J. Stark, Leicester Biocentre.         |



strains, 0.1ml of spore suspension was inoculated into 50ml YEME medium supplemented with 34%(w/v) sucrose and 0.5% glycine in a 250ml flask containing a stainless steel coiled spring to aid dispersed growth and aeration. Incubation was for 36-42h at 30°C in a New Brunswick orbital shaker at 250-300 rev min<sup>-1</sup>. For *Streptomyces griseofuscus* strains, YEME medium was supplemented with 1%(w/v) glycine.

### 1.3 Growth and Preservation of *E. coli*

*E. coli* were grown on LB agar for 15-20 h at 37°C. LB agar contained 1%(w/v) tryptone, 0.5%(w/v) yeast extract, 0.5%(w/v) sodium chloride and 2%(w/v) agar. Cells from an LB agar plate which contained 1,000-5,000 colonies were removed in 5ml minimal salts medium containing 0.2%(w/v) glucose, 0.1 mM CaCl<sub>2</sub>, 1mM MgSO<sub>4</sub>, 90mM Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 22mM KH<sub>2</sub>PO<sub>4</sub>, 9mM NaCl, and 19mM NH<sub>4</sub>Cl, and centrifuged in a Hereaus Christ centrifuge at 3500 rev min<sup>-1</sup> for 10 min at room temperature. The cells were then resuspended in 0.5ml minimal salts medium containing 20%(v/v) glycerol, and stored at -20°C.

Liquid cultures of *E.coli* were grown in LB medium at 37°C on an orbital shaker at 200-300 rev min<sup>-1</sup>.

## 2. Cell-free Protein Synthesis: Preparation of Components and Conditions for Assay

To prepare subcellular fractions for *in vitro* protein synthesis, the following methods were used. Except where stated, all the manipulations were carried out at 0-4°C and the final products were divided into small aliquots, rapidly frozen in an industrial methylated spirits-CO<sub>2</sub> bath and stored at -70°C.

## 2.1 Preparation of coupled transcription-translation systems from

*S.lividans* TK21.

This method is essentially as published (Thompson, et al.,1984) Mycelium from 6 X 1L cultures grown for 14-16 h at 30°C, were harvested by centrifugation at 9000 rev min<sup>-1</sup> for 10 min in a Beckman JA10 rotor. the mycelium were resuspended in 400ml BufferI ( 10mM HEPES-KOH pH7.6 at 20°C, 10 mM MgCl<sub>2</sub>, 1M KCl,10mM β-mercaptoethanol ) and washed by centrifugation as above. The washing procedure was repeated twice with Buffer I and twice with Buffer II ( 50mM HEPES-KOH pH7.6 at 20°C, 10mM MgCl<sub>2</sub>, 60mM NH<sub>4</sub>Cl, 5mM β-mercaptoethanol and 10% (v/v) glycerol). After the final wash, the mycelium was resuspended in 150ml buffer II, collected by filtration onto Whatman No.1 paper, weighed and resuspended in 2.5ml Buffer II per gramme wet weight. Typically, the yield of culture was about 2-3 grams per litre of culture. The suspension was then passed through a chilled French pressure cell at 10,000 - 12,000 psi. Unbroken mycelium and cell debris were cleared from the preparation by centrifugation at 15,000 rev min<sup>-1</sup> for 30 min in a Beckman SW27 rotor. The supernatant was removed and recentrifuged under identical conditions. The resulting supernatant was designated "S30" and was typically 200-300 A<sub>260</sub> units ml<sup>-1</sup>. Then, while the bulk of the preparation was held at 0°C, a small portion of S30 was treated with micrococcal nuclease over a series of time to determine the time necessary to remove endogenous DNA and RNA, that would otherwise contribute to the plasmid-independent activity of the system. S30 ( 30 A<sub>260</sub> units ) was incubated with 1μl nuclease (Stock solution; 150U μl<sup>-1</sup> in 50mM glycine-KOH pH 9.2 at 20°C, containing 5mM CaCl<sub>2</sub>) in 1mM CaCl<sub>2</sub> at 30°C. Sample containing 5 A<sub>260</sub> units were removed at 10 min intervals and EGTA-KOH (pH 7.0 at 20°C) was added to 2mM final

concentration, in order to chelate calcium ions and render the calcium-dependent nuclease inactive. A portion (2  $A_{260}$  units) of each nuclease treated sample was then assayed for coupled transcription-translation activity in the presence and absence of exogenous plasmid, to determine the minimum incubation time needed to remove plasmid-independent activity. The remainder of the S30 was then appropriately treated with nuclease prior to storage at  $-70^{\circ}\text{C}$ . Extracts prepared in this way retained activity for more than 12 months.

## 2.2 Preparation of S100 and Crude Ribosomes.

A high speed supernatant fraction was prepared from a nuclease-treated S30 by centrifugation at  $50,000 \text{ rev min}^{-1}$  for 2.5 hrs in a Beckman Ti70 rotor. The resulting supernatant (S100) was stored at  $-70^{\circ}\text{C}$ . The crude ribosome pellet was resuspended in Buffer III (10mM HEPES-KOH pH 7.6 at  $20^{\circ}\text{C}$ , 10mM  $\text{MgCl}_2$ , 60mM  $\text{NH}_4\text{Cl}$  and 5mM  $\beta$ -mercaptoethanol) and centrifuged for 5-16 hrs at  $40,000 \text{ rev min}^{-1}$  in a Beckman Ti75 rotor. The pellet was resuspended in Buffer III and stored at  $-70^{\circ}\text{C}$ .

## 2.3 Preparation of crude initiation factors for protein synthesis.

This method is based on that developed for the preparation of crude initiation factors from *Bacillus subtilis* [Legault-Demare and Chambliss, 1974]. *S. lividans* mycelia from cultures grown for 16-20 h at  $30^{\circ}\text{C}$  were harvested at  $9,000 \text{ rev min}^{-1}$  for 10 min in a Beckman JA10 rotor, washed twice in Buffer IV (Buffer I minus glycerol) and once in Buffer III. The pellet from the final wash was resuspended in a small volume of Buffer III and passed through a precooled French pressure cell at 10,000 - 12,000 psi. The resulting suspension was cleared by centrifugation at  $15,000 \text{ rev min}^{-1}$  for 30 min in a Beckman SW27 rotor. The supernatant was then

recentrifuged at  $45,000 \text{ rev min}^{-1}$  for 4 h in a Beckman Ti70 rotor. The crude ribosome pellet was resuspended in Buffer III, stirred slowly at  $0^{\circ}\text{C}$  for 16 hrs and recentrifuged at  $45,000 \text{ rev min}^{-1}$  as above. The pellet was then resuspended in Buffer V (10mM HEPES-KOH pH 7.6 at  $20^{\circ}\text{C}$ , 10mM  $\text{MgCl}_2$ , 1M  $\text{NH}_4\text{Cl}$  and 5mM  $\beta$ -mercaptoethanol) and slowly stirred for 16 h at  $0^{\circ}\text{C}$ . Ribosomes were removed from the preparation by centrifugation as above and the upper four-fifths of the supernatant collected. Seven volumes of saturated ammonium sulphate solution pH 7.0, were slowly added to three volumes of supernatant and kept at  $0^{\circ}\text{C}$  for 1.5 h with continuous stirring. The resultant precipitate was collected by centrifugation at  $15,000 \text{ rev min}^{-1}$  for 15 min in a Beckman JA21 rotor and the pellet resuspended in 1.5ml of Buffer III per 6L of original culture.

This "factor" preparation was extensively dialysed against Buffer III and stored at  $-70^{\circ}\text{C}$ .

## 2.4 Preparation of Salt Washed Ribosomes

Salt-washed ribosomes were obtained from 30,000 X g supernatants prepared as described in section 2.3 above, with one additional step: DNAase ( $5\mu\text{g ml}^{-1}$  final concentration) was added to the suspension after passage through the french pressure cell. The S30 was then layered over an equal volume of 20% (w/v) sucrose in Buffer VI (Buffer V with the  $\text{MgCl}_2$  concentration adjusted to 30mM) and centrifuged for 5 h or overnight at  $45,000 \text{ rev min}^{-1}$  in a Beckman Ti70 rotor. The supernatant was discarded and brown membranous material was removed from the ribosome pellet by gentle agitation with a glass rod, in a small volume of Buffer III. The ribosome pellet was then resuspended in the same buffer and stored as small aliquots at  $-70^{\circ}\text{C}$ .

## 2.5 Conditions for coupled transcription-translation assays

Assays were performed in a 34  $\mu$ l volume and contained 26% (v/v) synthesis mix (see below), 2-3.3  $\mu$ g plasmid DNA, ( $^{35}$ S) methionine (27  $\mu$ Ci  $\text{ml}^{-1}$ , 10 Ci  $\text{mmol}^{-1}$ ), magnesium acetate to give 12 mM final  $\text{Mg}^{2+}$  concentration and the components of cell-free extract to be tested. When S30 was used, 2A<sub>260</sub> units were included. Otherwise 24 pmol of salt-washed ribosomes were employed together with crude initiation factor preparation and S100. The inputs of the latter two components were optimised for each preparation.

Synthesis mix contained 200 mM HEPES-KOH pH 7.6 at 20°C; 140 mM ammonium acetate; 280 mM potassium acetate; 7mM DTT; 5mM ATP (sodium salt; pH 7.0 with Tris); 3.4 mM CTP, GTP, and UTP (all sodium salts; pH 7.0 with Tris); 100 mM PEP (trisodium salt; pH 7.0 with Tris); 19 amino acids (minus methionine) each at 1.4 mM; 7.5% (w/v) PEG 6000; 260  $\mu$ M calcium folinate and 100 units pyruvate kinase in 20% (v/v) glycerol.

Incubations were at 30°C. 5-10  $\mu$ l samples were removed into 0.1M KOH at various time intervals and heated at 95°C for 7 min to hydrolyse methionyl-tRNA. Following the addition of excess 10% (w/v) TCA, acid-precipitable material was collected on Whatman GF/C filters, extensively washed with 5% (w/v) TCA and dried under infra-red light. The radioactivity retained on the filters was estimated by liquid scintillation spectrometry using a toluene-based scintillation fluid (Fisofluor No.3).

## 2.6 Polyacrylamide gel analysis of the products of protein synthesis *in vitro*.

Samples for gel analysis were produced as above (section 2.5) except that ( $^{35}$ S) methionine (14 Ci  $\text{ml}^{-1}$ ; 800 Ci  $\text{mmol}^{-1}$ ) was used. After 20 min incubation at 30°C, 2 $\mu$ l of unlabelled methionine (44 mg  $\text{ml}^{-1}$ ) was added to the reaction and the incubation continued for 10 min, to allow completion of all radiolabelled peptides. Then a 2 $\mu$ l sample was removed to estimate the

radioactive content of the hot TCA-precipitable material. The samples were mixed with one third volume of loading buffer (375 mM Tris-HCl pH 8.8 at 20°C; 4% (w/v) SDS; 35% (v/v) glycerol; 2.7 M  $\beta$ -mercaptoethanol and 0.01% (w/v) BPB), heated for 10 min at 100°C prior to loading on to a 10 % polyacrylamide gel (14.5 x 10.5 x 1.5 cm) containing 0.1 % SDS, prepared according to the standard procedure of (Laemmli, *et al.*, 1970). Electrophoresis was typically for 16 h at 50V. The gels were then fixed in 7% (v/v) acetic acid, treated with "Amplify" and dried on to Whatman No.1 paper, prior to fluorography using Fuji RX film at -70°C.

## 2.7 Preparation of methylating extracts.

In experiments where S30 was used as the source of methylase activity, extracts were prepared as described above. However, in cases where a ribosomal wash fraction was required, the S30 was centrifuged at 50,000  $\text{rev min}^{-1}$  for 3 h at 4°C in a Beckman Ti70 rotor. The pellet of crude ribosomes was suspended in Buffer VI (2/3 vol. initial S30)[10mM HEPES-KOH pH7.6 at 20°C, 30mM  $\text{MgCl}_2$ , 1M  $\text{NH}_4\text{Cl}$ , 5mM  $\beta$ -mercaptoethanol] containing 10% glycerol and kept on ice for 5 hours. The salt washed ribosomes were then layered over half that volume of similar buffer containing 20% (w/v) sucrose and centrifuged at 35,000  $\text{rev min}^{-1}$  for 16 hours at 4°C in a Beckman Ti70 rotor. The resultant supernatant (ribosomal wash fraction) was dialysed four times against 100 volumes of Buffer III containing 10% glycerol and was stored at -70°C.

## 2.8 Preparation of ribosomal RNA

When required as a substrate for methylase activity, total RNA from salt washed ribosomes (RNA70) was prepared by extraction of the particles with LiCl and urea as described by (Fahnestock, *et al.*, 1974). Here, sucrose and salt

washed ribosomes were adjusted to an  $A_{260}$  of 300-500 in Buffer III and after adding an equal volume of freshly prepared solution I [8M urea, 4M LiCl], the mixture was placed on ice for 36-48 h. The RNA precipitate was then recovered by centrifugation at room temperature in an MSE bench top centrifuge for 5 min, and the resulting RNA pellet washed with a 1:1 mixture of Buffer III and solution I. Following re-centrifugation for 2 min at room temperature the RNA was resuspended in water to give a final concentration of approximately 5 pmol/ $\mu$ l. All RNA preparations were stored in water at -70°C.

## 2.9 Assays of rRNA methylase activity

Methylase assays were carried out in a buffer containing 50mM HEPES-KOH pH7.5 at 20°C, 7.5 mM  $MgCl_2$ , 37.5 mM  $NH_4Cl$ , and 3mM  $\beta$ -mercaptoethanol plus 5  $\mu$ Ci [ $^3H$ -methyl] S-adenosyl methionine (500 mCi mmol<sup>-1</sup>; 18.5 GBq mmol<sup>-1</sup>). Assays were performed in a total volume of 0.1ml containing 20 pmol of RNA together with 50  $\mu$ l of ribosomal wash fraction. The assay mix in the absence of RNA, was pre-incubated for 10 min at 30°C before initiating the reaction by adding the RNA substrate. Samples (20 $\mu$ l) were removed at intervals into approximately 1ml ice-cold 5% (w/v) TCA and incubated for a further 20 min at 0°C, before collecting acid precipitable material on Whatman GF/C filters. The filters were extensively washed with 5% (w/v) TCA, dried under infra-red light and the radioactivity retained on the filters estimated by liquid scintillation spectrometry using a toluene based scintillation fluid.

## 2.10 Analysis of methylated RNA by paper chromatography.

When radiolabelled RNA was to be analysed by descending paper chromatography, bulk methylations were carried out in which the normal

assay mixture (described above) was scaled up by a factor of 3 (to 300  $\mu$ l final volume) and 15  $\mu$ Ci of S-adenosyl [methyl- $^3$ H] methionine with increased specific radioactivity (15Ci/mmol; 555 GBq/mmol) was used. Labelled RNA was recovered by the addition of an equal volume of phenol:chloroform (50: 50), the mixture was agitated for a few seconds, and centrifuged at 3,000 rev min<sup>-1</sup> for 10 min in an MSE bench top centrifuge. The supernatant was removed and re-extracted with phenol: chloroform as described above. Following precipitation of the RNA from the aqueous phase with 3x volume of ethanol in the presence of 300mM sodium acetate at -20°C for 16 h, the RNA was collected by centrifugation at 3,000 rev min<sup>-1</sup> for 15 min and the RNA pellet dried in a vacuum dessicator for 3 min. The RNA was then resuspended in 50  $\mu$ l of distilled water and hydrolysed in the presence of 1M HCl at 100°C for 60 min and following incubation for 15 min at -70°C the sample was lyophilised for a further 1.5 h. The RNA was resuspended in 100  $\mu$ l of 5mM NH<sub>4</sub>OH and lyophilised until a volume of 40  $\mu$ l remained. The remaining sample was loaded on to 3MM paper (Whatman, Inc.) and the products separated by descending paper chromatography using as solvent either isopropanol/HCl/water (170:41:39) or butanol/ammonia /water (95:5:14) for 27 or 19 h respectively. The chromatograms were dried, markers detected under U.V. light and radioactivity was estimated by liquid scintillation spectrometry after the tracks were cut into strips 1 cm wide.

## 2.11 Analysis of methylated RNA by thin-layer chromatography.

Ribosomal RNA was radiolabelled as described above, however following phenol:chloroform extraction and ethanol precipitation the RNA pellet was resuspended in 30  $\mu$ l of 25mM sodium acetate pH 5.3 at 20°C and digested with nuclease P1 (10 units) for 3 hours at 37°C to generate nucleoside-5'-monophosphates. The sample was then loaded on to cellulose plates [Macherey-Nagel MN 300] and analysed by two-dimensional



thin-layer chromatography, using as solvent isobutyric acid:  $\text{NH}_4\text{OH}$  (5:3; vol: vol) in the first dimension and isopropanol:  $\text{HCl}$ : water (70: 15: 15; v: v: v) in the second. The chromatogram was run for 9 h and 1.5 h respectively. After visualising standards under UV light, the plates were sprayed with EnHance (New England Nuclear) and the radiolabelled products located by fluorography at  $-70^\circ\text{C}$ .

### 3. Isolation and Manipulation of DNA.

#### 3.1 Preparation of Total Genomic DNA.

The isolation of total DNA from strains of *Streptomyces* and *E. coli* was carried out using the lytic procedure of Smith (in Hopwood, *et al.*, 1985). A 25 ml *Streptomyces* culture was incubated at  $30^\circ\text{C}$  for 40-42 h and then harvested by centrifugation at  $3,000 \text{ rev min}^{-1}$  for 10 min at room temperature in a Hereus-Christ centrifuge. The mycelium was washed with 10.3% (w/v) sucrose, and centrifuged as above. The pellet was then resuspended in 4 ml "lysozyme solution" ( 2 mg  $\text{ml}^{-1}$  lysozyme, 25 mM Tris-HCl pH 8.0 at  $20^\circ\text{C}$ , 25mM EDTA-KOH pH 8.0, 10.3% (w/v) sucrose) and incubated for 10 min at  $37^\circ\text{C}$ . After the addition of 4 ml Kirby mix [2% (w/v) sodium triisopropyl naphthalene sulphonate, 12% (w/v) sodium 4-aminosalicylate, 0.1 M Tris-HCl pH 8.0 at  $20^\circ\text{C}$ , 6% (v/v) phenol equilibrated with 0.1M Tris-HCl pH 8.0 at room temperature], the preparation was agitated on a vortex mixer for 1 min. An equal volume of phenol (saturated as above): chloroform (50:50) was then added, the mixture agitated for a further 15S followed by centrifugation at  $3,000 \text{ rev min}^{-1}$  for 10 min. The supernatant was removed, re-extracted with an equal volume phenol:chloroform and centrifuged as above. Following the precipitation of DNA and RNA from the aqueous phase with an equal volume of isopropanol in the presence of 300mM sodium acetate, nucleic acid was spooled out using

a glass hook. The precipitate was then washed in 80% (v/v) ethanol and dissolved in 5ml TE buffer ( 10mM Tris-HCl pH 8.0 at 20°C, 1mM EDTA-KOH pH 8.0 at 20°C) containing 40µg ml<sup>-1</sup> RNAase (pre-heated to 90°C for 10 min to inactivate any contaminating deoxyribonuclease present). The preparation was then incubated at 37°C for 30 min, extracted with phenol: chloroform and reprecipitated with isopropanol and salt as above. The DNA was finally resuspended in 1 ml TE buffer and stored at 4°C.

### 3.2 Preparation of Plasmid DNA from *Streptomyces*

Since all the *Streptomyces* plasmids used in this study contained the thiostrepton resistance gene as a primary selectable marker, cultures were routinely grown in media supplemented with thiostrepton (20 µg ml<sup>-1</sup>). All plasmids were prepared by the alkaline lysis method of [Kieser *et al.*, 1984], from 50 ml cultures grown for 40-48 h at 30°C.

Mycelium was harvested by centrifugation at 3,000 rev min<sup>-1</sup> for 10 min at room temperature, washed by centrifugation through 10.3% (w/v) sucrose and resuspended in 5 ml final volume of "lysozyme solution" (section 3.1 above). Following incubation at 37°C for 20 min, 2.5 ml of freshly prepared alkaline SDS (0.3M sodium hydroxide, 2% (w/v) SDS) was added and the mixture agitated immediately on a vortex mixer. The cell lysate was incubated at 70°C for 20 min and then slowly cooled to room temperature. The preparation was then extracted with 2 ml unbuffered phenol: chloroform (50:50) and centrifuged for 15 min at 3,000 rev min<sup>-1</sup>. The aqueous phase was removed, extracted once with neutral phenol: chloroform and once with chloroform. Nucleic acid was precipitated by the addition of an equal volume of isopropanol in the presence of 300mM sodium acetate and incubated at room temperature for 20 min. The precipitate was then collected by centrifugation at 8,000 rev min<sup>-1</sup> for 10 min in a Sorvall HB4 rotor, washed with 80% (v/v) ethanol, dried *in vacuo* for 10 min and resuspended in 1 ml

TE buffer containing  $40\ \mu\text{g ml}^{-1}$  RNase. After incubation at  $37^\circ\text{C}$  for 30 min, the preparation was extracted with phenol: chloroform and reprecipitated as above. The DNA was dried and finally resuspended in 1 ml TE buffer.

Purer preparations of plasmid DNA were obtained by isolating supercoiled DNA from caesium chloride gradients. Plasmid DNA in less than 1 ml was diluted to 4 ml with TE buffer. Caesium chloride (4.2g) was dissolved in the DNA solution and ethidium bromide ( $10\ \text{mg ml}^{-1}$ ) added. This solution was then centrifuged at  $48,000\ \text{rev min}^{-1}$  for 16 h at  $20^\circ\text{C}$  in a Beckman VTi65.2 rotor. Bands of DNA were visualised under U.V. light and that corresponding to supercoiled DNA collected, extracted with an equal volume isopropanol (saturated with caesium chloride) until all the ethidium bromide had been removed and then precipitated with sodium acetate and ethanol at  $-20^\circ\text{C}$ . The DNA was collected by centrifugation for 10 min in an MSE Microcentaur microfuge, washed with 80% (v/v) ethanol, dried *in vacuo* and resuspended in 0.1 ml TE buffer.

### 3.3 Large scale preparation of *E.coli* plasmids

Cultures (50 ml) were grown for 16 h at  $37^\circ\text{C}$  in LB medium supplemented with  $200\ \mu\text{g ml}^{-1}$  ampicillin. Cells were harvested by centrifugation at  $3,000\ \text{rev min}^{-1}$  for 10 min at room temperature in a Hereaus-Christ centrifuge, resuspended in 2 ml of lysozyme solution (25 mM Tris-HCl pH 8.0 at  $20^\circ\text{C}$ ; 10 mM  $\text{Na}_2\text{EDTA}$  pH 8.0 at  $20^\circ\text{C}$ ; 50 mM glucose;  $5\ \text{mg ml}^{-1}$  lysozyme) and incubated at room temperature for 15 min. Cell lysis was achieved by the addition of 4 ml of freshly prepared alkaline SDS solution (0.2 M NaOH; 1% SDS). The mixture was inverted several times and incubated at  $0^\circ\text{C}$  for 10 min. Chromosomal DNA was precipitated by the addition of 3 ml of 3 M Potassium acetate (pH 4.8 at  $20^\circ\text{C}$ ; see materials). The contents of the universal were mixed thoroughly and incubated on ice for 10 min. The precipitate was removed by centrifugation at  $3,000\ \text{rev min}^{-1}$  for

10 min at room temperature in a Hereaus-Christ centrifuge. The aqueous phase was extracted with an equal volume of phenol:chloroform and chloroform before precipitating the nucleic acid by the addition of 0.6 volumes of isopropanol and incubation at room temperature for 15 min. The nucleic acid was collected by centrifugation at  $7,000 \times g$  in a Sorvall HB4 rotor for 30 min at room temperature. The pellet was washed with absolute ethanol, dried *in vacuo* and resuspended in 0.5 ml TE buffer containing  $40 \mu\text{g RNase ml}^{-1}$ . After incubation at  $37^\circ\text{C}$  for 30 min the preparation was extracted as above and precipitated with 3 volumes ethanol and 300 mM sodium acetate at  $-20^\circ\text{C}$  for 1 h or overnight. The plasmid DNA was collected by centrifugation for 20 min in a Sorvall HB4 rotor, dried *in vacuo* and resuspended in 0.1 ml TE buffer. When the plasmid was destined for use in the coupled transcription-translation system, or as probes for transcript mapping, RNase was not used.

#### 3.4 Small scale preparation of *E. coli* plasmids.

When a large number of plasmid was required for analysis by restriction mapping, a modification of the procedure described by (Birnboim, *et al.*, 1979) was employed.

Selected colonies were grown in 2 ml LB medium supplemented with  $200 \mu\text{g ampicillin ml}^{-1}$  in an 11.5 ml Sarstedt test tube, suspended horizontally and shaken vigorously at  $37^\circ\text{C}$  overnight. 1.5 ml of overnight culture were transferred to a 1.6 ml microcentrifuge tube the cells harvested by centrifugation for 2 min. The cells were resuspended in 0.1 ml lysozyme solution (see above) and incubated at room temperature for five min. Lysis was achieved by the addition of 0.2 ml freshly prepared alkaline SDS (see above) to the tubes which were sharply inverted 2-3 times and incubated, on ice, for 5 min. Chromosomal DNA was then precipitated by adding 0.15 ml 3M potassium acetate pH 4.8 at  $20^\circ\text{C}$  (see materials) and mixing the contents

of the tube by inverting it and agitating briefly (1-2 seconds) on a vortex mixer. After incubation on ice for 5 min the precipitate was collected by centrifugation in a Burkard microcentrifuge. The aqueous phase was extracted with an equal volume of phenol:chloroform and chloroform before the nucleic acid was precipitated using 2 volumes of isopropanol and incubation at room temperature for 5 min, followed by centrifugation in a Burkard microcentrifuge for 10 min. The pellet was washed with absolute ethanol (precooled at -20°C), dried *in vacuo*, and resuspended in 0.1 ml TE buffer containing 40 µg RNase ml<sup>-1</sup>. The preparation was incubated at 37°C for 1 h before extraction with an equal volume phenol:chloroform and chloroform. The aqueous phase was taken and the plasmid DNA precipitated by the addition of 3 volumes ethanol and 300mM sodium acetate. The DNA was collected, washed and dried as above, and the pellet resuspended in 40µl TE buffer. For restriction analysis 3 µl of the preparation was adequate.

### 3.5 Agarose gel electrophoresis of DNA samples.

DNA preparations were routinely analysed in horizontal 0.7% (w/v) agarose gels cast in TEA buffer (40mM Tris-acetate pH8.0 at 20°C, 2mM Na<sub>2</sub>EDTA). DNA samples containing 10% (v/v) sample buffer [TEA buffer supplemented with 50% (v/v) glycerol, 0.01% (w/v) xylene cyanol FF and 0.01% (w/v) BPB] were loaded on to gels (8 cm x 8 cm x 3mm) which were submerged in TEA and electrophoresed at 80V for 40-50 min. DNA was visualised by staining in 1µg ethidium bromide ml<sup>-1</sup> for 10 min and observation on a U.V. transilluminator. Lambda phage DNA fragments from digestion with the restriction endonucleases *HindIII* and *EcoRI* were used as size markers for linear molecules (Daniels, *et al.*, 1980).

### 3.6 Restriction, phosphatase treatment and ligation of DNA.

DNA was cleaved with various restriction endonucleases under the

conditions specified by the manufacturers. Reactions were typically in volumes of less than 50 $\mu$ l. When the DNA was required for further manipulation, it was subjected to the following treatment. After extraction with an equal volume of neutral phenol: chloroform and chloroform alone, the DNA was precipitated from the aqueous phase with 3 volumes of ethanol in the presence of 300mM sodium acetate, using an industrial methylated spirits-CO<sub>2</sub> bath for 20 min. The precipitate was collected by centrifugation for 10 min in an MSE Microcentaur microcentrifuge, washed in 80% (v/v) ethanol, centrifuged for 5 min, dried under vacuum for 10 min and finally resuspended in TE buffer.

In many experiments, vector DNA was also treated with calf intestinal alkaline phosphatase (CIAP) during restriction. This enzyme removes the terminal phosphates after cleavage and therefore abolishes the recircularisation of vector molecules in ligation mixtures. Typically, 1-2 units of enzyme were used to treat up to several microgrammes of DNA. Before phenol-chloroform extraction and subsequent precipitation, the CIAP was inactivated by heating the sample at 75°C for 15 min in the presence of 0.1% (w/v) SDS, 10 mM Tris-HCl pH8.0 at 20°C, 100mM sodium chloride and 1mM Na<sub>2</sub>EDTA.

For shotgun cloning experiments, 1 $\mu$ g linear vector (CIAP treated) was ligated with 5-6  $\mu$ g genomic DNA fragments at 40 $\mu$ g ml<sup>-1</sup> final concentration in 66 mM Tris-HCl (pH 7.5 at 20°C), 6.6 mM MgCl<sub>2</sub>, 1 mM DTT and 0.4 mM ATP. 1 unit of T4 DNA ligase was added and incubation was carried out at room temperature for 16-20 h. The DNA was precipitated with salt and ethanol as above and resuspended in 20 $\mu$ l TE buffer.

In subcloning experiments, vector and donor DNA fragments were ligated at approximately 1:2 molar stoichiometry, in 25-50  $\mu$ l under ionic conditions as described above. The ligation mixture was then used directly

for transformation experiments without recourse to precipitation.

### 3.7 Isolation of specific DNA molecules from agarose gels.

In some experiments, it was necessary to purify particular DNA fragments prior to ligation. The DNA species of interest were separated by electrophoresis in a 1% (w/v) low melting point agarose gel prepared in TEA buffer under the conditions described previously. The DNA was visualised on a transilluminator.

The method employed was that of (Langridge, *et al.*, 1980). Gel slices containing the required DNA were removed from the gel and incubated in a 1.6 ml microcentrifuge tube at 70°C for 30 min. The following manipulations were performed at 37°C. An equal volume of water/CTAB (see materials) and an equal volume of butanol/CTAB (see materials) were added and the contents of the tube agitated for 30 s using a vortex mixer. The phases were separated by centrifugation for 1 min in an MSE microcentaur microcentrifuge and the upper butanolic phase was retained. The aqueous phase was then re-extracted with half a volume of butanol/CTAB and, following centrifugation as above, the butanol phases were pooled. The preparation could now be returned to room temperature. The DNA was extracted from the butanol phase by the addition of 1/4 volume of 0.3M sodium acetate pH 7.0 at 20°C. The mixture was agitated on a vortex mixer for 30 sec and the phases separated by centrifugation for 1 min in a microcentrifuge. The lower, aqueous phase was removed, and as a consequence of an increase in volume, 3M sodium acetate was added to return the concentration of those ions to 300mM. The aqueous phase was extracted with chloroform before being precipitated with 3 volumes of ethanol and incubation in an IMS/CO<sub>2</sub> bath for 30 min or overnight at -70°C. The DNA was collected by centrifugation for 10 min in a Burkard microcentrifuge, dried *in vacuo* and resuspended in 10 µl TE buffer.

### 3.8 Southern hybridization of DNA .

#### a) Transfer of DNA

DNA samples were separated by electrophoresis in a 0.7% agarose gel in TEA buffer at  $6V\text{ cm}^{-1}$  for 3 h. To allow efficient transfer of large DNA molecules, the DNA in the gel was "acid nicked" by washing the gel in 0.25 M HCl for 7 min. The DNA was then denatured in a solution containing 0.5M NaOH and 1.5 M NaCl for 30 min at room temperature. Finally the gel was neutralised by washing in 3 M NaCl and 0.5 M Tris-HCl (pH 7.4 at 20°C) for 30 min at room temperature.

The blotting apparatus was set up with a wick of Whatman 3MM paper supported on a glass plate. The ends of the wick were placed in a solution of 3M NaCl and 0.3M sodium citrate and the gel placed on top of this, with the top surface uppermost. Hybond-N nitrocellulose membrane (Amersham) was cut to the size of the gel, pre-soaked in a solution of 0.045M sodium citrate and 0.45 M NaCl and placed on top of the agarose gel. The gel was then surrounded by spacers composed of double layered Saranwrap to prevent the buffer from bypassing the gel and membrane. A sheet of Whatman N°1, pre-soaked as above, was placed on top of the membrane and a 10 cm stack of absorbant paper towels were held in position above these, under a 1 kg weight. Wet paper towels were replaced every 5 minutes for the first 30 min and every 15 min for the next 1.5 hours.

The membrane was then removed, washed briefly in 0.045 M sodium citrate, 0.45 M NaCl and allowed to dry at room temperature. The membrane was wrapped in a single layer of Saranwrap and the DNA cross linked to the membrane by placing it on a UV transilluminator for 2 min, with the side having been in contact with the gel facing downwards.

#### b) Preparation of radiolabelled DNA fragments



Radiolabelled probes for Southern hybridization were prepared by a method using random hexadeoxynucleotide primers ([Feinberg, et al.,1984])

The DNA fragment to be labelled was excised from a 1% (w/v) low melting point agarose gel after electrophoresis at 0.5V cm<sup>-1</sup> for 16-20 h and visualisation in ethidium bromide on a U.V. transilluminator. The weight of the gel slice was determined and 1.5 ml distilled water added per gram of agarose. From this an approximate value for the DNA concentration was calculated. The gel slice was incubated at 100°C for 7 min and then kept at 37°C for 60 min. 25 ng DNA fragment was radiolabelled in a 25 µl reaction mixture at room temperature for 16 h. The reaction contained 50 mM Tris-HCl (pH8.0 at 20°C), 5mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 20µM each of dATP, dGTP, and TTP, 200mM HEPES-NaOH pH 6.6 at 20°C, 1.35 A<sub>260</sub> units hexadeoxynucleotides, 0.4 mg ml<sup>-1</sup> enzyme grade BSA, 25 µCi [α-<sup>32</sup>P] dCTP (3,000-4,000 Ci mmol<sup>-1</sup>) and 2 units large fragment of DNA polymerase I.

The enzyme was inactivated by heating to 70°C and unincorporated nucleotides were removed by gel filtration through a Sephadex G50 column. The column was prepared as described by [Maniatis *et al.*, 1982]. A suspension of Sephadex G50 in water was autoclaved at 15 psi for 10 min and used to prepare a column in a 1 ml syringe plugged with siliconized glass wool. The column was packed by centrifugation at 2,000 rev min<sup>-1</sup> in an MSE bench top centrifuge and rinsed with sterile water as described above to prepare the column for use.

The radiolabelled DNA was then passed through the column by centrifugation at 2000 rev min<sup>-1</sup> for 1 min at room temperature, and collected in a 1.6 ml microcentrifuge tube. The DNA was then denatured by incubation at 95°C for 5 min.

### c) Hybridization of DNA

The membrane was prehybridised in 25 ml buffer containing 0.27M NaCl, 0.015 M  $\text{NaH}_2\text{PO}_4$  (pH 7.4 at 20°C), 1.5 mM  $\text{Na}_2$  EDTA (pH7.4 at 20°C), 1% (w/v) SDS, 6% (w/v) PEG 6000 and 0.5% (w/v) dried milk, in a perspex chamber for 1hour at 65°C. The membrane was then transferred to a second chamber containing 25 ml of the same buffer, 25 ng of radiolabelled DNA probe was added, and hybridisation continued for 16 hours with constant agitation. The membrane was then washed briefly in 0.045 M sodium citrate, 0.45 M NaCl at room temperature before being washed three times in 200 ml of the same solution containing 0.1% (w/v)SDS at 65°C for 10 min and finally three times in 200 ml of a solution containing 7.5 mM sodium citrate, 0.075 M NaCl and 0.1% SDS at 65°C for 10 min. The membrane was allowed to dry and the radioactive bands visualised by autoradiography.

#### 4. Preparation of bacteria for transformation with plasmid DNA.

##### 4.1 Preparation, transformation and regeneration of *S. lividans* protoplasts.

This method is that of Bibb, *et al.* (1978), modified by Thompson, *et al.* (1982). Since transformation is *via* protoplasts and these are highly susceptible to traces of detergent, all glassware was acid-washed and sterile plastic items were used.

Mycelium from a 25 ml culture incubated at 30°C for 38-40 h, was harvested by centrifugation at 3,000 rev min<sup>-1</sup> for 10 min in a Hereaus Christ centrifuge and washed twice by centrifugation through 10.3% (w/v) sucrose at room temperature. Protoplasts were generated by incubation of the washed mycelium in 4 ml lysozyme solution (1mg lysozyme ml<sup>-1</sup> in L buffer) at 30°C for 30 min. L buffer contained 10.3% (w/v) sucrose, 2.5 mM  $\text{MgCl}_2$ , 2.5 mM  $\text{CaCl}_2$ , 1.4 mM  $\text{K}_2\text{SO}_4$ , 0.4 mM  $\text{KH}_2\text{PO}_4$ , 25 mM TES-NaOH (pH 7.2 at 20°C) and 0.2% (v/v) trace element solution. The trace element solution contained (per litre) 40 mg  $\text{ZnCl}_2$ ; 200 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ; 10 mg

$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ; 10 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 10 mg  $\text{Na}_2\text{B}_4\text{O}_7$  and 10 mg  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ . The suspension was triturated three times, incubated for a further 15 min at  $30^\circ\text{C}$  and then diluted with 5 ml P buffer (as L buffer, but with 10 mM  $\text{MgCl}_2$  and 25 mM  $\text{CaCl}_2$ ). Protoplasts were separated from residual mycelium by passage through a cotton wool plug, and collected by centrifugation at  $3,000 \text{ rev min}^{-1}$  for 10 min in an MSE bench top centrifuge. The protoplast pellet was resuspended in 0.5 ml P buffer and the concentration of protoplasts determined by measurement of the optical density at 600 nm (1 OD unit is equivalent to  $1.5 \times 10^9$  protoplasts).

Protoplasts ( $4 \times 10^9$ ) were diluted with 5 ml P buffer and centrifuged as above, immediately prior to transformation. The pellet was resuspended in a minimal volume of P buffer (approximately 0.1 ml), to which 25 ng supercoiled plasmid DNA or 20-30  $\mu\text{l}$  ligation mix was added, followed by 0.5 ml T buffer [25% (w/v) PEG 1000, 2% (w/v) sucrose, 1 mM  $\text{K}_2\text{SO}_4$ , 75 mM  $\text{CaCl}_2$ , 35 mM Tris-maleic acid pH 8.0 at  $20^\circ\text{C}$  and 0.2% (v/v) trace element solution]. Not later than 30 s after 5 ml P buffer was added. The protoplasts were harvested by centrifugation, resuspended in a minimal volume of P buffer and then diluted to 1 ml with the same buffer.

Protoplasts ( $4 \times 10^8$ ) were spread onto each plate of regeneration medium (R2YE), since this number has been reported to be optimal for regeneration (Thompson, et al., 1982). R2YE agar contained 10.3% sucrose, 1.4 mM  $\text{K}_2\text{SO}_4$ , 50 mM  $\text{MgCl}_2$ , 1% (w/v) glucose, 0.4 mM  $\text{KH}_2\text{PO}_4$ , 20 mM  $\text{CaCl}_2$ , 0.3% (w/v) L-proline, 0.5% (w/v) yeast extract, 0.01% (w/v) casamino acids (Difco), 25 mM TES-NaOH pH 7.2 at  $20^\circ\text{C}$ , 5 mM NaOH, 0.2% (v/v) Trace element solution and 2.2% (w/v) Difco agar. The plates were dried in a laminar flow hood for 3-4 h prior to use. In this time, they had lost approximately 15% weight.

After 18-22 h incubation at  $30^\circ\text{C}$ , primary transformants were selected

by flooding the plates with 2 ml thiostrepton suspension ( $0.5 \text{ mg ml}^{-1}$  in water). After further incubation for 4-5 days, the number of transformants was estimated. In all the *Streptomyces* transformation experiments in this work, thiostrepton resistance was used as the selectable marker.

#### 4.2 Preparation and transformation of competent cells from *E.coli*

Strain NM522 was used as the cloning host for all the manipulations in *E.coli*. This strain does not possess a functional  $\beta$ -galactosidase gene since the  $\alpha$ -peptide portion is absent. However, when plasmid vectors containing the  $\alpha$ -peptide region are introduced into this strain,  $\beta$ -galactosidase function is restored by intragenic complementation. Expression of this gene is increased in the presence of an inducer such as isopropyl- $\beta$ -D-thiogalacto-pyranoside (IPTG). This system is particularly useful when organisms are grown in a medium containing X-gal in addition to IPTG. X-gal is chromogenic substrate for  $\beta$ -galactosidase, the product of which is blue in colour. As a consequence, *E. coli* NM522 carrying a plasmid that contains a gene coding for the  $\alpha$ -peptide of  $\beta$ -galactosidase are blue in colour when grown in the presence of IPTG and X-gal. However, if DNA is inserted in the  $\alpha$ -peptide gene then no functional  $\beta$ -galactosidase is produced and the colonies are white. Thus there is a convenient colour test to determine whether plasmid vectors contain inserts.

The method for obtaining competent cells is that of (Mandel, *et al.*, 1970). *E.coli* NM522, 5  $\mu\text{l}$  stored in glycerol at  $-20^\circ\text{C}$ , was used to inoculate 3 ml LB medium, which was incubated overnight at  $37^\circ\text{C}$ . Overnight culture, 2 ml was added to 100 ml LB liquid and shaken vigorously at  $37^\circ\text{C}$  until the absorbance at 600nm reached 0.2-0.25. Cultures were kept on ice for 10 min and the centrifuged in a Hereaus-Christ centrifuge at  $3,000 \text{ rev min}^{-1}$  for 10 min at  $4^\circ\text{C}$ . The cell pellet was resuspended in 40 ml ice-cold  $0.1\text{M CaCl}_2$  and

incubated on ice for 30 min. The cells were harvested again at 4°C and resuspended in 0.1M CaCl<sub>2</sub> to 1 ml final volume. These were then incubated for 1-24 h at 0°C prior to the addition of 0.25 volumes 0.1M CaCl<sub>2</sub>: 50% (v/v) glycerol and storage as 0.2 ml aliquots in 1.5 ml Nunc vials at -70°C.

In order to determine the transformation efficiency of a preparation of competent cells, 10 ng supercoiled pUC18 DNA (Yanisch-Perron, et al., 1985) in a volume less than 30 µl was added to 0.2 ml competent cells and incubated at 0°C for 30 min. The transformation mixture was transferred to 42°C for 2 min, diluted with 1 ml LB and incubated for 1 hour at 37°C. A portion of the transformation mixture (0.2 ml) was spread over the surface of an LB agar plate supplemented with 100 µg ml<sup>-1</sup> ampicillin, to select transformants. After 16 hours incubation at 37°C, the transformation efficiency was determined.

When DNA had been inserted into the α-peptide encoding DNA of pUC18, the ligation mix was used directly to transform competent cells as described above. However, the LB agar plates contained, in addition to 100 µg ml ampicillin, 0.3M IPTG and 50µg ml<sup>-1</sup> X-gal.

After overnight growth at 37°C, white transformants were selected and spread on to LB agar plates containing 200 µg ml<sup>-1</sup> ampicillin and incubated at 37°C. This secondary selection was required as the secreted β-lactamase inactivated enough of the ampicillin in the transformation plates to allow the growth of plasmid-free cells.

## 5. Transcript Mapping

### 5.1 Preparation of total RNA

*S. lividans* strains were grown in YEME medium supplemented with 34%

sucrose and 0.5% glycine at 30°C for 40-48 hours. Mycelium was harvested by centrifugation at 3,000 rev min<sup>-1</sup> in a Hereaus-Christ centrifuge for 10 min at room temperature. The cells were washed twice with 10.3% sucrose and centrifuged as above. The mycelial pellet was resuspended in 8 ml lysozyme solution [1 mg ml<sup>-1</sup> lysozyme in P buffer] and incubated for 30 min at 30°C. The mycelium was then broken up by pipetting and incubated for a further 20 min at 30°C. Following the separation of protoplasts from mycelium by centrifugation at 1,000 rev min<sup>-1</sup> for 15 sec, the protoplasts were collected by centrifugation at 3,000 rev min<sup>-1</sup> for 10 min in a Hereaus-Christ bench top centrifuge.

The protoplasts were then resuspended in 8 ml lysis solution [25 mM Tris-HCl pH 7.4 at 20°C; 10 mM Na<sub>2</sub>EDTA pH 8.0 at 20°C; 0.1 M NaCl; 1% (w/v) SDS] and agitated on a vortex mixer for 2-3 min. The lysate was extracted with an equal volume of neutral phenol:chloroform and agitated on a vortex mixer for 5 min and the two phases separated by centrifugation at 3,000 rev min<sup>-1</sup> for 10 min at room temperature. The aqueous phase was then extracted a further two times with neutral phenol:chloroform followed by two subsequent chloroform extractions. The nucleic acid was precipitated using 300 mM ammonium acetate and 2 volumes ethanol, and incubated at -20°C for 3-16 h. The precipitate was collected by centrifugation in 30 ml Corex tubes at 7,000 rev min<sup>-1</sup> for 20 min at 4°C in a Sorvall HB4 rotor. The pellet was dried *in vacuo* and resuspended in 2 ml sterile water. Specific precipitation of the RNA was achieved by the addition of 3 volumes 4 M sodium acetate pH 6.0 at 20°C and incubation at 4°C for 16 hours. The precipitated RNA was collected as above and the supernatant discarded. The pellet was left to dry and resuspended in 1 ml sterile water.

The RNA's were stored as isopropanol precipitates in 350 µg aliquots at

-20°C.

## 5.2 Labelling the 5' end of DNA.

The principle of this procedure is to label the DNA at one specific site by replacement of the 5' terminal phosphate with  $^{32}\text{P}$ . This was achieved by restriction of the DNA with a single enzyme followed by CIAP treatment. The DNA was then restricted by a second enzyme and the appropriate fragment was extracted from an agarose gel. In this way, DNA fragments with only one 5' terminal phosphate removed and therefore suitable for radiolabelling were obtained.

The DNA was restricted as described earlier, extracted with an equal volume of phenol:chloroform and precipitated with 300 mM sodium acetate, 3 volumes ethanol and incubated in an IMS/CO<sub>2</sub> bath for 15 min.

The DNA was collected by centrifugation for 10 min at room temperature in a Burkard microcentrifuge, dried *in vacuo* and resuspended in 50 µl buffer containing 50 mM Tris-HCl (pH 9.0 at 20°C), 1 mM MgCl<sub>2</sub> and 0.1 mM ZnCl<sub>2</sub>. The DNA was treated with CIAP by the addition of 1 unit of enzyme and incubation at 37°C for 1 hour. The alkaline phosphatase was inactivated by the method described earlier, and the DNA precipitated as described above. The DNA was then cleaved by a second restriction endonuclease and extracted, following electrophoresis, from a 1% low melting point agarose gel by the method described in section 3.7

The DNA fragment was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.6 at 20°C), 10 mM MgCl<sub>2</sub>, 5 mM DTT and 0.1 mM Na<sub>2</sub>EDTA pH 8.0 at 20°C, with 70 µCi [ $\gamma$ - $^{32}\text{P}$ ] ATP (3000 Ci mmol<sup>-1</sup>) and 10 units T4 polynucleotide kinase at 37°C for 1 hour in a reaction volume of 0.1 ml. The enzyme was inactivated by extraction with phenol:chloroform and the aqueous phase separated by centrifugation for 10 min at room temperature

in a microcentrifuge. The unincorporated radiolabelled ATP was removed by passage through a Sephadex G50 column as described in section 3.8 (c). The unincorporated radiolabelled ATP, inorganic salts and any contaminating phenol:chloroform is retained on the column, while large molecules pass through. The DNA was precipitated by the addition of 300 mM sodium acetate and 3 volumes ethanol and stored at  $-20^{\circ}\text{C}$ .

### 5.3 Hybridisation

The radiolabelled probe DNA and the appropriate RNA preparation were collected by centrifugation for 15 min at  $4^{\circ}\text{C}$  in a Burkard microcentrifuge. The pellets were dried *in vacuo* and resuspended in 10  $\mu\text{l}$  and 20  $\mu\text{l}$  of sterile water respectively. The nucleic acids were then combined, and following the addition of 0.24 ml deionised formamide, heated to  $100^{\circ}\text{C}$  for 3 min and placed immediately on ice. 30  $\mu\text{l}$  of 10 X concentration hybridisation buffer [0.4 M NaCl; 20 mM PIPES-NaOH pH 6.4 at  $20^{\circ}\text{C}$ ; 2mM  $\text{Na}_2\text{EDTA}$  pH 8.0 at  $20^{\circ}\text{C}$ , final concentration] was added and the preparation incubated for 30 min at  $75^{\circ}\text{C}$ . It was then cooled slowly to  $65^{\circ}\text{C}$ , whereupon incubation was continued for a further 2 hours at  $65^{\circ}\text{C}$  (Favarolo, et al., 1980). The hybridisation mix was then placed on ice for 2-3 minutes before the nucleic acid was precipitated by the addition of 3 volumes of ethanol (precooled to  $-20^{\circ}\text{C}$ ) and incubated overnight at  $-20^{\circ}\text{C}$ .

### 5.4. Digestion with nuclease S1

The DNA/RNA hybrids were collected by centrifugation for 15 minutes in a Burkard microcentrifuge. The pellet was washed with 80% ethanol (precooled to  $-20^{\circ}\text{C}$ ), dried *in vacuo* and resuspended in 87.4  $\mu\text{l}$  sterile water. S1 buffer components were added to give a final buffer concentration of 50 mM sodium acetate pH 4.6 at  $20^{\circ}\text{C}$ , 280 mM NaCl, 5 mM  $\text{ZnCl}_2$  and 20  $\mu\text{g ml}^{-1}$



denatured salmon sperm DNA. Nuclease S1 (400 units) was added and the reaction incubated at room temperature for 30 min, when the reaction was stopped by extraction with an equal volume of phenol:chloroform. The phases were separated by centrifugation for 2 min in a Burkard microcentrifuge, and the hybrids were precipitated by the addition of 300 mM sodium acetate, 3 volumes ethanol and 1µg carrier DNA. This was then incubated overnight at -70°C.

## 6. Generation of Nested Deletions.

Plasmid (2.5µg), purified with caesium chloride was initially linearised with restriction endonucleases to generate an ExoIII nuclease sensitive end adjacent to the target sequence (5'-overhanging end), and a nuclease resistant end (3' overhanging end). Following digestion the DNA was precipitated with 3 volumes ethanol and 300mM sodium acetate in an IMS/CO<sub>2</sub> bath for 15 min, and the precipitate collected by centrifugation for 10 min in a Burkard microcentrifuge. The pellet was washed with 80% ethanol (precooled to -20°C), dried *in vacuo* and resuspended in 25µl distilled water. Double-digested DNA (20µl) was then added to an equal volume of ExoIII buffer [150mM sodium chloride, 200mM Tris-HCl (pH8.0 at 20°C), 20mM MgCl<sub>2</sub>], and following the addition of 1µl ExoIII nuclease (100U/µl), digested at 30°C. Aliquots (2µl) were removed from the reaction mix at preselected times (see Table 2.2) and added to 3µl S1 nuclease buffer [50 mM sodium acetate pH 4.6 at 20°C, 280 mM NaCl, 5 mM ZnCl<sub>2</sub>, 1.5U S1 nuclease; final concentration]. Tubes were kept on ice until all aliquots had been removed from the ExoIII reaction, at which time they were incubated simultaneously at 20°C for 30 min. Following inactivation of the S1 nuclease by incubating samples at 65°C for 10 min, the extent of the deletions was

**Table 2.2 Guidelines for the production of nested deletions**

For reactions in 75 mM NaCl at 30°C.

| Spacing <sup>1</sup> |                       | 1kb | 2kb | 3kb | 4kb | 5kb |
|----------------------|-----------------------|-----|-----|-----|-----|-----|
| 200bp                | Interval <sup>2</sup> | 6-7 | 6-7 | 2   | 2   | 2   |
|                      | Samples <sup>3</sup>  | 5   | 10  | 15  | 20  | 25  |
| 300bp                | Interval              | 10  | 3   | 3   | 3   | 3   |
|                      | Samples               | 3   | 7   | 10  | 14  | 17  |
| 400bp                | Interval              | 4   | 4   | 4   | 4   | 4   |
|                      | Samples               | 3   | 5   | 8   | 10  | 13  |
| 500bp                | Interval              | 6   | 6   | 6   | 6   | 6   |
|                      | Samples               | 2   | 4   | 6   | 8   | 10  |

<sup>1</sup> Spacing required between successive deletions, in base pairs.

<sup>2</sup> Interval between timed samples, in minutes.

<sup>3</sup> Number of timed samples required.

analysed by electrophoresis of each sample (1/2 total volume) on a 1% agarose gel. The DNA in the appropriate remaining samples was then ligated in a total volume of 20  $\mu$ l containing 66 mM Tris-HCl (pH 7.5 at 20°C), 6.6 mM  $MgCl_2$ , 1 mM DTT, 0.4 mM ATP, 5% PEG and 1 unit of T4 DNA ligase for 16 hours at 20°C. The ligated DNA samples were then used to transform *E.coli* NM522 as described previously.

## 7. DNA Sequencing.

All sequencing was carried out using the T7 sequencing<sup>TM</sup> kit from Pharmacia LKB, based on the dideoxy chain termination method (Sanger, et al., 1977). The DNA (5-10  $\mu$ g of miniprep DNA, as described in section 3.4; 2-3  $\mu$ g CsCl purified DNA) was denatured by incubation with 0.2M sodium hydroxide and 2mM  $Na_2EDTA$  (pH8.0 at 20°C) for 5 min at room temperature in a reaction volume of 40  $\mu$ l. The DNA was then precipitated by the addition of 200 mM ammonium acetate (pH4.6 at 20°C) and 90  $\mu$ l ethanol and incubated for 15 min in an  $IMS/CO_2$  bath. The precipitate was collected by centrifugation for 10 minutes at room temperature in a Burkard microcentrifuge, washed with 70% (v/v) ethanol in TE buffer and dried *in vacuo*. The pellet was resuspended in 14  $\mu$ l solution containing  $MgCl_2$ , DTT, and 5 ng oligonucleotide primer, and incubated at 37°C for 20 min. The reaction mixture was then incubated for 10 min at 20°C, followed by the addition of 3 units of T7 RNA polymerase, 3  $\mu$ l labelling mix (containing dCTP, dGTP, and TTP), and 1  $\mu$ l (10  $\mu$ Ci) of [ $\alpha$ -<sup>35</sup>S]dATP and a further 5 min incubation at 20°C. A portion of this hybridization mix (4.5  $\mu$ l) was then added to each of four tubes containing 2.5  $\mu$ l A mix, C mix, G mix, or T mix and incubated at 37°C for 5 min (the concentrations of dideoxynucleotides or deoxynucleotides in these mixes cannot be quoted here since they are not

given in the instruction manual). The reactions were terminated by the addition of 5  $\mu$ l stop solution [90% (v/v) deionized formamide, 10 mM Na<sub>2</sub> EDTA pH 8.3, 0.3% (w/v) BPB, 0.3% (w/v) xylene cyanol], and stored at -20°C until required. Samples were heated to 95°C for 3 min prior to loading on a 6% urea-polyacrylamide gel. The gels were fixed with a solution of 10% (v/v) acetic acid and methanol and dried on to Whatman N°1 paper, prior to fluorography at 20°C.

## MATERIALS

### 1. Enzymes.

The following enzymes were obtained from the Sigma Chemical Company: lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and pyruvate kinase (type III from rabbit muscle). Calf intestinal alkaline phosphatase (molecular biology grade), deoxyribonuclease I (from bovine pancreas) and micrococcal nuclease (from *Staphylococcus aureus*) were purchased from Boehringer Mannheim. Restriction endonucleases, T4 DNA ligase, large fragment of DNA polymerase I and nuclease S1 were from Bethesda Research Laboratories.

### 2. Biochemicals.

The following biochemicals were purchased from Sigma Biochemical Company: ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, TTP, PEP, DTT, L-amino acids, calcium folinate, spermidine trihydrochloride, IPTG, HEPES, TES, Tris,  $\beta$ -mercaptoethanol. Urea, low melting point agarose and BSA were obtained from Bethesda Research Laboratories. SDS and acrylamide were from Serva Ltd., while Sephdex G50 and hexanucleotides (cat N° 2166) were from Pharmacia fine chemicals, PEG1000 (Koch-light), sodium triisopropyl-

naphthalene sulphonate (Kodak), sodium 4-aminosalicylate (Aldrich), and X-gal (Anglia Biotechnology) were obtained from the sources indicated.

### 2.1 Preparation of 3 M potassium acetate, pH 4.8.

This was prepared by mixing the following solutions: 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml water. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

### 2.2 Preparation of Butanol/CTAB and water/CTAB.

Butanol (125 ml) and water (125 ml) were mixed and the phases allowed to separate. 1 g of CTAB was then dissolved in 100 ml of the water saturated butanol. 100 ml of the butanol-saturated water was added, mixed thoroughly and allowed to separate at room temperature (approximately 16-20 hours). The upper, butanolic phase was removed and both solutions kept in tightly capped bottles at 37°C.

## 3. Reagents for radiochemical analysis

[ $^{35}\text{S}$ ] methionine, [ $\alpha$ - $^{32}\text{P}$ ] dCTP, [ $\gamma$ - $^{32}\text{P}$ ] ATP, [ $^{14}\text{C}$ ] methylated protein mixture and AMPLIFY were obtained from the Radiochemical Centre, Amersham. Polaroid Type 57 4 x 5" land film was used to photograph agarose gels and Fuji RX film was used for autoradiography and fluorography. Fisofluor N<sup>o</sup>3 (Fisons) was used for liquid scintillation spectrometry.

## 4. Antibiotics

The antibiotics used in this work were obtained from the sources indicated: ampicillin and erythromycin (Sigma Chemical Co.); thiostrepton (Squibb Institute, Princeton, N.J., USA); tylosin (Eli Lilly Co., Indianapolis),

lincomycin and celesticetin (Upjohn Co., Kalamazoo, Mich., USA), carbomycin (Pfizer), spiramycin (May & Baker).

## **CHAPTER 3.**

### **CHARACTERISATION OF AN ANTIBIOTIC RESISTANCE MECHANISM IN *STREPTOMYCES LIVIDANS***

## Introduction

Lincomycin is a member of the lincosamide group of antibiotics, that inhibit bacterial protein synthesis (Josten and Allen, 1964),(Vazquez, 1966), *via* an interaction with the 50S ribosomal subunit (Vazquez, 1966b). The mode of action of lincomycin has been studied in some detail and is discussed in Chapter 1.

Inactivation of lincosamides has been detected in a variety of *Streptomyces* species, with detoxification of the drug being due to phosphorylation or nucleotidylation of the hydroxyl group in position 3 (see Fig 1.8), though resistance in other species has also been attributed to nucleotidylation at position 4 (Arthur, et al., 1987). In addition, lincomycin resistance has been linked to the so called 'MLS' resistance phenotype found in a wide range of organisms. As discussed in detail in Chapter 1, resistance to this diverse group of antibiotics has been ascribed to rRNA methylation, and more specifically to the existence of N<sup>6</sup>,N<sup>6</sup>, dimethyladenosine at position 2058 within 23S rRNA (Skinner, et al., 1983)(Zalacain and Cundliffe, 1989).

This chapter describes the analysis of an inducible lincomycin resistance phenotype found in the widely used organism *S.lividans*, strain TK21 (Hopwood, et al., 1983). The observation was made some years ago that *S.lividans* became highly resistant to lincomycin when grown in the presence of sub-inhibitory concentrations of erythromycin. (R. Skinner; unpublished data). Since *S.lividans* is currently the favourite host for genes isolated from other actinomycetes study of this intrinsic resistance mechanism became of considerable interest, not least because production of an antibiotic by this organism has not been described.

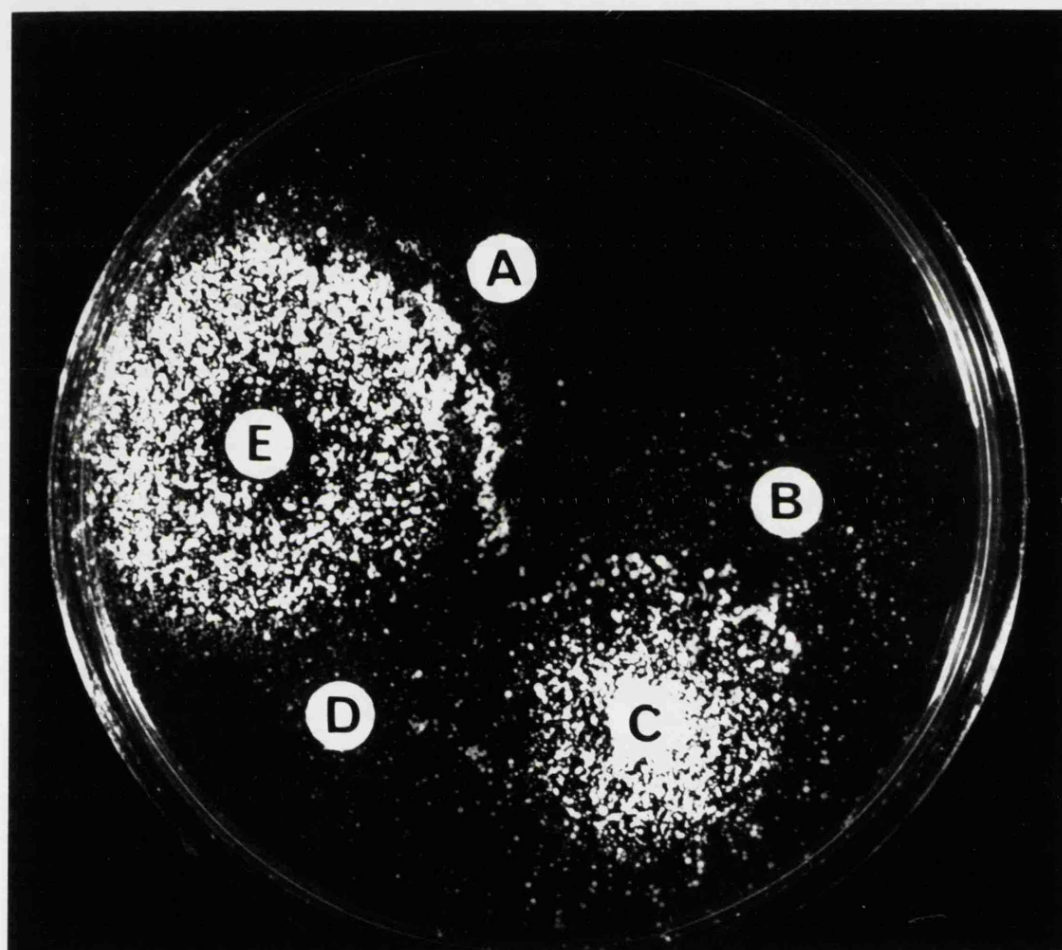


### 3.1. Resistance phenotype of *S.lividans*

As lincomycin resistance has previously been linked with cross resistance to other members of the MLS group of antibiotics, MIC values of lincomycin and a number of macrolides, for *S.lividans* were determined. Following growth on NE plates containing subinhibitory concentrations ( $10\text{ }\mu\text{g ml}^{-1}$ ) of erythromycin, *S.lividans* became significantly more resistant to subsequent challenge with lincomycin and macrolides (Table 3.1), though resistance was predominantly expressed against lincomycin. In addition, the observation was made that erythromycin is not the only antibiotic capable of inducing resistance. Using a rapid disc assay system (Fujisawa and Weisblum, 1981), various MLS drugs were shown to induce resistance to lincomycin ( $200\text{ }\mu\text{g ml}^{-1}$ ) on NE plates, though not as efficiently as erythromycin on a weight for weight basis (Fig 3.1). In descending order of induction efficiency, celesticetin, tylosin, and spiramycin were all shown to have an effect on the system, with the latter drug being a very poor inducer. Interestingly, lincomycin (or its semi-synthetic derivative clindamycin) and carbomycin did not act as inducers. It was however noted at this stage that the specificity of induction was identical to that described for the inducible MLS resistance phenotype found in *Staphylococcus aureus* (Weaver and Pattee, 1964)(Allen, 1977).

Since MLS antibiotics are known to bind to bacterial ribosomes and inhibit their function, experiments were carried out to determine the sensitivity or otherwise of ribosomes from both induced and uninduced cultures of *S.lividans*, to lincomycin and macrolide antibiotics. Salt washed ribosomes from *S.lividans*, grown in the absence and presence of erythromycin, were prepared and assayed for their ability to direct cell free protein synthesis in a coupled transcription-translation system in the

Fig. 3.1. Induction of Lincomycin Resistance in *S.lividans*.



Spores of *S.lividans* were plated onto NE media containing lincomycin at 200  $\mu\text{g/ml}$  and discs containing 3  $\mu\text{g}$  of either carbomycin (A), tylosin (B), celesticetin (C), spiramycin (D), or erythromycin (E) were placed on the agar. Growth was observed after incubation at 30°C for 3 days.

**Table 3.1. MIC values of various antibiotics for *S. lividans* TK21.**

| Antibiotic   | MIC (ug/ml)                     |                               |
|--------------|---------------------------------|-------------------------------|
|              | <i>S. lividans</i><br>uninduced | <i>S. lividans</i><br>induced |
| Lincomycin   | 30-50                           | >5000                         |
| Erythromycin | 30-50                           | 250-300                       |
| Tylosin      | 25-50                           | 300-400                       |
| Carbomycin   | 50-100                          | >1000                         |

*S. lividans* TK21 was grown on NE media at 30°C, and MIC values were determined after 4 days of growth. For induction purposes the strain was grown on 20 ug/ml erythromycin, prior to determination of MIC values.

presence of lincomycin and the macrolides erythromycin, carbomycin, and tylosin. All other sub-cellular components were derived from uninduced mycelium of *S. lividans*. Clearly (Fig. 3.2), ribosomes from induced *S.lividans* were highly resistant to lincomycin and were also significantly more resistant to macrolides than were similar reactions containing ribosomes from uninduced strains of *S.lividans*.

When ribosomes from strains exhibiting the classical MLS resistance phenotype are assayed in a similar *in vitro* system, they appear to be more highly resistant to macrolide antibiotics than was observed in the above case. (Zalacain and Cundliffe, 1990). Indeed, such ribosomes remain unaffected in their ability to direct cell free protein synthesis in the presence of tylosin or carbomycin at  $10 \mu\text{g ml}^{-1}$ , whilst those from induced *S.lividans* become totally inhibited at such concentrations. Whilst the data do not prove that the inducible resistance phenotype exhibited in *S.lividans* is due entirely to events affecting the ribosome, it appeared that resistance at the level of the ribosome is due to a novel modification system not previously described.

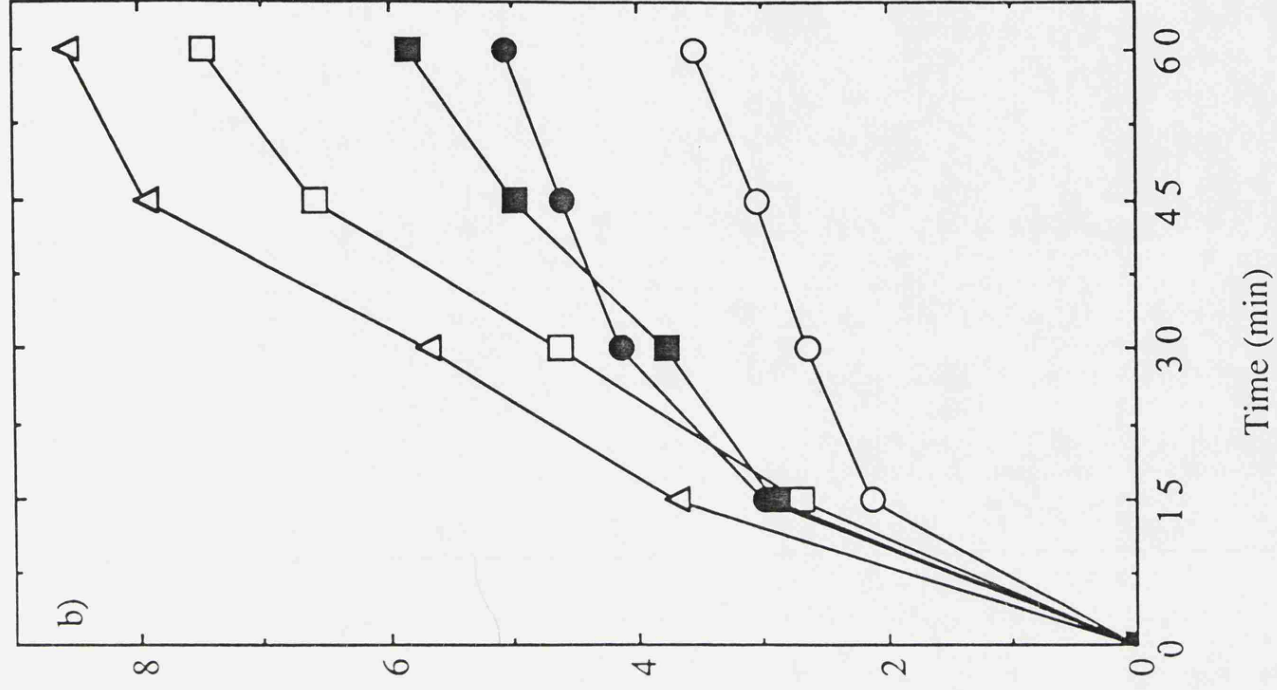
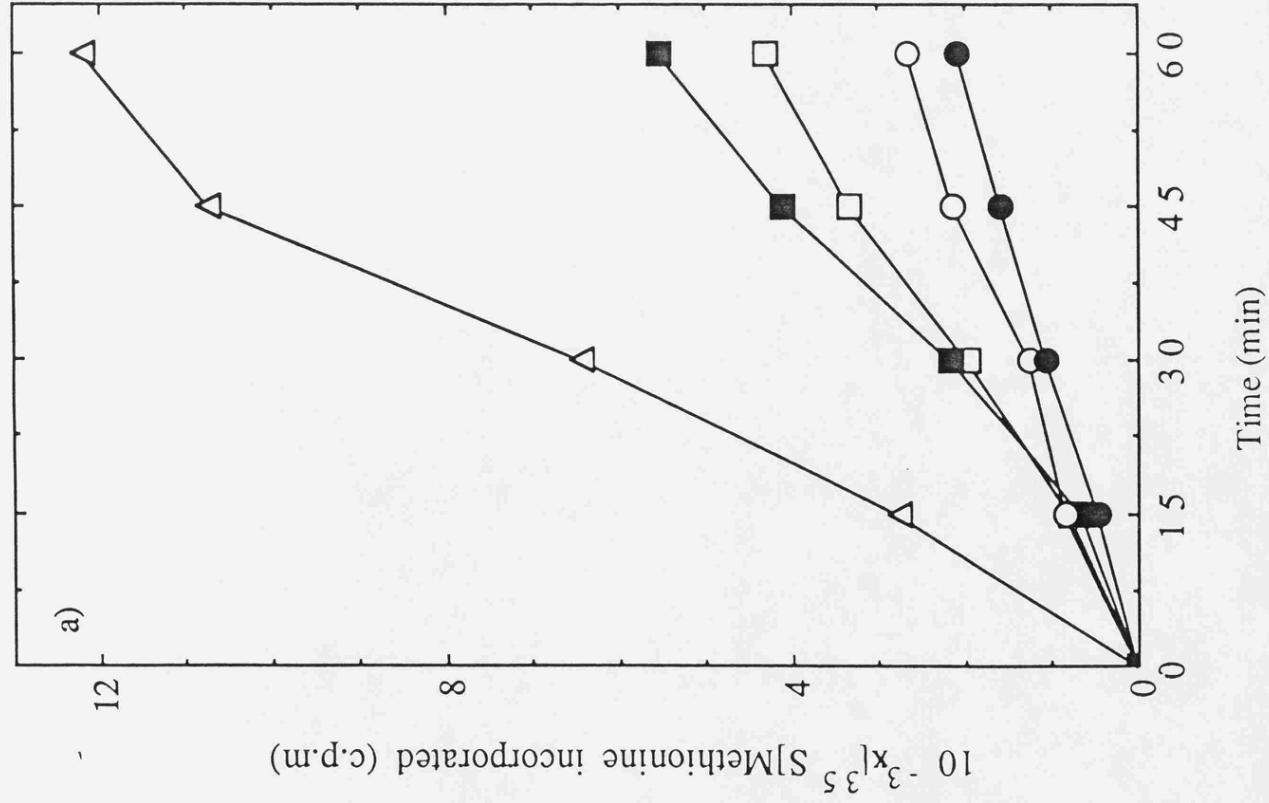
### 3.2. Identification of a Ribosomal RNA Methylase

Ribosomal resistance mechanisms described so far in antibiotic producing strains have commonly been associated with specific methylation of rRNA (see Chapter 1), and this is particularly true of the MLS resistance phenotype. Experiments were therefore undertaken to examine the possibility that methylation of rRNA might underlie resistance to lincosamides and macrolides in induced *S.lividans*.

In preliminary experiments, when total rRNA (RNA 70) from uninduced mycelium of *S.lividans* was incubated with crude S30 extracts prepared

**Fig.3.2 Sensitivity of salt-washed ribosomes from induced *S.lividans* TK21.** Reactions (34 $\mu$ l) contained S100 and crude initiation factors from *S.lividans* TK21. Salt washed ribosomes (20 pmol) from uninduced *S.lividans* (a), and induced *S.lividans* (b) were incubated with DMSO ( $\Delta$ ), lincomycin [10  $\mu$ g/ml] ( $\square$ ), erythromycin [30  $\mu$ g/ml] ( $\blacksquare$ ), tylosin [0.5  $\mu$ g/ml] (O), and carbomycin [0.5  $\mu$ g/ml] ( $\bullet$ ).

Fig. 3.2

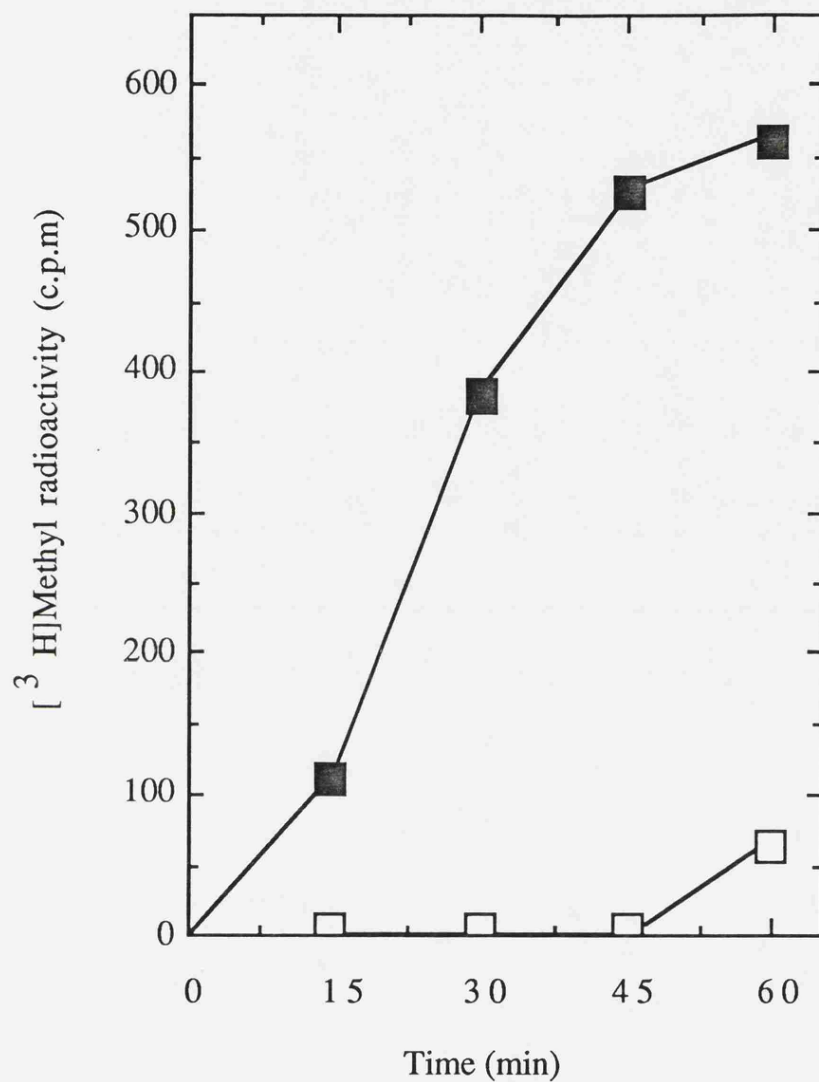


from induced mycelium, in the presence of [methyl- $^3\text{H}$ ]-S-adenosyl-methionine ([methyl- $^3\text{H}$ ] SAM), radiolabelled methyl groups were incorporated into the RNA. However, RNA70 prepared from induced mycelia, as a negative control, was shown not to act as a substrate for methylation. Subsequently, a ribosomal wash fraction was used as the source of methylase (Fig 3.3) indicating that under inducing conditions *in vivo*, the methylating enzyme is most probably associated with the ribosome. Methylation of RNA70 from the uninduced strain occurred with a stoichiometry of 0.45 when crude S30 extract was used as the source of methylase, this value increased however to 0.6 when a ribosomal wash fraction was used. The rise in stoichiometry was presumably due to the increased concentration and purity of the enzyme.

In separate experiments, 23S rRNA was shown to be the specific substrate for the methylase (Fig. 3.4). When 16S rRNA, total ribosomal particles, 50S or 30S subunits were used as the added substrate, no incorporation of tritiated methyl groups was detected (ribosomal subunits were a kind gift from Dr. M. Zalacain). This suggested that *in vivo*, the rRNA methylase acts upon 23S rRNA prior to the assembly of the ribosome, and that intact ribosomes cannot subsequently be modified by the inducible methylase.

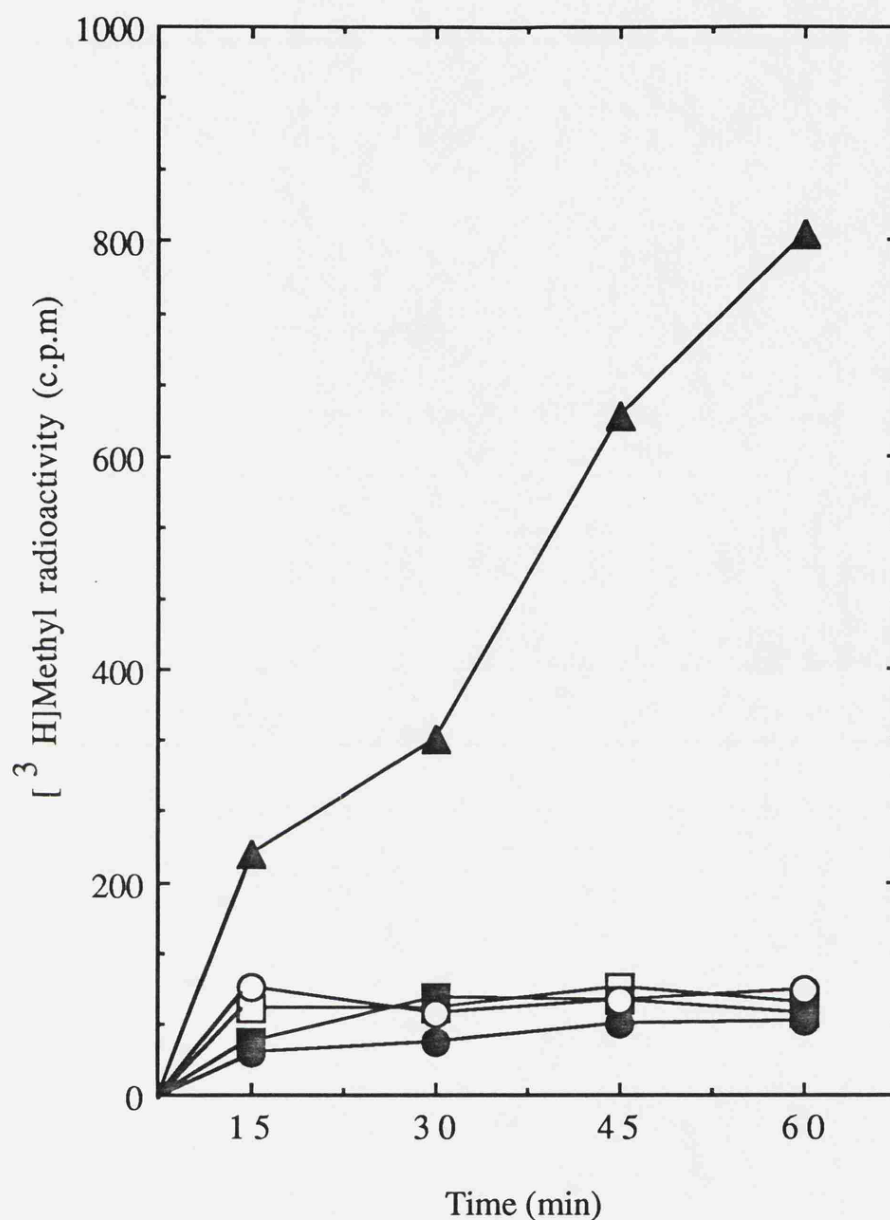
Having detected the appearance of a 23S rRNA specific methylating enzyme following induction of *S.lividans*, it was next sought to identify the type of modification involved and to establish a causal connection between that event and resistance.

### 3.3. Specificity of rRNA methylation



**Fig. 3.3 Methylation of rRNA *in vitro*.** A ribosomal wash fraction from induced *S. lividans* was used as the source of methylase, together with [methyl-<sup>3</sup>H] SAM as cofactor and RNA from uninduced *S. lividans* TK21 (■), and induced *S. lividans* (□)





**Fig. 3.4 Methylation of rRNA and Ribosomal Particles**

A ribosomal wash fraction from induced *S.lividans* TK21 was used as the source of methylase, together with [methyl- $^3\text{H}$ ] SAM as cofactor and 23S rRNA (▲), 16S rRNA (□), 50S subunits (■), 30S subunits (○), and 70S particles (●).

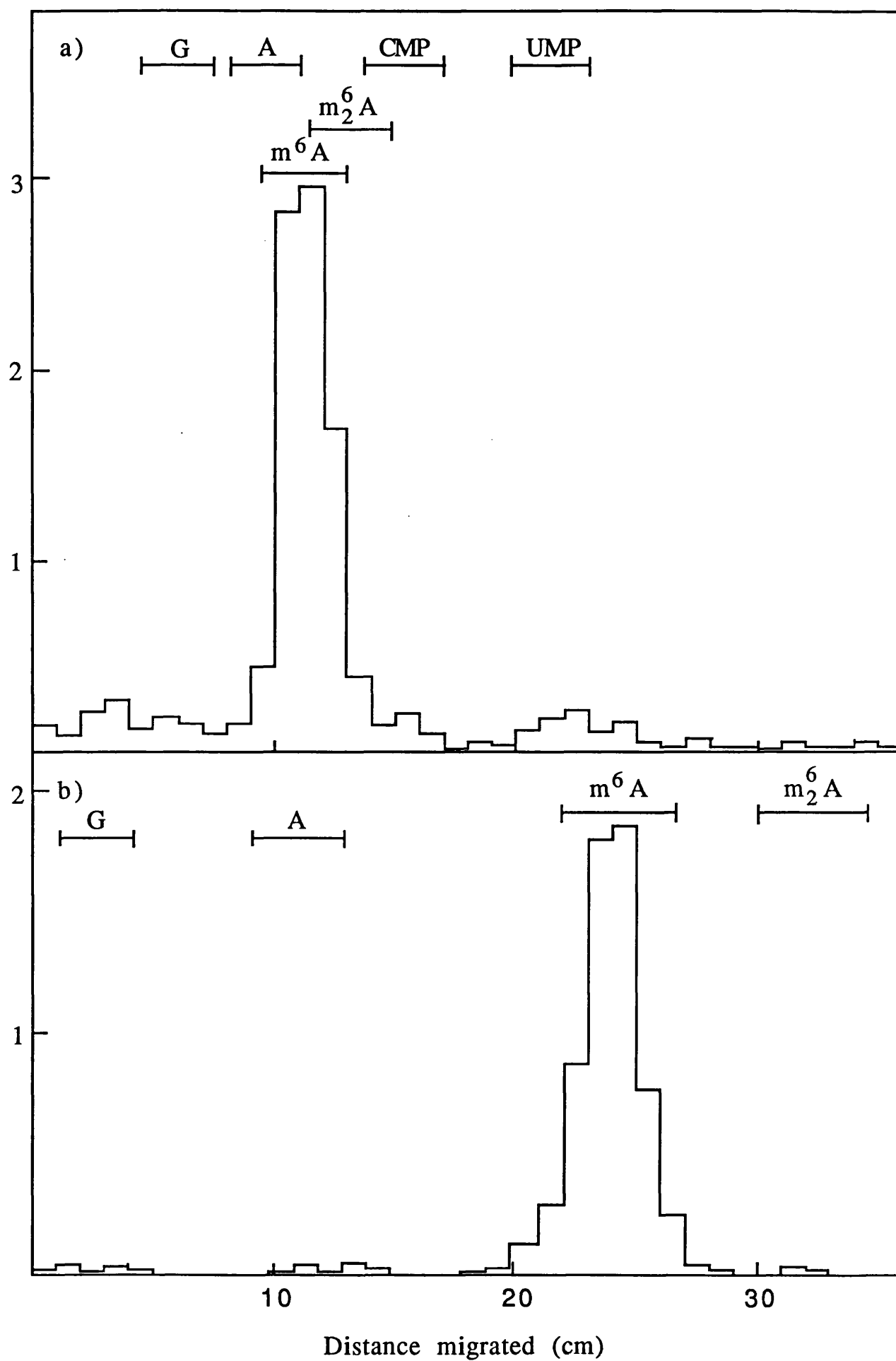
The nature of the modified residue was initially determined using descending paper chromatography. Ribosomal RNA from uninduced strains of *S.lividans* was radioactively labelled using a ribosomal wash fraction as the source of methylase and [methyl-<sup>3</sup>H]SAM as cofactor. Routinely, the stoichiometry of methylation was checked at this stage and was commonly around 0.2 - 0.3. The RNA was subsequently subjected to acid hydrolysis, liberating purine bases and pyrimidine-3'-monophosphates, and the products separated by descending paper chromatography. Initially isopropanol and HCl was used as the solvent, and in this system the products migrate in the order, adenine, guanine, cytosine-3'-monophosphate, and uridine-3'-monophosphate, with the latter moving the furthest distance.

Following chromatography of the hydrolysed RNA in this system, a single radiolabelled species was observed running ahead of the adenine marker, and co-migrating with N<sup>6</sup>-monomethyladenine and N<sup>6</sup>,N<sup>6</sup>-dimethyladenine standards (Fig. 3.5a). In this solvent the latter two markers are incompletely resolved, however in organic phases purine bases have a markedly enhanced chromatographic mobility when the amino group is methylated, this gave a clear indication that the radiolabelled species was in fact a methylated adenine. This was confirmed when the radiolabelled material was eluted from the paper using 20 mM HCl, and re-run on a paper chromatogram using as the solvent butanol and ammonia. Here it co-migrated with an internal standard of N<sup>6</sup>-monomethyladenine and was fully resolved from other modified adenine standards such as 1-methyladenine, 2-methyl adenine, 3-methyladenine, and N<sup>6</sup>,N<sup>6</sup>-dimethyladenine (Fig. 3.5b).

In a separate analysis, RNA70 from uninduced *S.lividans* was radiomethylated as described above, and digested with ribonuclease P<sub>1</sub>,

**Fig. 3.5. Identification on a methylated base from 23S rRNA.** Ribosomal RNA from uninduced *S.lividans* TK21 was modified *in vitro* with an extract from induced *S.lividans*, plus [methyl-<sup>3</sup>H] SAM as cofactor. Methylated RNA was hydrolysed with HCl, and the products separated by descending paper chromatography in isopropanol/HCl solvent (a) and butanol/NH<sub>3</sub> solvent (b).

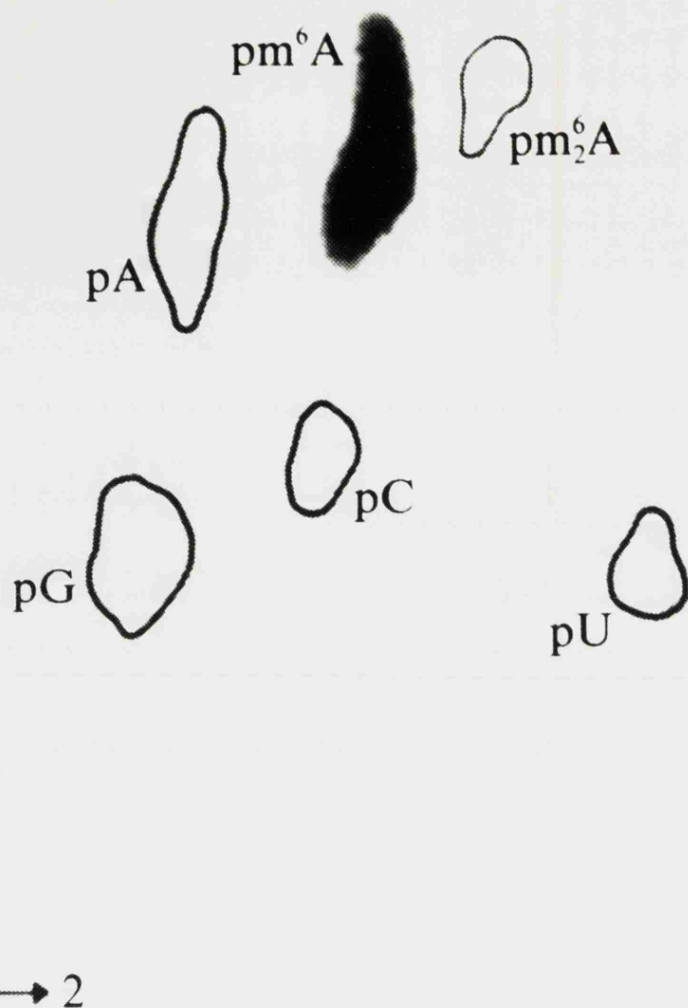
Fig 3.5.



thereby generating nucleoside-5'-monophosphates. The products were then separated by two-dimensional thin layer chromatography using HCl and isopropanol in the first dimension and isobutyric acid and ammonia in the second. Again a single radiolabelled species was observed comigrating with N<sup>6</sup>-monomethyladenosine-5'-monophosphate (Fig. 3.6).

### 3.4 Location of the modified residue within 23S rRNA

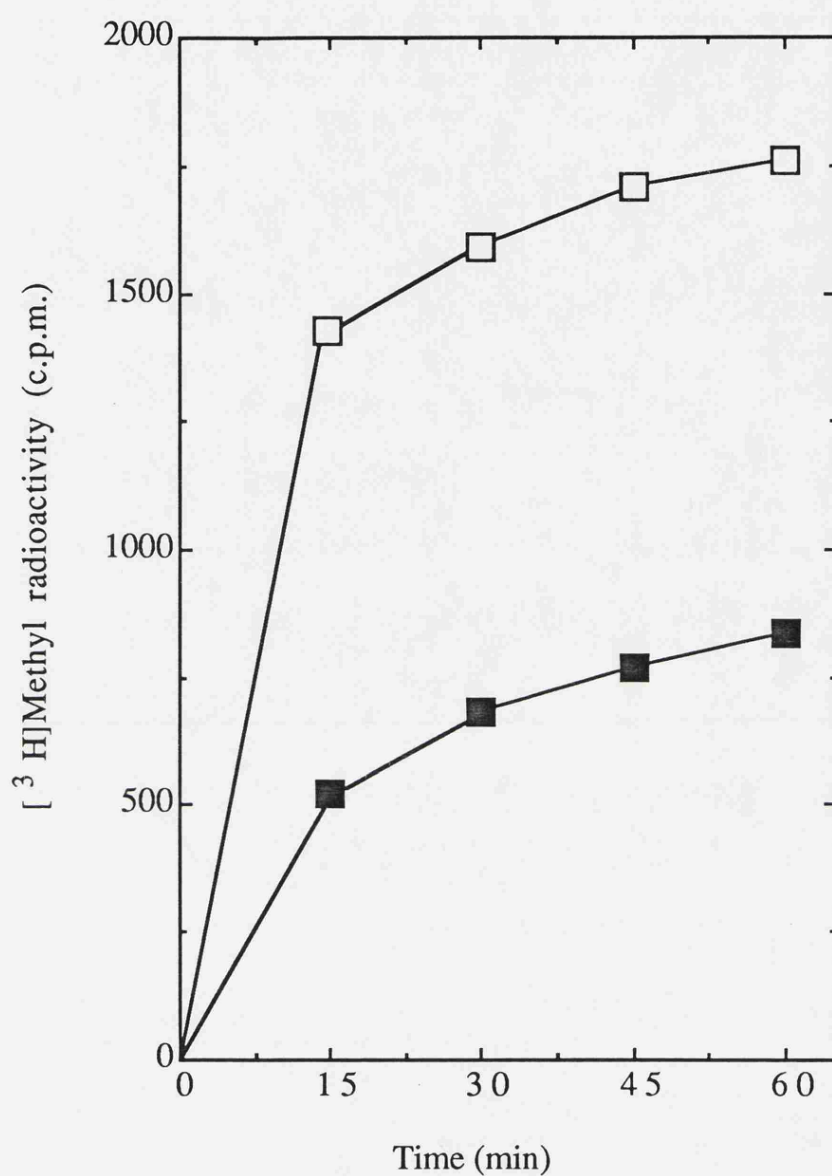
Having determined that the inducible methylase from *S.lividans* monomethylated the N<sup>6</sup>-amino group of an adenine base within 23S rRNA, experiments were undertaken to determine the precise location of this base within the rRNA. Previously when methylated residues have been located within rRNA (for a review see Cundliffe, 1989) they have been identified with the aid of reverse transcriptase. This method depends upon the ability of modified residues to cause the enzyme to pause or stop while transcribing an RNA template. In particular modifications of the latter that interfere with base pairing are thought to be effective in preventing cDNA extension by reverse transcriptase (Youvan and Hearst, 1981). After annealing a primer to the rRNA downstream of the suspected modified base, reverse transcriptase is used to run back through the sequence and the cDNA transcripts are then analysed by gel electrophoresis. By comparing the RNA sequence with the position at which reverse transcriptase pauses, the site of methylation can be determined. The enzyme does not however pause at all methylated bases within rRNA. Pausing at 7-methylguanosine and 1-methyladenosine have been reported (Beauclerk and Cundliffe, 1987), in addition to N<sup>6</sup>,N<sup>6</sup>,-dimethyladenosine (Zalacain and Cundliffe, 1989), but N<sup>6</sup>-monomethylation is known to have little effect on the action of reverse transcriptase (M. Zalacain; unpublished data).



**Fig. 3.6. Identification of a methylated nucleotide from rRNA.** Ribosomal RNA from uninduced *S. lividans* TK21 was modified *in vitro* using an extract from induced *S. lividans* as the source of methylase, plus [methyl-3H]SAM as cofactor. Methylated RNA was digested with nuclease P1 and the products separated by two-dimensional TLC. The position of dimethyl-adenine was determined in a parallel experiment and has been added. The origin (O) and the direction of migration in both dimensions are indicated.

In order to locate the methylated base within the sequence of *S.lividans* 23S rRNA (approximately 3120 nucleotides in length; J.L. Pernodet, personal communication), advantage was taken of the known specificity of another methylating enzyme isolated from *Sacc. erythraea*. At this time, classical MLS resistance had in three cases been associated with dimethylation of the same base, A-2058, within 23S rRNA. The *ermE* methylase of *S.erythraeus*, the *ermC* methylase of *Staph. aureus*, and the *tlrA* methylase of *S.fradiae* had all been shown to act specifically at this site. Given this association between residue A-2058 of 23S rRNA and the MLS resistance phenotype, the action of the *ermE* methylase on rRNA from induced and uninduced *S.lividans* was compared (Fig. 3.7). In this case RNA70 from uninduced mycelium was methylated *in vitro* by the *ermE* methylase with a stoichiometry of 1.6, using S30 from *S. lividans* AT391 as the source of enzyme. However, when RNA from induced mycelium was the added substrate the stoichiometry of methylation was reduced to 0.6, suggesting that *ermE* product was only capable of adding one methyl group to this RNA as opposed to two added to RNA from uninduced *S.lividans*. Such values indicated that residue A-2058 or a closely adjacent site in the 3 dimensional structure of the ribosome may be inducibly monomethylated to confer lincomycin resistance in *S.lividans*.

To test this hypothesis directly, rRNA from uninduced *S.lividans* was methylated *in vitro* using a ribosomal wash fraction from induced mycelium as the source of methylase activity and [methyl-<sup>3</sup>H] SAM as cofactor. During this 60 minute incubation period, radiolabelled methyl groups were incorporated into adenine within 23S rRNA. A 150 fold excess of non-radiolabelled SAM at a final concentration of 0.5mM was then added, to prevent further radiomethylation, together with crude S30 extract from *S.lividans* AT391 (i.e. containing *ermE* methylase activity) and incubation continued for a further 30 min. As a negative control, rRNA was



**Fig. 3.7 Methylation of rRNA by the *ermE* methylase.**  
A crude extract from *S. lividans* AT391 was used as the source of methylase, together with [methyl-  $^3\text{H}$ ] SAM as cofactor and RNA from uninduced *S. lividans* TK21 (□) and induced *S. lividans* (■) as substrate



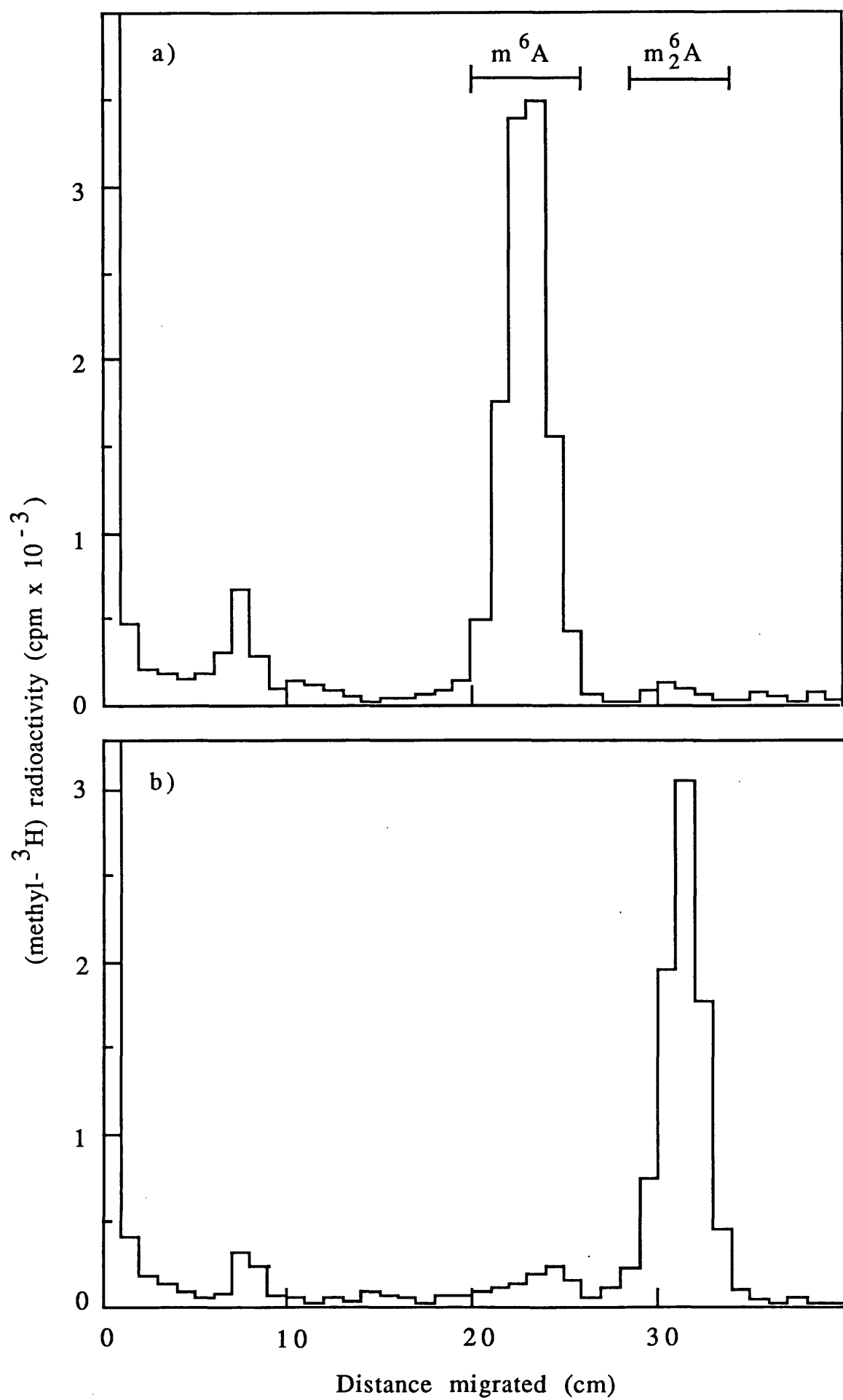
radiolabelled as above and incubation continued with excess unlabelled cofactor in the presence of additional methylase from induced *S.lividans*.

Both batches of RNA were subjected to acid hydrolysis with HCl and their products separated by paper chromatography in butanol and ammonia solvent. When RNA had only been modified by the inducible methylase from *S.lividans*, the radiolabelled product was seen to co-migrate with the N<sup>6</sup>-monomethyladenine standard as expected (Fig. 3.8a). However, when such monomethylated RNA had been subjected to further modification by the *ermE* methylase the radiolabelled species now comigrated with the N<sup>6</sup>,N<sup>6</sup>-dimethyladenine marker (Fig. 3.8b). Clearly, the adenine residue that was initially monomethylated by the *S.lividans* enzyme could be further methylated by the *ermE* product, indicating that both enzymes acted at the same site within 23S rRNA, namely A-2058. The *ermE* methylase has been shown conclusively to act at a single site within 23S rRNA (Skinner, et al., 1983) and since all the radioactivity was shifted to dimethyladenine following incubation with the *ermE* product, it would appear that the *S.lividans* enzyme, subsequently designated the *lrm* methylase, must also act exclusively at one site.

Further evidence of this being the exact site of modification by the *lrm* product came when a similar experiment was conducted using a ribosomal wash fraction from *S.lividans tlrA* (Fig.3.9). The *tlrA* product also dimethylates 23S rRNA exclusively at A-2058, though its mode of action differs slightly from that of the *ermE* methylase. Unlike the latter enzyme the *tlrA* product methylates rRNA, at least *in vitro*, in a two step reaction. (Zalacain and Cundliffe, 1989). RNA from induced *S.lividans* was again radiolabelled with the inducible methylase, followed by subsequent incubation with an excess of unlabelled cofactor and either a ribosomal wash fraction from *S.lividans tlrA* or further *S.lividans* TK21 methylase. As before,

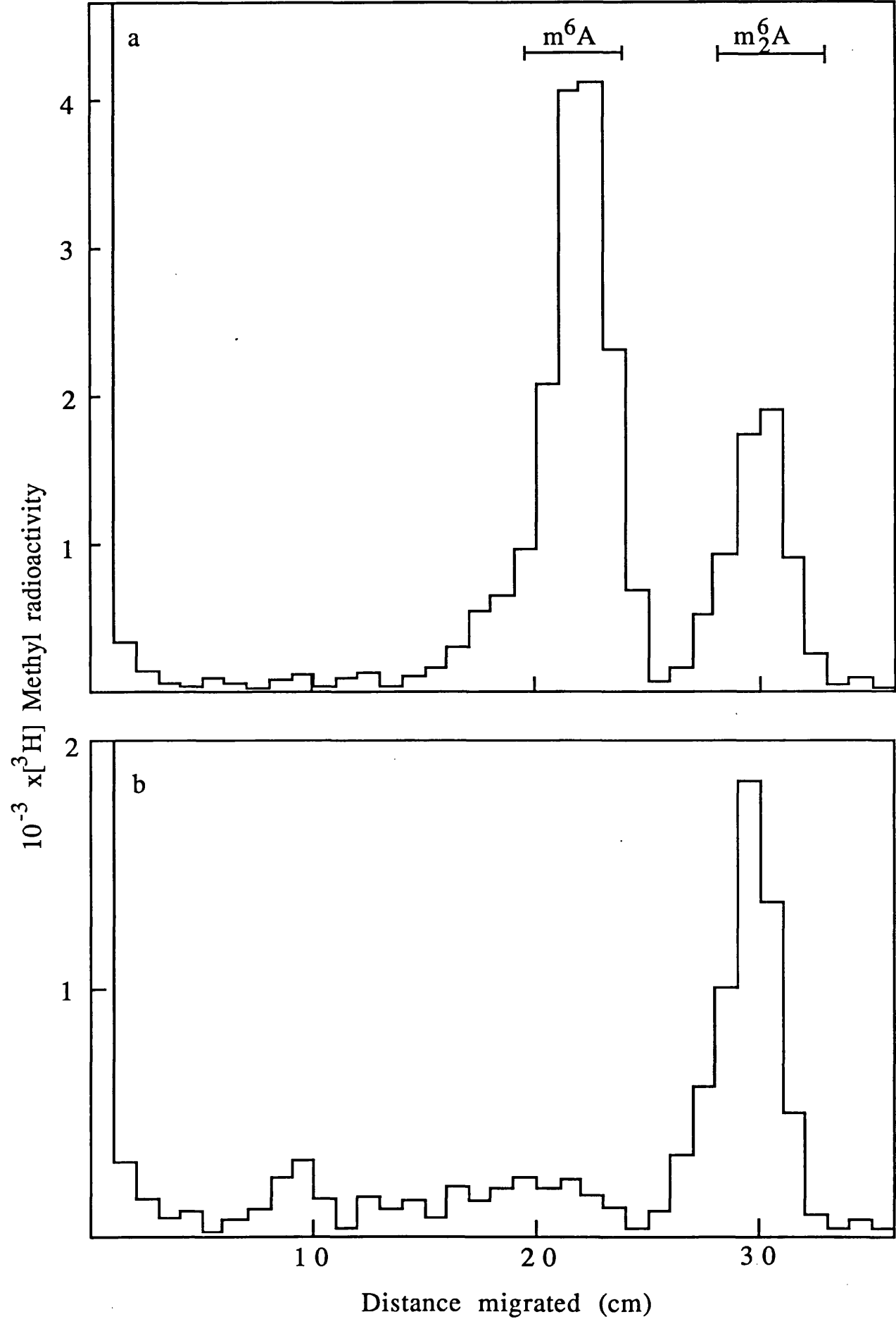
**Fig. 3.8 Biphaseic methylation of rRNA *in vitro*.** Ribosomal RNA from uninduced *S.lividans* was incubated with extract from induced *S.lividans*, using [methyl-<sup>3</sup>H] SAM as cofactor (a). A 150 fold excess of unlabelled cofactor was the added (b), and incubation continued for a further 45 min. Methylated RNA was subjected to acid hydrolysis, and the products separated by paper chromatography in a butanol/NH<sub>3</sub> solvent.

Fig. 3.8



**Fig. 3.9 Methylation of rRNA *in vitro* by the *tlrA* methylase.** Ribosomal RNA from uninduced (a) and induced (b) *S.lividans* TK21 was incubated with extract from induced *S.lividans* *tlrA*, using [methyl-<sup>3</sup>H] SAM as cofactor. Methylated RNA was subjected to acid hydrolysis, and the products separated by paper chromatography in a butanol/NH<sub>3</sub> solvent.

Fig. 3.9



following acid hydrolysis and paper chromatography, the radiolabelled species was seen to shift from monomethyladenine to a peak at dimethyladenine following incubation with the *tlrA* methylase, indicating again that both enzymes act at the same site.

Prior to this work, only one mechanism of ribosomal modification leading to lincomycin resistance had been described in detail, namely dimethylation of residue A-2058 within 23S rRNA. However during a survey of various *Streptomyces* species, phenotypes differing somewhat from that associated with the classical MLS resistance were observed (Graham and Weisblum, 1979), and it was suggested that different mechanisms of resistance did in fact exist in these strains, resulting in the varied phenotypes. The above data have confirmed that idea, revealing the existence of a second ribosomal modification system, that of monomethylation at A-2058 which leads to high level lincomycin resistance, but affords only modest protection against macrolides. This is in contrast with dimethylation at the same site which leads to high level macrolide and lincosamide resistance:- the classical MLS resistance phenotype, although ribosomes modified in this way are still not totally immune to such drugs (Zalacain and Cundliffe, 1990). This observation probably explains why a number of macrolide producing *Streptomyces* have been found to possess multiple resistance genes, which afford protection against the drug they are producing. Four resistance genes have been isolated from *S. fradiae* (Birmingham, et al., 1986)[Zalacain & Cundliffe; in press], and there are strong indications that a fifth gene exists in that strain (M. Zalacain, personal communication). Of the genes isolated, two (*tlrA* and *tlrD*) encode ribosomal methylases which act upon 23S rRNA at position 2058, dimethylating and monomethylating the residue respectively (Zalacain and Cundliffe, 1989)[Zalacain, in press]. The products of the remaining two genes *tlrB* and

*tlrC* are as yet uncharacterised, though it is currently suspected that *tlrC* encodes part of an active membrane transport system (C. Hershberger, unpublished data) that functions by exporting tylosin from the cell as it is made.

The carbomycin producer, *S.thermotolerans*, is also known to possess two resistance genes; *carA*, which is thought to encode a protein that constitutes part of a membrane transport system, and *carB* which encodes a ribosomal methylase that acts in an identical manner to the *lrn* methylase (Zalacain and Cundliffe, 1990).

Monomethylating enzymes that modify position A-2058 have subsequently been detected in two further antibiotic producing strains; *S.caelestis*, the producer of the lincosamide celesticetin and the spiramycin producer *S.ambofaciens* (Calcutt and Cundliffe, 1990) (J.L. Pernodet; unpublished data). Thus, as expected the originally novel modification system described for *S.lividans* is by no means unique and the discovery of monomethylating enzymes which act at A-2058 can also be used to explain some of the different MLS resistance phenotypes observed in *Streptomyces* spp. (see Chapter1).

## **CHAPTER 4**

### **GENERATION AND CHARACTERISATION OF LINCOMYCIN RESISTANT CLONES OF *STREPTOMYCES LIVIDANS***



## Introduction

Gene cloning in *actinomycetes* has been limited almost exclusively to the genus *Streptomyces*, and it was the discovery that they harboured plasmids that enabled the construction of vectors for cloning. The first extrachromosomal element identified from *Streptomyces* was SCP1 of *Streptomyces coelicolor* (Vivian, 1971) which was later found to be a linear plasmid encoding the biosynthetic and resistance genes for methylenomycin. However, SCP1 could not be physically isolated due to its large size (> 200 kb), its low copy number, and more to the point the fact that it is linear (Chater and Bruton, 1983)(Kinashi, et al., 1987). The first *Streptomyces* plasmid to be characterised therefore was SCP2, a fertility determinant from *S.coelicolor*, which enhances chromosomal recombinations in matings (Bibb, et al., 1977). Spontaneous plasmid mutants (SCP2\*) can also be isolated from *S.coelicolor* which further increase chromosomal recombination.

Since then a variety of plasmids have been identified in a great many strains (Table 4.1) and used to construct cloning vectors. The vectors most routinely used have been developed from the 3 plasmids SLP1.2, SCP2\*, and pIJ101, those based on the former two being of low copy number and those on the latter of high copy number. The element SLP1.2 was one of several episomes, known as the SLP1 family, obtained after genetic crosses between *S.coelicolor* and *S.lividans*, and although it forms an integral part of the chromosome in *S.coelicolor*, SLP1.2 is an autonomous replicon in *S.lividans* (Bibb, et al., 1981). The 8.8 kb plasmid pIJ101 was isolated from *S.lividans* ISP5434 and plasmids derived from it have a broader host range than those derived from SLP1.2 in addition to a much higher copy number of between 40 and 300. Low copy number plasmids do however have the advantage of greater stability when containing DNA inserts of a large size and such vectors

Table 4.1. Naturally occurring *Streptomyces* plasmids

| Strain                               | Plasmid | Size (kb) | Copy N° | Remarks  | References   |
|--------------------------------------|---------|-----------|---------|--|--|
| <i>S.coelicolor</i><br>A3(2)         | SCP1    | 410-590   | low     | Linear plasmid encoding biosynthesis of and resistance to methylenomycin | Vivian <i>et. al.</i> 1971                                 |
|                                      | SCP2    | 31        | 1-2     | Fertility factor   | Kinashi <i>et. al.</i> 1987                                |
|                                      | SLP1    | 9.4-14.5  | 1       | Autonomous plasmids derived from the chromosome                          | Schrempf <i>et. al.</i> 1975                               |
|                                      | SLP4    | -         | -       | Detected by pock formation   | Bibb <i>et. al.</i> 1981                                   |
| <i>S.lividans</i><br>ISP5434         | pIJ101  | 8.9       | 40-300  | Replication, transfer and pock-forming functions have been mapped        | Kieser <i>et. al.</i> 1982                                 |
|                                      | pIJ102  | 4.0       | 40-300  |  |  |
|                                      | pIJ103  | 3.9       | 40-300  |  |  |
|                                      | pIJ104  | 4.9       | 40-300  |  |  |
| <i>S.ambofaciens</i><br>KA-1028      | pSA1    | 8.0       | 2       | High proportion of plasmid associated with chromosome or membrane        | Omura <i>et. al.</i> 1981<br>Ikeda <i>et. al.</i> 1982     |
| <i>S.bikiniensis</i><br>KA-421       | pSB1    | 29        | -       |  | Omura <i>et. al.</i> 1981                                  |
|                                      | pSB2    | 57        | -       |  | Omura <i>et. al.</i> 1987                                  |
| <i>S.ribosidificus</i><br>ATCC 21294 | pSR1    | 79        | -       | Present also in ribostamycin non-producing mutants                       | Okanishi <i>et. al.</i> 1980<br>Noriji <i>et. al.</i> 1980 |
| <i>S.venezuelae</i><br>3022a         | pUC3    | 32        | -       | Does not code for chloramphenicol biosynthesis.                          | Malik <i>et. al.</i> 1977<br>Ahmed and Vining . 1983       |

have proved important in the cloning of entire biosynthetic pathways, such as that of actinorhodin from *S.coelicolor* (Malpartida and Hopwood, 1984).

The development of efficient cloning systems has allowed the investigation and isolation of many streptomycete genes. Typically, these have been genes involved in antibiotic production and resistance although those involved in primary metabolism and differentiation are also now being isolated. In addition it is now possible to pursue the nucleotide sequences that interact with RNA polymerase, ribosomes, and other nucleic acid binding moieties, the study of which has already answered some of the questions concerning the switches which control development in *Streptomyces*.

The ability to 'shotgun clone' antibiotic resistance genes from producing organisms in sensitive strains has greatly facilitated the characterisation of some resistance mechanisms and it was hoped that just as the isolation of antibiotic resistance genes had aided other studies, the cloning of the lincomycin resistance determinant from *S.lividans* would also facilitate the study of this gene. In this instance, the lincomycin resistance phenotype expressed by *S.lividans* had in part already been characterised without prior isolation of the resistance determinant responsible. However, studies of *lrm* expression had not been carried out in a negative background, and at the beginning of this study there was no direct evidence to demonstrate that it was responsible for lincomycin resistance in strain *S.lividans* TK21. In addition, isolation of the rRNA methylase gene was necessary to facilitate studies of *lrm* induction, and to this end attempts were made to isolate the gene responsible for lincomycin resistance in *S.lividans* by the use of shotgun cloning techniques.

A method by which DNA could be introduced into *Streptomyces* at high

frequency was developed some years ago (Bibb, *et al.*, 1978). However, the techniques described are related to transformation of *S.lividans* or its close relative *S.coelicolor*. Transformation of other *Streptomyces* species such as *S.griseofuscus* and *S.albus* have been performed in this laboratory using similar techniques, but the frequencies of transformation obtained are substantially lower than that needed for shotgun cloning experiments. This initially presented the problem of having to isolate an inducible gene from *S.lividans* using the same strain as host, in that induction of primary transformants with erythromycin to select the correct recombinant would result in all the transformants expressing the lincomycin resistance phenotype. Since selection in this way was impossible, a mutant of *S.lividans* TK21 was isolated that expressed the resistance phenotype constitutively, thereby enabling the mutated gene to be cloned in *S.lividans* TK21 whilst avoiding the need for prior induction of the cells.

The isolation of such constitutive mutants has been described previously in the case of *Staphylococcus aureus* containing *ermC*, a resistance gene which encodes a rRNA dimethylating enzyme that also acts at position A-2058 in 23S rRNA. During initial studies of *ermC* induction, mutational analysis was carried out on the wild type strain in which constitutively resistant mutants of *Staph. aureus* were generated by selection on inhibitory concentrations of MLS antibiotics with low inducing activity. The resistance determinants were cloned from eleven of these mutants, and by comparison of the mutant DNA sequences with that of the wild type gene, a model for the regulation of MLS resistance was proposed, that of translational attenuation. The mutants obtained of *Staph. aureus* were found to express resistance constitutively due to various deletions and base substitutions in the mRNA of the leader peptide as discussed in Chapter1. Given the apparent similarities in induction specificity between *ermC* and *lrm* it was proposed

that induction of these two resistance determinants may be controlled by a similar mechanism.

The generation of a mutant of *S.lividans* TK21, possessing constitutive resistance to lincomycin, and the cloning of the mutated gene are described in this chapter. In addition the isolation of the wild type gene from *S.lividans* TK21 is also recorded.

#### **4.1. Generation of a Mutant of *S.lividans*, constitutively resistant to lincomycin**

The procedure used to generate the constitutive mutant was that of U.V. mutagenesis, chosen because of its simplicity of use. Spores of *S.lividans* TK21, were initially irradiated with U.V. light at a distance of 20 cm, for a period of 60 sec, 80 sec, and 2 min. This exposure results in a survival percentage of 5%, 1%, and 0.1% respectively, as determined from a killing curve constructed prior to the experiment. In determining the time of exposure, such a percentage survival rate is required that ensures the remaining viable spores have received sufficient U.V. light to allow the required mutational event to occur, but that this exposure is not so excessive as to result in the formation of sick strains. A 1 - 5% rate of survival has previously been found to be the most useful for obtaining mutants [Fautini, A.A. 1975].

To allow for the expression of any altered resistance phenotype, spores were plated on to media containing no selection pressure following exposure to U.V. light. After growth at 30°C for 4 days, the colonies were replica plated onto media containing lincomycin (200  $\mu\text{g ml}^{-1}$ ), and incubated for a further 3 days. After this time, 10 primary colonies were selected and restreaked

onto media containing lincomycin (200  $\mu\text{g ml}^{-1}$ ).

In initial experiments, ribosomes were isolated from two of the mutant strains MNT13 and MNT14, and assayed for their ability to direct *in vitro* coupled transcription - translation in the presence and absence of lincomycin (Fig 4.1). Systems containing ribosomes from MNT13 were inhibited by lincomycin to a lesser extent than those containing ribosomes from wild type *S.lividans*. Indeed, when the results of ribosomes isolated from strain MNT13 were compared to the resistance obtained with ribosomes from induced *S.lividans*, the response of both sets of ribosomes to lincomycin was shown to be identical. Whilst systems containing ribosomes from strain MNT14 were substantially more resistant to lincomycin than those containing control ribosomes from *S.lividans* TK21, the ability of these ribosomes to direct cell-free protein synthesis was impaired to a greater extent, by lincomycin, than that of ribosomes isolated from MNT13. For this reason strain MNT13 was selected for further study.

#### 4.2. Characterisation of Lincomycin Resistance in *S.lividans* MNT13.

The hypothesis that ribosomal resistance in strain MNT13 was due to the constitutive expression of *lrm* was subsequently tested directly and the strain was initially examined for the presence of a rRNA methylating enzyme. A ribosomal wash fraction from *S.lividans* MNT13, was incubated with RNA70 from uninduced *S.lividans* TK21 in the presence of [methyl- $^3\text{H}$ ] SAM as cofactor. The results however gave no indication of a constitutively expressed rRNA methylase (data not shown), which in itself was surprising since ribosomes from the strain had demonstrated resistance to lincomycin (Fig.4.1). These ribosomes and in particular the rRNA were therefore

**Fig. 4.1 Effect of MLS antibiotics on *in vitro* protein synthesis by cell extracts of *S.lividans* MNT13 and MNT14.** Reactions (34μl) contained S100 and crude initiation factors from *S.lividans* TK21. Salt washed ribosomes (20 pmol) from *S.lividans* TK21 (a), *S.lividans* MNT14 (b), and *S.lividans* MNT13 (c) were incubated with DMSO (□), lincomycin [10 μg/ml] (■).

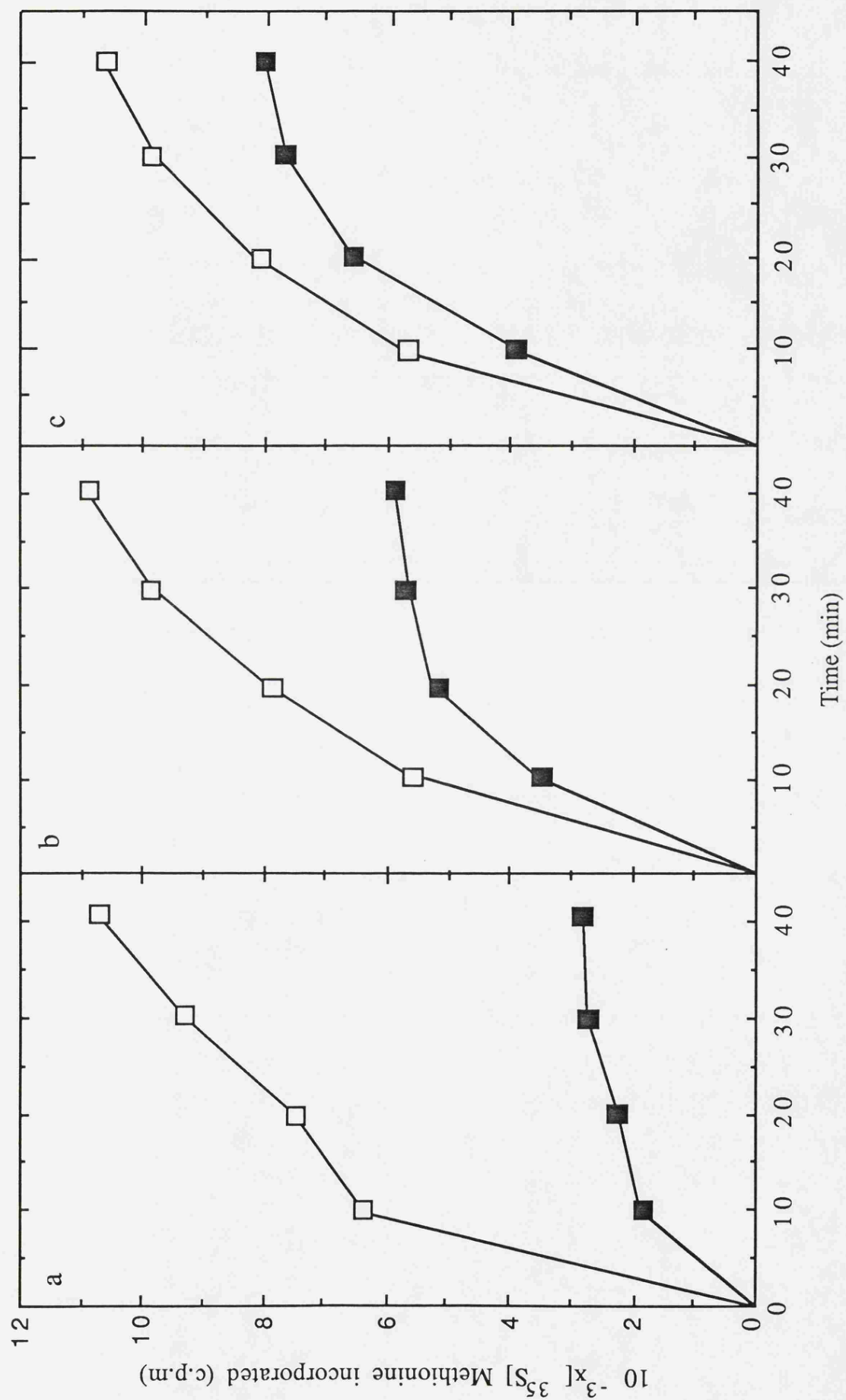


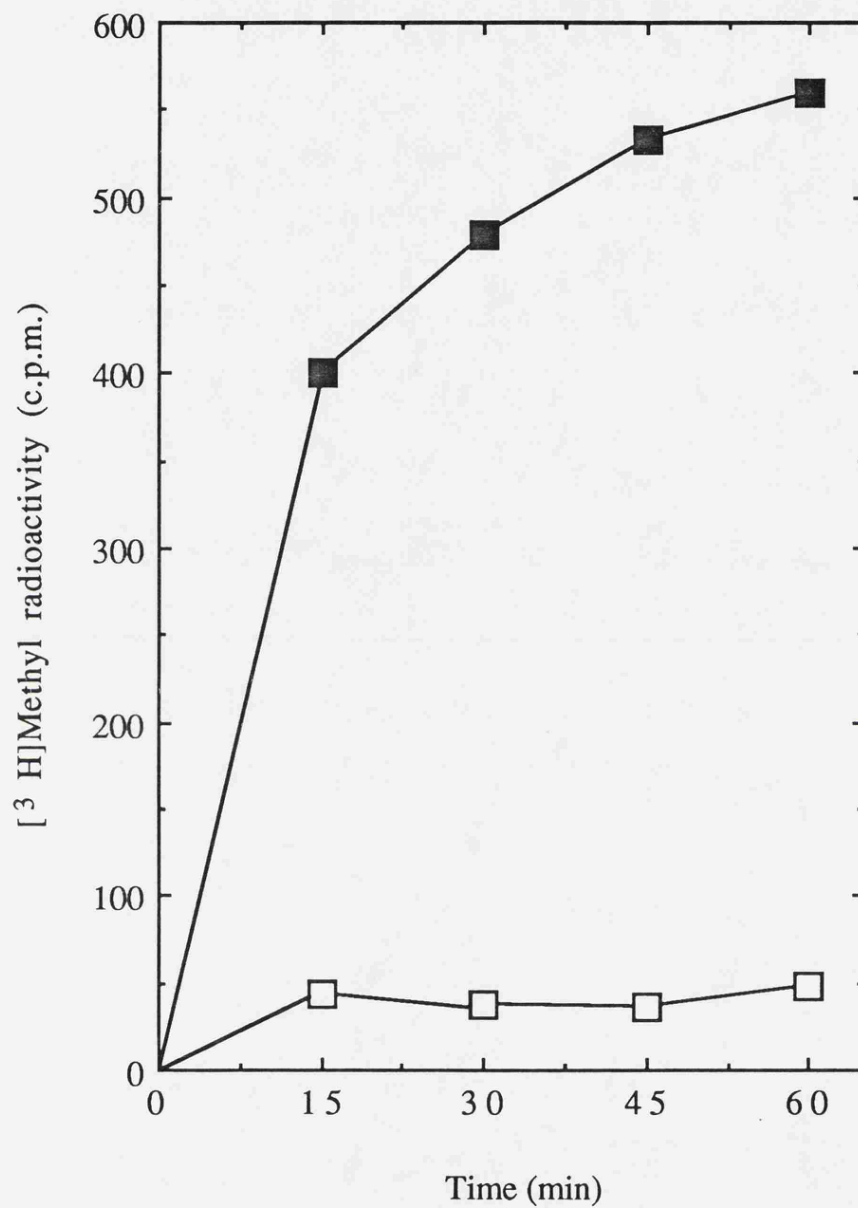
Fig. 4.1



examined in closer detail.

In initial experiments, RNA70 from the mutant strain was incubated with a ribosomal wash fraction from induced *S.lividans* TK21 as the source of methylase, and [methyl-<sup>3</sup>H] SAM as the usual cofactor. As a positive control an additional assay was set up using RNA70 from uninduced *S. lividans* TK21 as the added substrate. Whilst incorporation of methyl groups into this latter RNA substrate was clearly evident at the normal stoichiometry of 0.45 (Fig 4.2), no significant methylation was observed of rRNA from *S. lividans* MNT13, implying that the residue at position 2058 in 23S rRNA from the latter strain was unavailable for methylation by the *lrm* product. One obvious explanation was that *lrm* was indeed being expressed constitutively in this strain, and as a result the adenine moiety at position 2058 was permanently monomethylated, thereby preventing further action by *lrm in vitro*. Alternatively, since methylase activity could not be detected in the strain, the possibility of course remained that a mutation had occurred within one or more of the 6 rRNA operons present in *S.lividans*, causing the residue at 2058 to be replaced by guanine or one of the purine bases. However, in this event a certain proportion of RNA would still be expected to contain an adenine residue at position 2058, which would be reflected in the extent of methylation. Since significant modification of rRNA from *S.lividans* MNT13 was not detected in these experiments such an explanation seems unlikely.

This latter possibility was indeed discounted when rRNA from *S.lividans* MNT13 was incubated with the *tlrA* methylase of *S.fradiae*, in the presence of [methyl-<sup>3</sup>H] SAM. As a positive control, RNA70 from uninduced *S.lividans* was also used as a substrate. Following incubation and acid hydrolysis the products of methylation were separated by descending paper



**Fig. 4.2 Methylation of RNA from *S. lividans* MNT13**

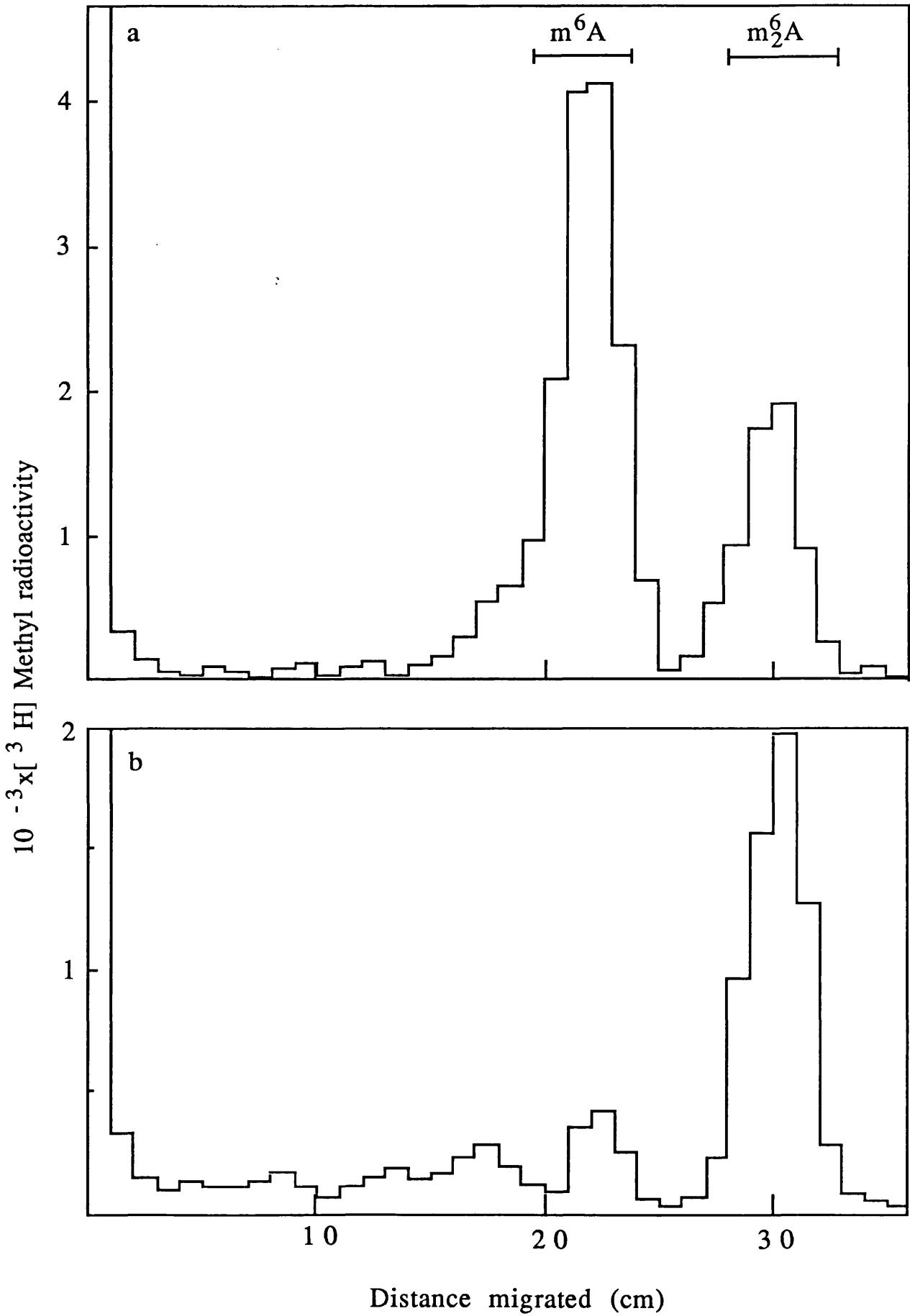
A ribosomal wash fraction from induced *S. lividans* TK21 was used as the source of methylase together with [Methyl- <sup>3</sup>H] SAM as cofactor and rRNA from uninduced *S. lividans* TK21 (■), and *S. lividans* MNT13 (□).

chromatography in butanol/ammonia solvent (Fig 4.3). A similar experiment has been described in detail in Chapter 3.4. If the adenine residue at position 2058 in strain MNT13 had been replaced by one of the other 3 bases, it would not have been expected to act as a substrate for the *tlrA* methylase, and by comparing the action of the *tlrA* enzyme on the 2 substrates, it is clear that rRNA from MNT13 does act as a substrate for methylation but by far the majority of product was di-methyladenine. This was in contrast with the positive control which demonstrated that the *tlrA* enzyme initially monomethylates rRNA and then subsequently adds a second methyl group. The data clearly suggested that rRNA from MNT13 was already monomethylated *in vivo* such that *tlrA* was only able to add the second methyl group. If resistance had been due to a base substitution within the ribosomal RNA a different pattern of methylation would have been observed; RNA containing the altered base would not have acted as a substrate for the *tlrA* enzyme, whilst the remaining 'wild type' rRNA would have been primarily monomethylated as in the positive control. This was not the case.

The disparity remained that methylase activity had not been detected in strain MNT13, it was therefore proposed that constitutive resistance in MNT13 was as a result of constitutive methylase expression in that strain, but that levels of the enzyme, despite being adequate to generate resistant ribosomes, were too low to be detected in the assays described here. This putative low level of methylase expression could have been a consequence of the way in which induction is controlled in the strain. If as expected, translational attenuation is responsible, then a mutational event may have occurred in the proposed leader sequence that favours the so called 'active' conformation (i.e. that which favours expression of *lrm*). However if this mutation only involved one or a few base changes as opposed to a substantial deletion then formation of the 'inactive' configuration would still

**Fig. 4.3. Methylation of mutated rRNA *in vitro* by the *tlrA* methylase.** Ribosomal RNA from *S.lividans* MNT13 was incubated with an extract from induced *S.lividans* TK21 (a) and *S.lividans tlrA* (b), using [methyl-<sup>3</sup>H] SAM as cofactor. Methylated RNA was subjected to acid hydrolysis, and the products separated by paper chromatography in a butanol/NH<sub>3</sub> solvent

Fig 4.3

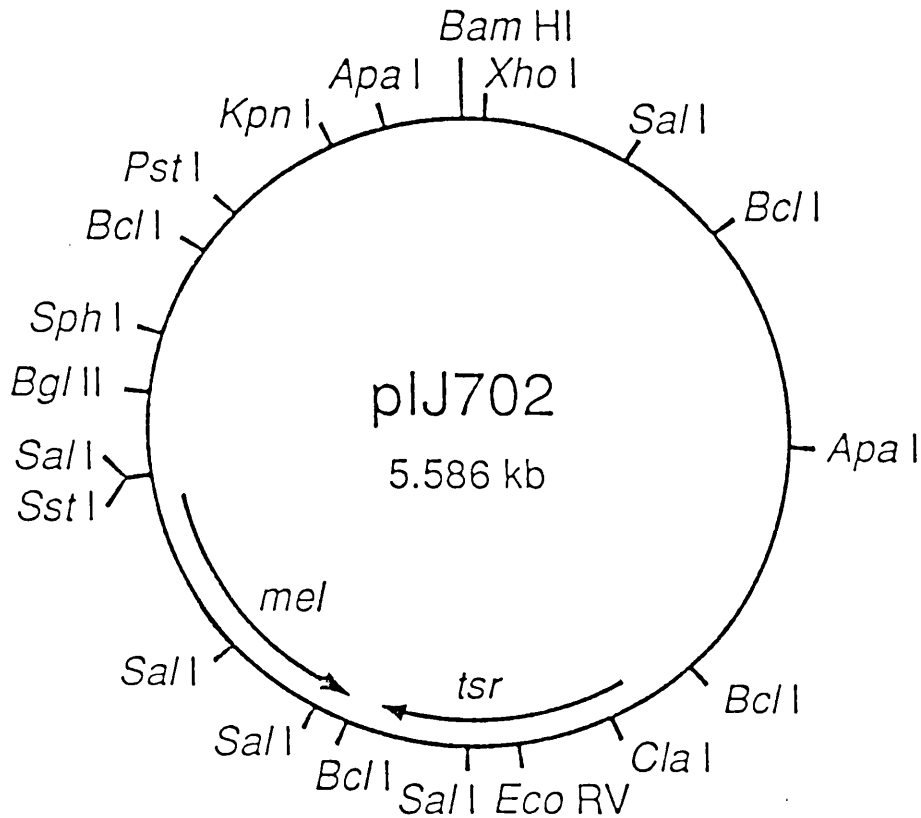


be possible with methylase only being produced when the leader sequence flipped to the 'active' conformation. This conformational alteration in the wild type strain requires induction by exogenous drug, but in the mutant may be able to occur spontaneously resulting in the production of low levels of methylase. Attempts were subsequently made to clone the resistant determinant from strain MNT13.

#### 4.3. Cloning of a Lincomycin Resistance Determinant from *S.lividans* MNT13

In isolating the resistance determinant from this mutant strain, genomic DNA fragments from MNT13 were cloned into the high copy number *Streptomyces* plasmid, pIJ702 (Katz, et al., 1983). This vector (Fig.4.4) is one of the most commonly used *Streptomyces* plasmids, the replicon of which is derived from pIJ101 a multi-copy plasmid isolated from *S.lividans* (Kieser, et al., 1982). In addition to the minimal replicon of pIJ101, plasmid pIJ702 contains both the thiostrepton resistance gene (*tsr*) from *Streptomyces azureus* (Thompson, et al., 1982) and a tyrosinase gene (*mel*) from *Streptomyces antibioticus* (Katz, et al., 1983). Thiostrepton is used as a selection for primary transformants whilst the *mel* gene allows for the identification of recombinants. Strains of *S.lividans* harbouring pIJ702 produce a brown pigment when grown on media supplemented with tyrosine, due to the action of the cloned tyrosinase gene. Insertion of DNA fragments into the *mel* gene, using any of the three unique restriction sites, leads to insertional inactivation and colonies containing recombinant plasmids therefore appear white.

In the vital shotgun cloning experiment, genomic DNA fragments from MNT13 were generated by partial digestion with *Sau*3A and ligated with



**Fig.4.4 Restriction endonuclease map of the high copy number plasmid pIJ702.** The vector pIJ702 is a derivative of the high copy number plasmid pIJ101, and contains the thiostrepton resistance gene (*tsr*) from *S.azureus* and the tyrosinase gene (*mel*) from *S.antibioticus*. Insertion of DNA fragments into the SphI, BglII, or SstI restriction sites usually prevents pigment production.

pIJ702 that had been linearised with BglII and terminally dephosphorylated using CIAP. During the partial digestion with Sau3A, DNA from MNT13 was incubated with enzyme over varying periods of time from 5 - 30 min and that reaction which generated fragments of between 1 and 8 Kb in size was determined by electrophoresis in a 0.7% agarose gel. Fragments of the stated size were purified by extraction from a 1% LMP agarose gel as described in Methods, and ligated DNA was then used to transform protoplasts of *S.lividans* TK21. Following regeneration, primary transformants were selected by overlaying plates with thiostrepton ( $20 \mu\text{g ml}^{-1}$ ).

After incubation of the plates for a further 4 days, to allow sporulation, approximately 50,000 primary transformants (on 10 plates) were replica-plated onto an equal number of plates containing both thiostrepton ( $20 \mu\text{g ml}^{-1}$ ) and lincomycin ( $200 \mu\text{g ml}^{-1}$ ), and incubated for 40 hours. This short incubation period was due to the high rate at which spontaneous mutants of *S.lividans*, expressing the resistance phenotype constitutively, were found to occur. If incubated for a period of greater than 48 hours, approximately 5% of initial transformants became constitutively resistant to lincomycin. However, plasmid from these recombinants would not re-transform *S.lividans* to constitutive lincomycin resistance, indicating that such strains had acquired chromosomal mutations. As indicated earlier this kind of spontaneous mutation has been described in the case of *Staphylococcus aureus*, and such a phenomenon appears to be a consequence of the induction mechanism in *Staph. aureus* with control of methylase induction being attributed to translational attenuation. The possibility also arises that the mutant isolated (MNT13) may have been generated spontaneously without the need for U.V. irradiation, since colonies were selected on a non-inducing antibiotic.



In the cloning experiment described above, only one resistant colony (designated GJ1) was obtained after incubation for 36 hours. Spores and aerial mycelium were removed from this colony and inoculated into 25 ml liquid medium supplemented with thiostrepton ( $20\mu\text{g ml}^{-1}$ ) and lincomycin ( $200\mu\text{g ml}^{-1}$ ). Following incubation at  $30^\circ\text{C}$  for 40 hours, plasmid DNA was prepared by the alkaline lysis procedure (See Methods), analysed by agarose gel electrophoresis using supercoiled pIJ702 as a size marker, and shown to contain a DNA species slower in mobility than pIJ702.

The plasmid DNA (pGJ1) isolated from *S.lividans* GJ1 was subsequently tested for its ability to transform *S.lividans* TK21 to lincomycin resistance. Approximately 50 ng of pIJ702 and pGJ1 were introduced into protoplasts of *S.lividans*, spread on plates containing regeneration media and incubated overnight at  $30^\circ\text{C}$ . Primary transformants were again selected by overlaying plates with thiostrepton, and after a period of 4 days, colonies were replica-plated onto media containing lincomycin ( $200\mu\text{g ml}^{-1}$ ). After a further incubation period of 2 days no growth was observed of *S.lividans* transformed with pIJ702, whilst protoplasts transformed with pGJ1 grew as a confluent lawn.

Restriction analysis of pGJ1 revealed that it consisted of pIJ702 with a 3.6 Kb insert at the BglII site (Fig. 4.7).

#### **4.4. Biochemical Characterisation of a Constitutively Lincomycin Resistant Clone of *S. lividans*.**

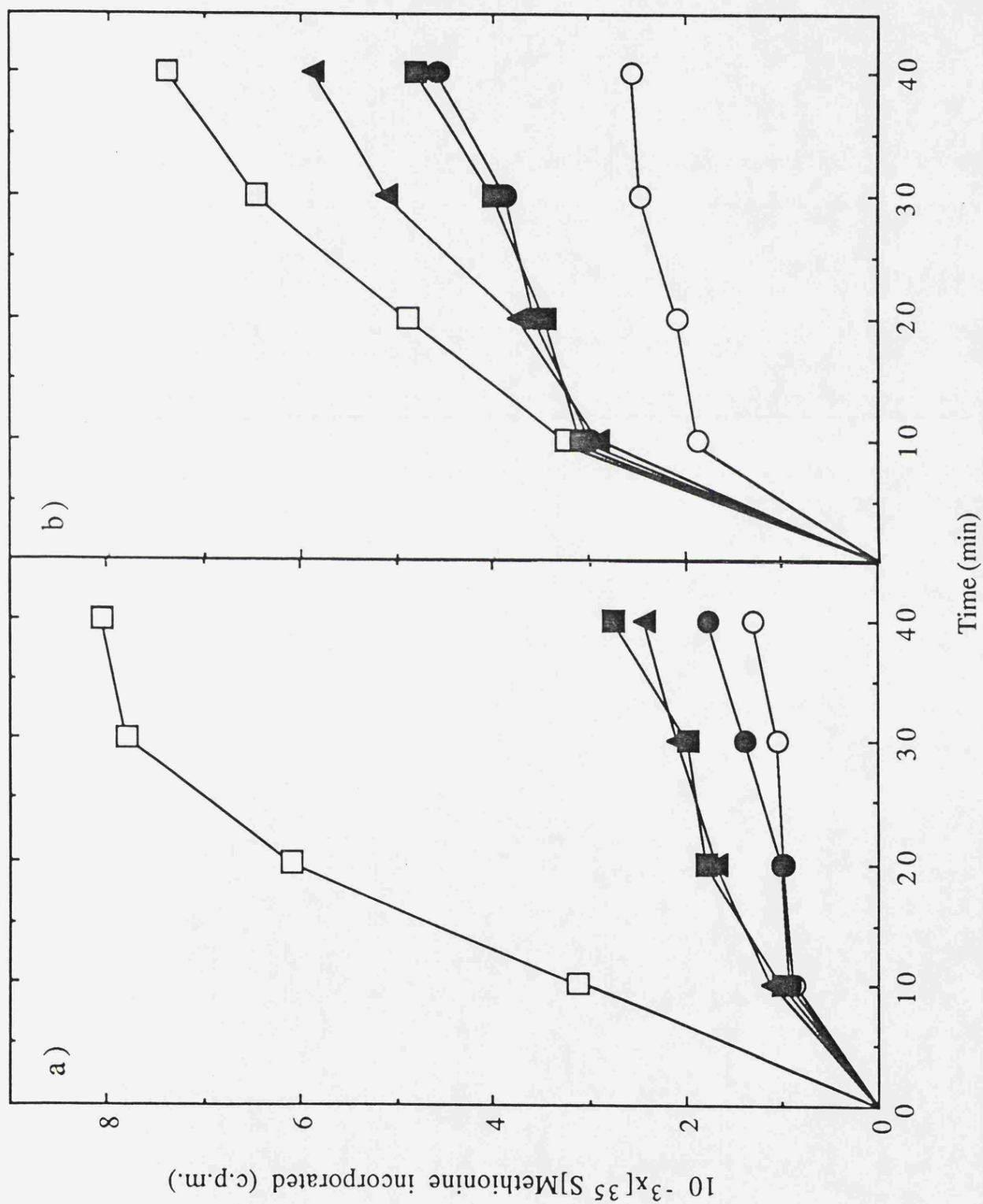
Prior to the cloning of the lincomycin resistant determinant, it had been established that induction of *S.lividans in vivo* resulted in the production of a methylase enzyme that modified 23S rRNA. There had however only been

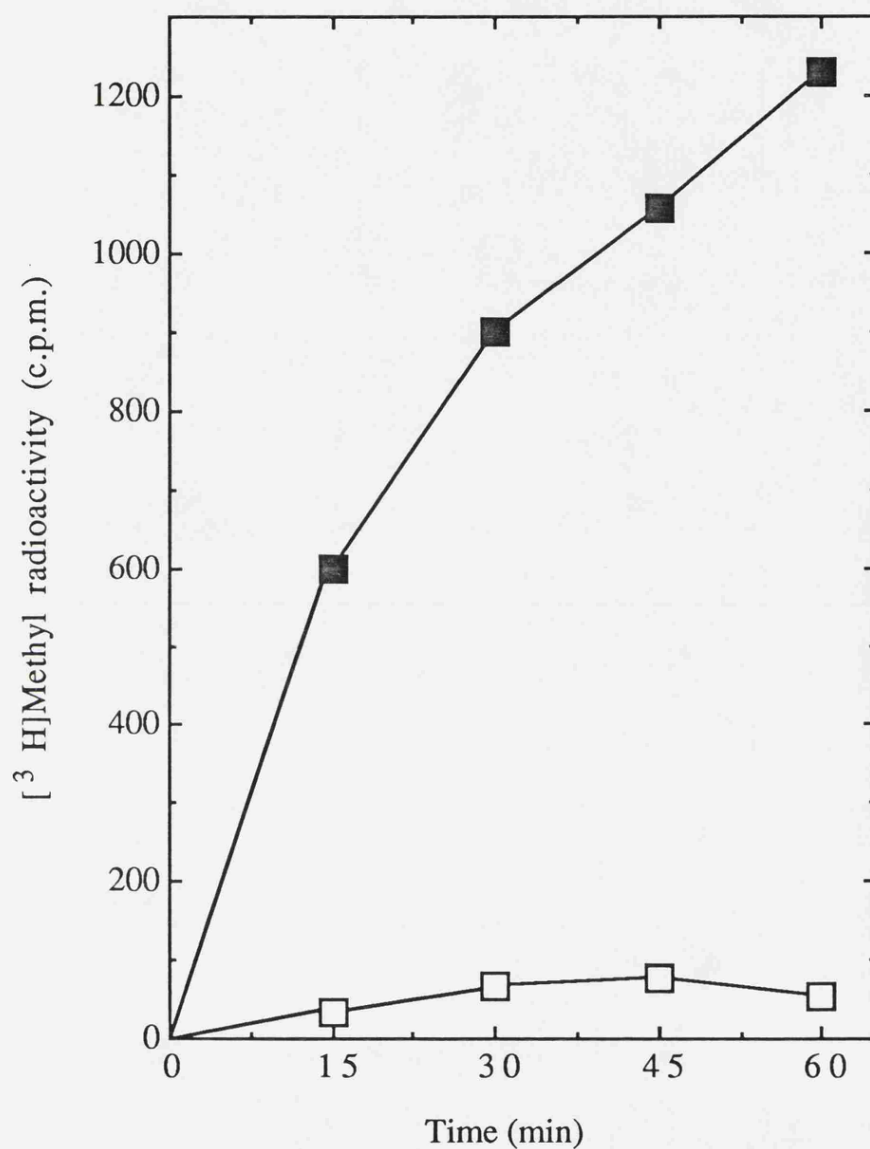
circumstantial evidence linking this event with the resistance phenotype observed. Having cloned a constitutively expressed resistance determinant from *S.lividans* MNT13, it was now possible to study the effect of such a gene on a sensitive strain (uninduced *S.lividans* in this case) and to show that the cloned gene does indeed encode an inducible methylase, which when expressed results in ribosomes of the host strain becoming resistant to lincomycin and various macrolides. For this purpose salt-washed ribosomes from *S.lividans* GJ1 were assayed for lincomycin resistance in a coupled transcription-translation reaction, with S100 and crude initiation factors prepared from uninduced *S.lividans* TK21. The ribosomes were found to be resistant to lincomycin *in vitro*, and this resistance was partially extended to macrolides (Fig. 4.5); a pattern identical to that seen with ribosomes from induced *S.lividans* TK21 (Fig 3.11). However ribosomes from *S.lividans* TSK1 were totally inhibited by the antibiotics tested, in their ability to direct cell-free protein synthesis.

Subsequently, *S.lividans* GJ1 was examined for the presence of a constitutively expressed ribosomal RNA methylase. A ribosomal wash fraction was therefore prepared from *S.lividans* GJ1, following growth of the strain in liquid media containing only thiostrepton ( $20\ \mu\text{g ml}^{-1}$ ) as a selection pressure. Lincomycin was not added to the media to prevent any possible induction of the chromosomally located resistance gene native to *S.lividans*, although earlier studies had suggested that *lrm* was not inducible by this drug. As a negative control a ribosomal wash fraction and RNA70 were also prepared from uninduced *S.lividans* TSK1 (i.e. containing pIJ702). When total rRNA from uninduced *S.lividans* TSK1 was incubated with a ribosomal wash fraction from *S.lividans* GJ1, in the presence of [methyl- $^3\text{H}$ ] SAM, radiolabelled methyl groups were incorporated into the RNA with a stoichiometry approaching 1.0 (Fig. 4.6). However, if RNA70 from *S.lividans*

**Fig.4.5 Effect ofMLS drugs on *in vitro* protein synthesis by cell extracts of *S.lividans* GJ1.** Reactions (34 $\mu$ l) contained S100 and crude intitation factors from uninduced *S.lividans* TK21. Salt-washed ribosomes from *S.lividans* TK21 (a), and *S.lividans* GJ1 (b) were assayed in the presence of DMSO ( $\square$ ), lincomycin [30  $\mu$ g/ml] ( $\blacktriangle$ ), erythromycin [30  $\mu$ g/ml] ( $\blacksquare$ ), tylosin [0.5  $\mu$ g/ml] (O), and carbomycin [0.5  $\mu$ g/ml] ( $\bullet$ )

Fig 4.5





**Fig. 4.6 Methylation of rRNA by the *lrm* methylase**  
A ribosomal wash fraction from *S. lividans* GJ1 was used as the source of methylase, together with [methyl-  $^3\text{H}$ ]SAM as cofactor and rRNA from uninduced *S. lividans* TK21 (■) and *S. lividans* GJ1 (□).

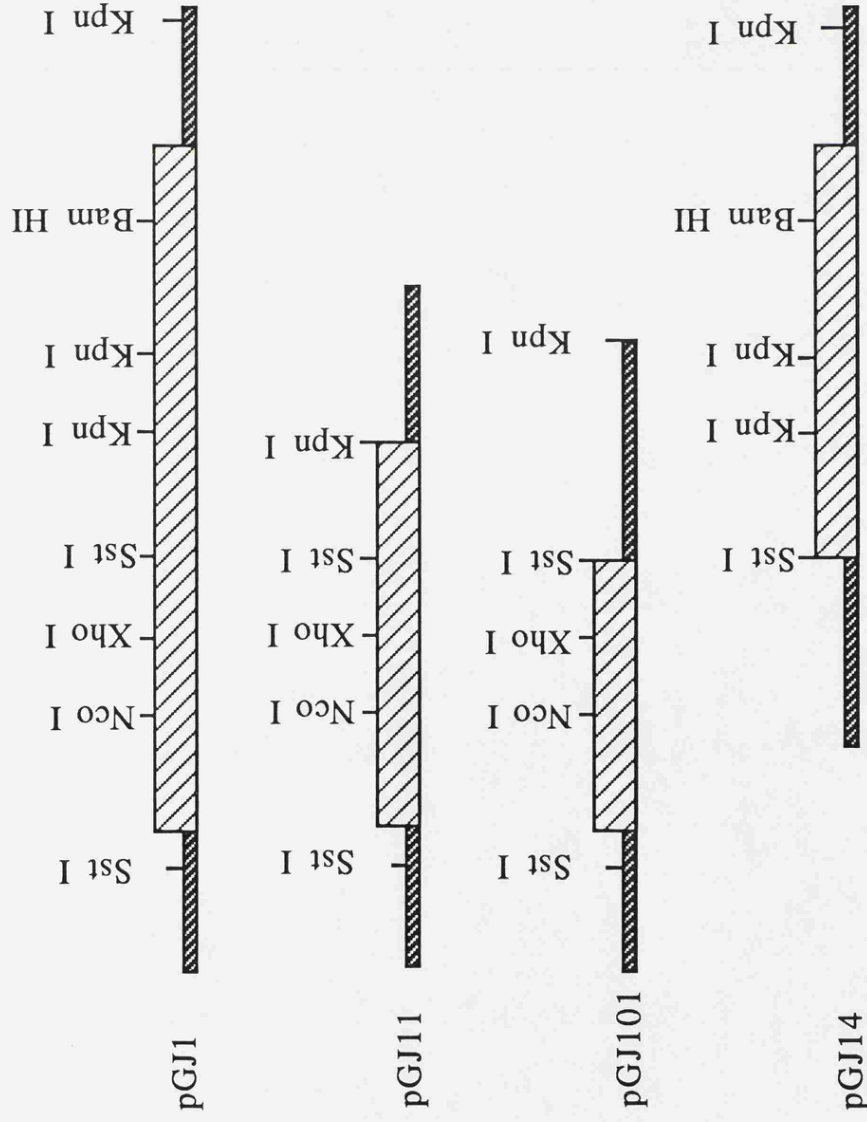
GJ1 was the added substrate, no significant methylation was observed. In reactions containing a ribosomal wash fraction from the control strain *S. lividans* TSK1, no incorporation of [methyl-<sup>3</sup>H] groups was observed into either of the 2 RNA substrates (data not shown). The results therefore indicate that the plasmid pGJ1 contains the gene *lrm*, encoding the lincomycin resistance methylase native to *S. lividans*.

As described in Section 4.1 constitutive methylase activity could not be detected in strain MNT13. However, isolation of the resistance determinant (*lrm*) on a multi-copy plasmid resulted in high methylase activity now being detected. Presumably, when present as a single copy in the chromosome, only small amounts of methylase, undetectable in the enzyme assays carried out here were produced. However when the gene was cloned onto a high copy number vector such as pIJ702, production of methylase was vastly increased. The previous hypothesis was therefore still appropriate.

#### 4.5. Sub-cloning of the Constitutively Expressed Lincomycin Resistance Determinant in *Streptomyces*.

The initial sub-cloning experiments in *Streptomyces* were designed to locate the exact position of *lrm* within the 3.6 kb of DNA initially cloned, and subsequently to reduce the size of that fragment to include only *lrm*. In one experiment pGJ1 was digested with SstI, the larger fragment being extracted from a LMP agarose gel and religated to generate pGJ14. In addition pGJ1 was incubated with KpnI, and again the larger of the two fragments was isolated and religated, resulting in pGJ11 (Fig. 4.7). When used to transform *S. lividans* TK21, pGJ11 still conferred lincomycin resistance demonstrating that it still contained the entire coding sequence of *lrm*. Strains harbouring pGJ14 however, were not able to grow on plates containing  $>25 \mu\text{g ml}^{-1}$

Lincomycin resistance phenotype  
conferred upon *S. lividans*



**Fig. 4.7** Restriction maps of pIJ702 containing DNA inserts from *S. lividans* MNT13. The resistance phenotypes conferred by the plasmids are indicated as R [resistant to lincomycin (100 ug/ml)] or S [sensitive to lincomycin (100 ug/ml)]

hatched box *S. lividans* MNT13 DNA  
solid black box pIJ702 DNA

1 kb

lincomycin. These results suggested that the *lrm* coding sequence lay to the left of the KpnI site and possibly before the SstI site. Further subcloning experiments involved digestion of pGJ1 with SstI, and re-cloning of the 1.3 Kb fragment into the SstI site of pIJ702, to generate pGJ101 (Fig 4.7). Strains of *S.lividans* transformed with pGJ101 were again shown to express lincomycin resistance, indicating that the *lrm* coding sequence was located within this SstI fragment, and that the gene had now been isolated on a 1.3 kb fragment of DNA.

At this stage, a further lincomycin resistance gene *clr* had been isolated and characterised from the celesticetin producer *Streptomyces caelestis* (Calcutt and Cundliffe, 1990). This resistance determinant encoded on pTB700 had been shown to encode a rRNA monomethylase that had exactly the same specificity for A-2058 as the *lrm* product. Obviously, since the *lrm* and *clr* products have an identical mode of action, both *S.lividans* GJ1 and *S.lividans* TB700, would be expected to exhibit identical resistance phenotypes. However, when the MIC values for lincomycin and a number of macrolide antibiotics of both strains were compared, obvious discrepancies became apparent (Table 4.2). The level of resistance towards lincomycin is the same in both strains, but *S.lividans* GJ1 clearly exhibits a higher level of resistance towards the macrolide antibiotics tested. The most noticeable difference is a marked increase in resistance towards tylosin.

The situation became somewhat clearer when the MIC values for *S.lividans* GJ101 were determined (Table 4.2), and shown to be identical to those for *S.lividans* TB700. Moreover, similar values were also obtained when the *carB* gene (encoding another monomethylase whose site of action is A-2058) from the carbomycin producer *S.thermotolerans* was cloned in *S.lividans* (Zalacain and Cundliffe, 1990)(Epp, et al., 1987). Clearly, the values



| MIC (ug/ml)  |                           |                          |                            |                            |
|--------------|---------------------------|--------------------------|----------------------------|----------------------------|
| Antibiotic   | <i>S.lividans</i><br>TK21 | <i>S.lividans</i><br>GJ1 | <i>S.lividans</i><br>TB700 | <i>S.lividans</i><br>GJ101 |
| Lincomycin   | 30-50                     | >5000                    | >5000                      | >5000                      |
| Erythromycin | 30-50                     | 250-300                  | 100-150                    | 100-150                    |
| Tylosin      | 25-50                     | 300-400                  | 50-75                      | 50-75                      |
| Carbomycin   | 50-100                    | >1000                    | 500-1000                   | 500-1000                   |

**Table 4.2. Minimal Inhibitory Concentrations of MLS antibiotics for various strains of *S.lividans*. All strains were grown on NE media at 30°C and MIC values were determined after 4 days of growth**

shown for these strains are indicative of monomethylation at A-2058 in 23S rRNA. The apparent discrepancy in MIC values between *S.lividans* GJ1 and *S.lividans* GJ101, suggests the presence of a further resistance gene on the larger of the two plasmids pGJ1. This extra resistance determinant when expressed together with the rRNA monomethylase appears to have no effect on lincomycin resistance (though monomethylation confers such high levels of resistance to lincomycin that any increase in these levels may be undetectable), but confers a somewhat higher level of resistance towards macrolides, and in particular tylosin.

Since the two genes had been cloned on the same DNA fragment, it is likely that they are located adjacent to each other in the chromosome of *S.lividans* MNT13, and similarly in TK21. The possibility existed however that two separate Sau3A fragments from different parts of the chromosome had ligated during the initial shotgun cloning experiment, resulting in the 3.6 kb fragment present in pGJ1. The likelihood of this occurring however is quite low.

Whilst a second resistance gene had been detected on pGJ1, no resistance phenotype had been attributed to that resistance determinant alone. As already discussed in this chapter, pGJ14 (a plasmid in which *lrm* was totally deleted), conferred no resistance to lincomycin and when the response of *S.lividans* harbouring pGJ14 towards macrolides was subsequently tested, no significant resistance was observed. However, since there was no evidence to indicate that the entire second resistance gene was present in pGJ14, no definitive conclusions could be made from these data.

In view of the possibility that the second resistance gene might be co-transcribed with *lrm*, further sub-clones from pGJ1 were not generated.

Instead, in an effort to determine a resistance phenotype attributable to the second gene, plasmid pGJE10 (an *E. coli* plasmid containing the same insert as GJ1 and described in section 4.7) was digested with Xho; a unique site thought to lie within the *lrn* coding sequence, and treated with large fragment DNA polymerase to generate blunt ends. The plasmid was then religated and used to transform *E. coli*, generating pGJE15:- a plasmid in which *lrn* had been inactivated. The latter plasmid was subsequently digested with PstI and partially with SstI, and the products separated on a 1% LMP agarose gel. The 3.8 Kb Pst/Sst fragment was extracted and ligated with pIJ702, previously digested with PstI and SstI and terminally dephosphorylated. The resulting ligation mix was used to transform *S.lividans* protoplasts and the transformants selected with thiostrepton. One such strain, *S.lividans* GJ15, was examined for its sensitivity to lincomycin and various macrolides and found to be sensitive to all the drugs tested.

This result could indicate that the product of the second gene confers no detectable resistance phenotype in the absence of monomethylated ribosomes. In other words, only when it is expressed in the presence of a more powerful resistance determinant such as a rRNA methylase does it affect resistance to macrolide antibiotics. However, if the two genes are co-transcribed, filling in of the XhoI site may have resulted in a shift in the reading frame causing the TGA stop codon of the methylase gene to be out of frame thus generating a translational fusion. If so, unless further stop codons appear within the new reading frame before the coding sequence of the second gene starts, any ribosomes translating the methylase would continue into the second open reading frame resulting in the production of an inactive fusion protein. These ribosomes engaged in translation of the methylase would conceivably block initiation of protein synthesis from the ribosome binding site of the second gene. An alternative explanation is that the second

gene may be expressed constitutively in *S.lividans* TK21, and it is the product of this gene that is responsible for the relatively high basal level of resistance detected in uninduced strains of *S.lividans* (Table 3.1), though if this were true the presence of the gene on a multi-copy plasmid would perhaps be expected to confer higher levels of resistance than in the wild type strain:- an observation not made. The question as to what the second gene encoded however remained undetermined.

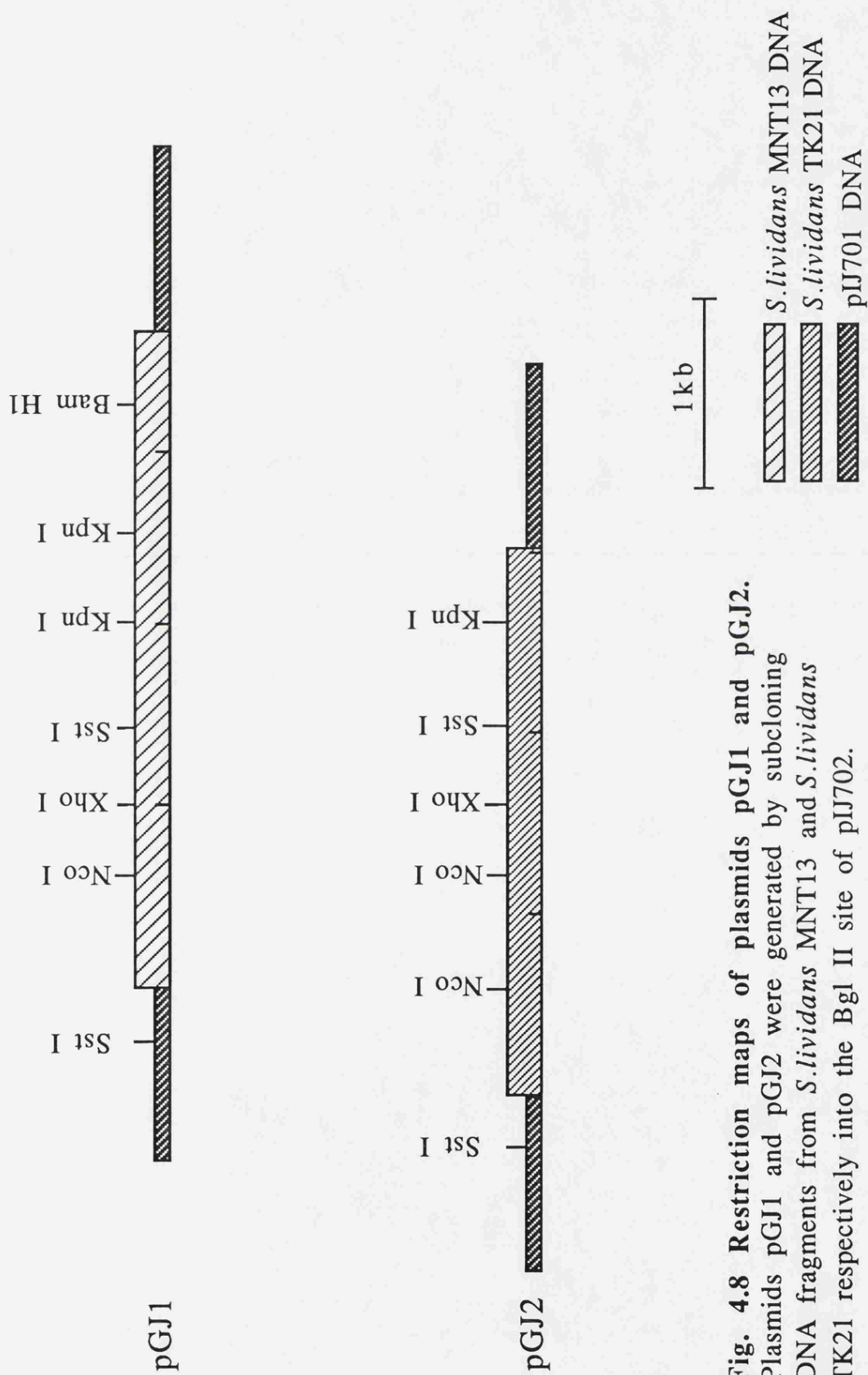
#### 4.6. Cloning of the Inducible Lincomycin Resistance Determinant from *S.lividans* TK21.

Prior to the present work, the tylosin resistance determinant *tlrA*, had been isolated from *S.fradiae* in *S.lividans* (Birmingham, et al., 1986), and shown to encode a dimethylase, that acts at A2058 in 23S rRNA (Zalacain and Cundliffe, 1989). The expression of this enzyme is inducible and a possible attenuator, similar to that described for *ermC* has been proposed (see Chapter 1). However, when cloned in *S.lividans* on a multi-copy plasmid, *tlrA* appeared to be expressed constitutively without prior induction. This was subsequently explained by the suggestion that the attenuator system is leaky, a phenomenon now thought to be common to genes controlled by translational attenuation. It is thought that such systems are not under tight control, in that the 'inactive' mRNA conformation can occasionally flip into the 'active' conformation without prior induction, resulting in accidental translation of supposedly inactive mRNA (i.e. 'leakiness'). When the gene is present as only one copy in the chromosome, the levels of methylase produced are minimal. However when present as multiple copies, any leakiness in the attenuator system is grossly exaggerated.

As already discussed, control of *lrm* induction is thought to be *via*

translational attenuation and is therefore possibly prone to the leakiness described above. Indeed, the ease with which constitutive mutants of *S.lividans* are obtained and the fact that constitutive methylase activity is only detected when the resistance determinant of MNT13 is present on a high copy number vector are highly suggestive of such a system operating. This being the case, when *lrn* is cloned in *S.lividans* TK21 on a multi-copy plasmid, a proportion of the cells harbouring the gene may well be expected to express the associated phenotype without prior induction. This hypothesis was put to the test, and attempts were made to clone lincomycin resistance determinants from *S.lividans* TK21 in an identical manner to that described for the mutated gene. Chromosomal DNA fragments from *S.lividans* TK21, generated by partial *Sau*3A digestion, were introduced into the *Bgl*II site of the high copy number vector pIJ702. Ligated DNA was then used to transform protoplasts of *S.lividans*, and primary transformants were selected for thiostrepton resistance. After further incubation to allow for subsequent sporulation, the primary transformants (50,000 on 10 plates) were replica-plated onto media containing both thiostrepton ( $20\ \mu\text{g ml}^{-1}$ ) and lincomycin ( $200\ \mu\text{g ml}^{-1}$ ) and incubation continued for a further 40 hours. One lincomycin resistant colony was obtained after this time.

Following inoculation of spores and aerial mycelium from this colony, into liquid media supplemented with thiostrepton ( $20\ \mu\text{g ml}^{-1}$ ) and lincomycin ( $200\ \mu\text{g ml}^{-1}$ ), and subsequent incubation for 40 hours at  $30^\circ\text{C}$ , plasmid DNA (pGJ2) was prepared. Restriction analysis revealed that a DNA fragment similar to that cloned from MNT13 had been isolated (Fig. 4.8). The insert (3kb) was however smaller than that in pGJ1 and a greater portion of the DNA upstream from *lrn* had been cloned. The fact that the two DNA fragments were so similar also indicated that no re-arrangement had occurred in cloning the two resistance determinants isolated on pGJ1 and



**Fig. 4.8** Restriction maps of plasmids pGJ1 and pGJ2. Plasmids pGJ1 and pGJ2 were generated by subcloning DNA fragments from *S. lividans* MNT13 and *S. lividans* TK21 respectively into the Bgl II site of pIJ702.

that the two genes must, indeed lie next each other in the chromosome.

The plasmid pGJ2 was subsequently tested for its ability to re-transform *S.lividans* TK21 to lincomycin resistance. Approximately 50 ng of that plasmid and pIJ702 were introduced into protoplasts of *S.lividans* TK21, and following overnight incubation primary transformants were selected by overlaying plates with thiostrepton. After allowing regeneration of protoplasts, colonies were replica plated onto media containing lincomycin (200  $\mu\text{g ml}^{-1}$ ) and incubated for a further two days. After this period no growth of *S.lividans* transformed with pIJ702 was observed, whilst a large number of colonies harbouring pGJ2 were seen to grow on lincomycin. It was however noted that the number of colonies transformed with pGJ2 was greater on media containing thiostrepton alone than on medium containing lincomycin plus thiostrepton, indicating that not all the cells harbouring pGJ2 were expressing resistance to lincomycin. Furthermore the MIC values of *S.lividans* GJ2 for lincomycin and several macrolides were determined, and found to be identical to those obtained for *S.lividans* GJ1. The second resistance gene first observed on pGJ1 must, therefore by comparison, also be present on pGJ2.

It appeared that cloning *lrm* onto a high copy number vector had indeed, resulted in 'constitutive' expression of the resistance phenotype in a portion of the cells. As stated earlier, this anomaly has previously been attributed to 'leaky' attenuator systems. The hypothesis was therefore tested by comparing the efficiency of plating of *S.lividans* GJ2 on plates containing thiostrepton (20  $\mu\text{g ml}^{-1}$ ) or both thiostrepton and lincomycin (500  $\mu\text{g ml}^{-1}$ ). An equal number of spores were spread on the above plates and the number of colonies observed after 3 days of growth. At this time only 10% of the colonies growing on thiostrepton were also able to grow on lincomycin, a

figure too high to be attributed to spontaneous mutation. Furthermore, when spores were collected from colonies growing on lincomycin and the latter experiment repeated, it was again observed that only 10 % of the colonies growing on thiostrepton were also able to grow on lincomycin. If mutation were the correct explanation for their initial growth on lincomycin, all the colonies should have grown on the drug when replated. It would therefore appear that the kind of 'leakiness' previously observed in *S.lividans tlrA* is also evident in *S.lividans* GJ2.

A similar experiment was conducted with *S.lividans* GJ1. In this case all of the colonies growing on thiostrepton appeared to grow on media containing lincomycin. This provided further evidence that a mutation had occurred in pMNT13 that affected expression of *lrm*.

The data presented in this section highlight the possibility that induction of *lrm* is controlled by translational attenuation . This is addressed directly in subsequent chapters.

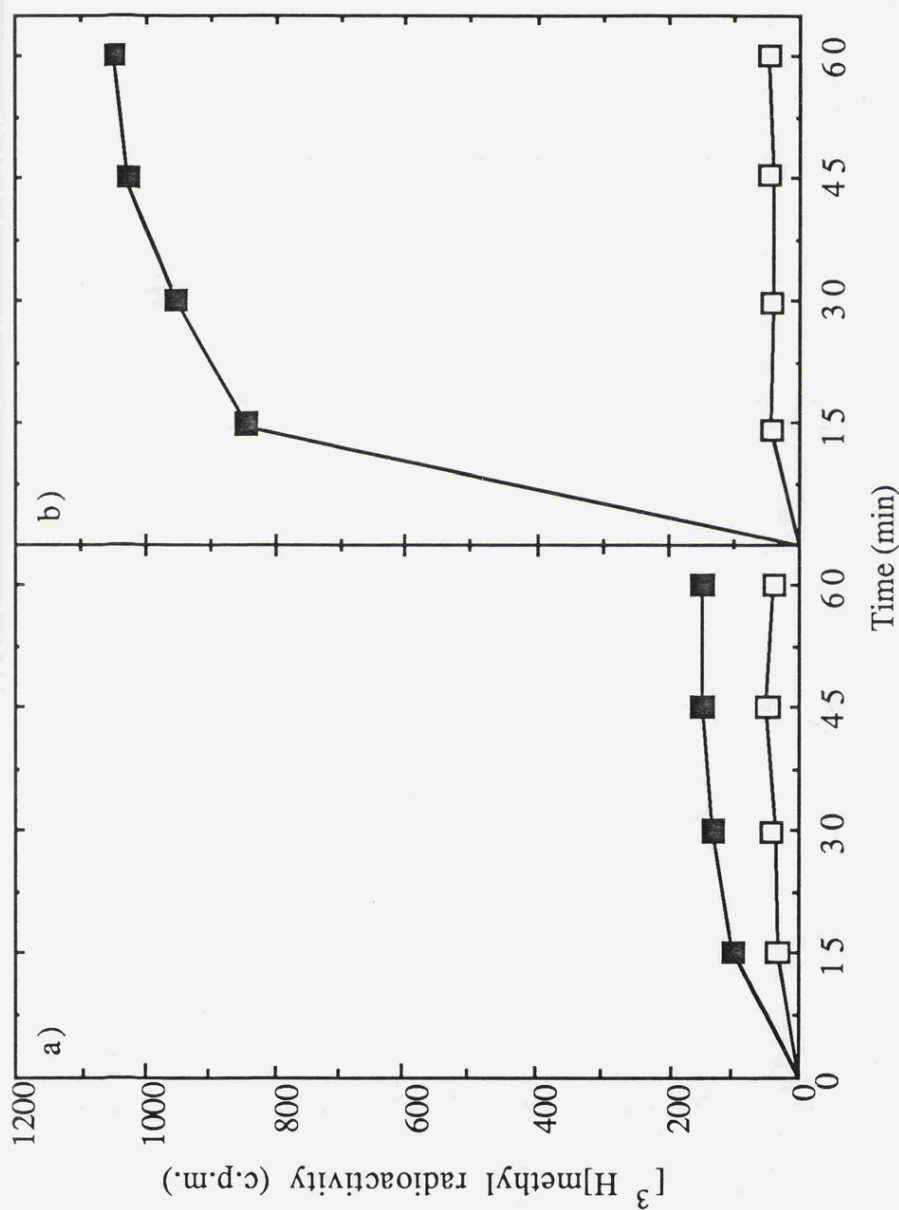
#### 4.7. Biochemical Characterisation of Lincomycin Resistant Clones from *S. lividans* TK21.

Having cloned a resistance determinant from *S.lividans* TK21, it was now possible to transfer that gene into a negative background such as *S.griseofuscus*, a strain which possesses no MLS resistance phenotype. It was then possible to show that the cloned gene does indeed encode an inducible methylase (i.e. the cloned gene was *lrm*), which when expressed results in the ribosomes of the host strain becoming resistant to lincomycin and various macrolides.



For this purpose, supercoiled plasmid pGJ2 was used to transform protoplasts of *S.griseofuscus*. The preparation of such protoplasts and the transformation procedure followed was identical to that described in the previous section. Transformants were again selected with thiostrepton and after a period of regeneration, colonies were scraped from the plate and streaked onto media containing lincomycin ( $200\mu\text{g ml}^{-1}$ ) to check that the cloned gene was being expressed in *S.griseofuscus*. Transformants were not replica-plated in this case as colonies of *S.griseofuscus* do not sporulate on regeneration media.

To determine whether a ribosomal resistance mechanism, mediated by induction of a methylase enzyme was indeed operating in the clones, ribosomal wash fractions and salt-washed ribosomes were prepared from *S.griseofuscus* GJ2, both prior to and following induction of that strain with erythromycin ( $20\mu\text{g ml}^{-1}$ ). As a negative control, similar cellular fractions were prepared from *S.griseofuscus* pIJ702. The strains were initially examined for the presence of a rRNA methylating enzyme (Fig. 4.9). When total rRNA from uninduced *S.lividans* pIJ702 was incubated with ribosomal wash fraction from induced *S.griseofuscus* GJ2, in the presence of [methyl- $^3\text{H}$ ] SAM, radiolabelled methyl groups were incorporated into the RNA with a stoichiometry approaching 1.0. However, if a ribosomal wash fraction from uninduced *S.griseofuscus* GJ2 was used as the source of methylase in a similar reaction, only marginal incorporation of  $^3\text{H}$ -methyl groups was observed. In both cases, background was considered to be the level of methylation obtained when RNA70 from induced cultures of *S.lividans* pIJ702 was the added substrate. In control reactions using ribosomal wash fraction from *S.griseofuscus* induced and uninduced, no significant incorporation of  $^3\text{H}$ -methyl groups was observed (data not shown).



**Fig. 4.9 Methylation of rRNA by *S. griseofuscus* GJ2.** Ribosomal wash fractions from uninduced a) and induced b) strains of *S. griseofuscus* containing pGJ2 were used as the source of methylase, together with  $[^3\text{H}]$  SAM as cofactor plus RNA70 from induced *S. lividans* (□) and uninduced *S. lividans* (■).

The results would therefore indicate that the plasmid pGJ2 contains the gene *lrm*, encoding the lincomycin resistance methylase native to *S.lividans*. In addition, when present in high copy number under non-inducing conditions, *lrm* is expressed at a low level. Upon induction with erythromycin, the expression becomes greatly enhanced.

Subsequently salt-washed ribosomes from *S.griseofuscus* GJ2 grown under inducing conditions, were assayed for activity and lincomycin sensitivity in a coupled transcription-translation reaction, with S100 and crude initiation factors prepared from *S.lividans* TK21 (Fig. 4.10) The ribosomes were indeed found to be resistant to lincomycin *in vitro*, and resistance was partially extended to macrolides, a pattern identical to that seen in ribosomes from induced *S.lividans*. (Fig 3.11). The ability of ribosomes from *S.griseofuscus* pIJ702 to direct cell-free protein synthesis was totally inhibited by all of the antibiotics used in the assay at the concentration indicated (Fig 4.10a), demonstrating that the strain possessed no intrinsic ribosomal modification system that conferred MLS resistance. Ribosomes from uninduced *S.griseofuscus* GJ2 were assayed in similar systems, and whilst the data are not shown, their response was identical to that of ribosomes isolated from *S.griseofuscus* pIJ702. The finding that induction of a rRNA methylase resulting in lincomycin resistant ribosomes was specific to *S.griseofuscus* strains harbouring pGJ2 provided direct evidence that *lrm* acted as a resistant determinant.

#### 4.8. Subcloning of *lrm* in *E.coli*.

To enable the construction of more detailed restriction maps, and to facilitate the subsequent manipulation of the cloned DNA fragments, the inserts from plasmids pGJ1 and pGJ2 were cloned into the *E.coli* expression

**Fig. 4.10 Sensitivity of salt-washed ribosomes from induced *S.griseofuscus* GJ2.** Reactions (34 $\mu$ l) contained S100 and crude initiation factors from *S.lividans* TK21. Salt washed ribosomes (20 pmol) from *S.griseofuscus* pIJ702 (a), and *S. griseofuscus* GJ1 (b) were incubated with DMSO ( $\square$ ), lincomycin [10  $\mu$ g/ml] ( $\Delta$ ), erythromycin [30  $\mu$ g/ml] ( $\blacksquare$ ), tylosin [0.5  $\mu$ g/ml] (O), and carbomycin [0.5  $\mu$ g/ml] ( $\bullet$ ), prior to assay for coupled transcription-translation.

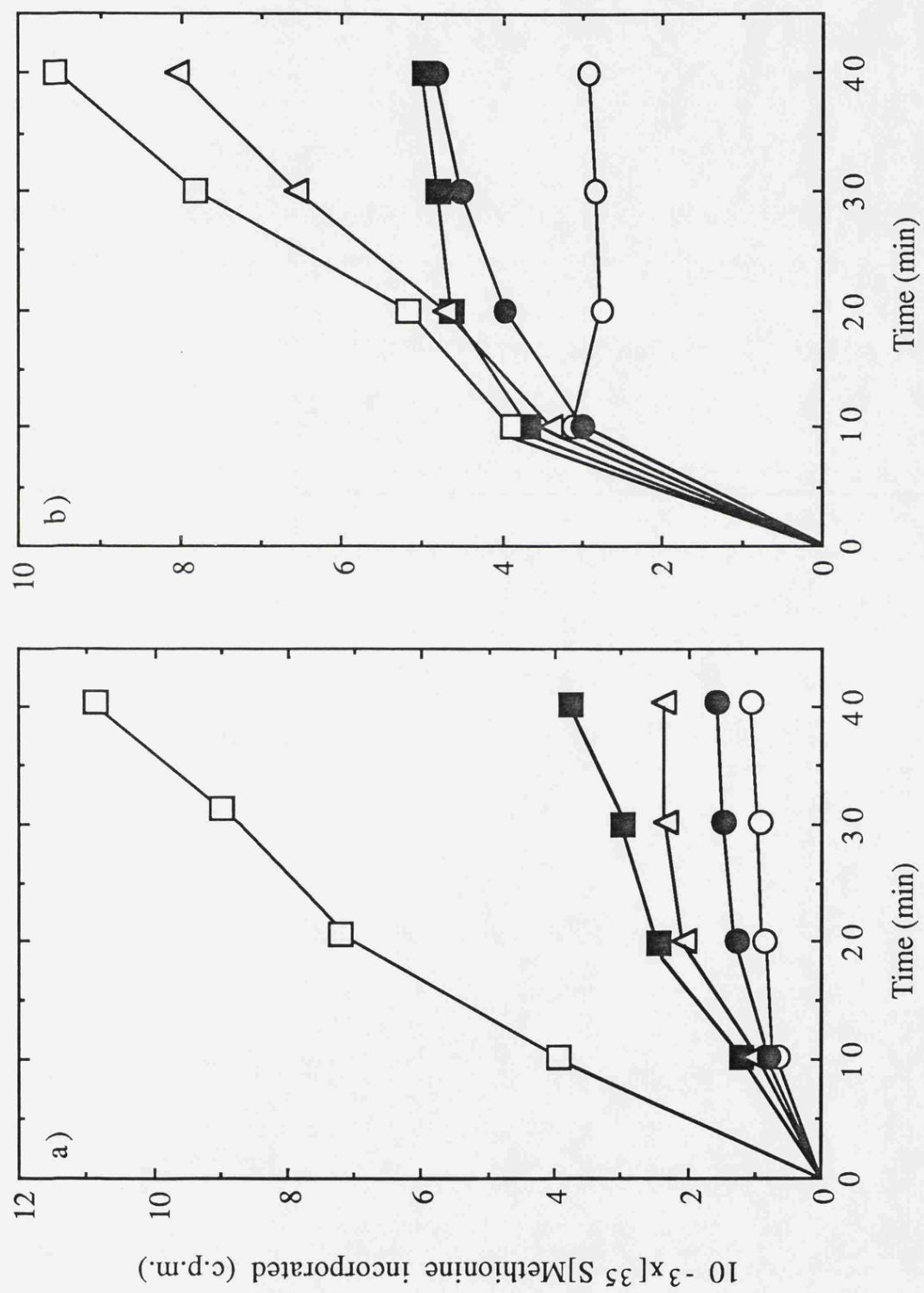
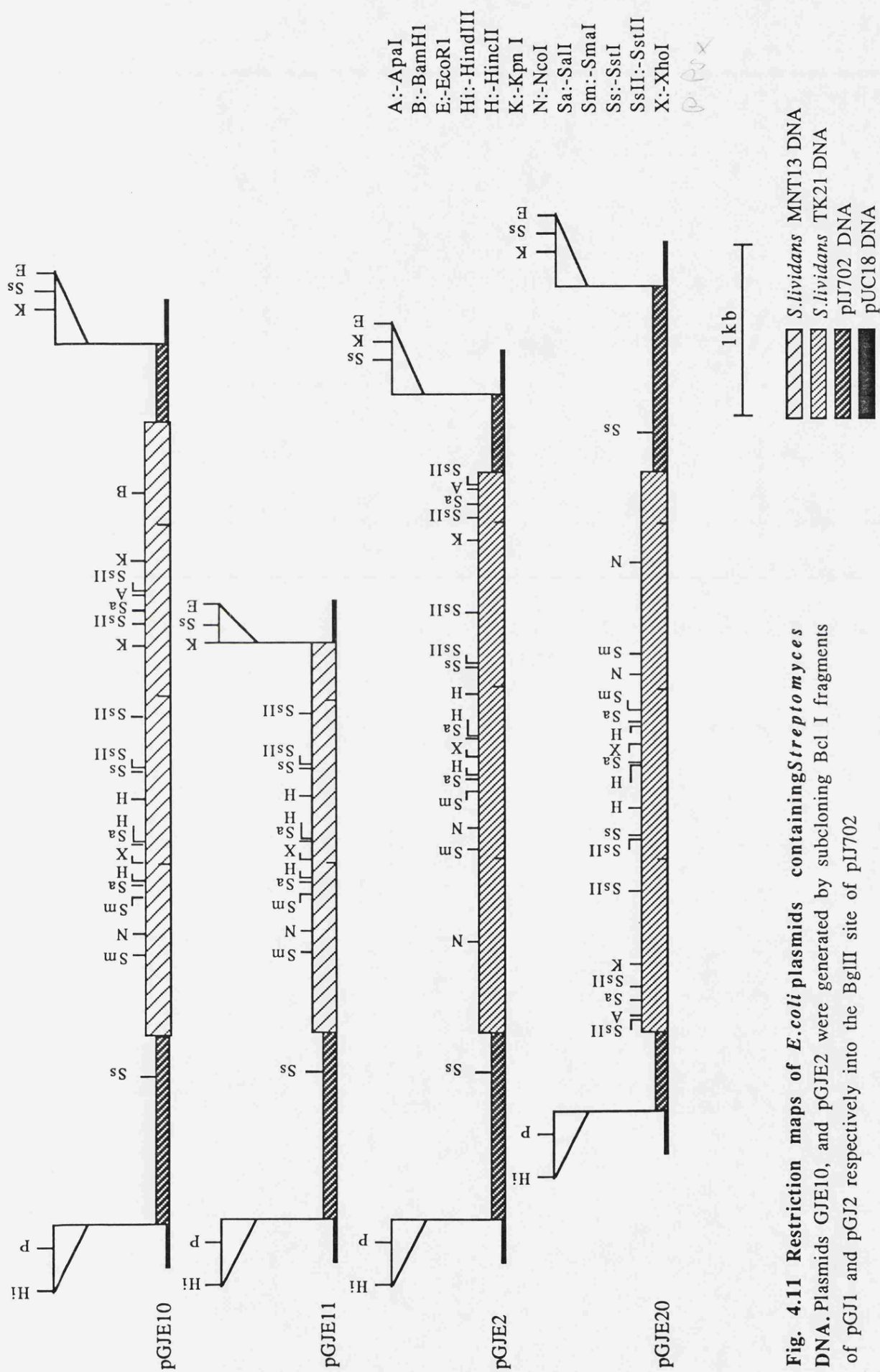


Fig. 4.10

vector pUC18. Cloning of the two inserts was carried out in an identical manner but only that involving the insert of pGJ1 will be described here.

Following digestion of 2  $\mu\text{g}$  of pGJ1 with BclI, and separation of the fragments by electrophoresis on a 1% LMP agarose gel, the appropriate 5.2 kb fragment was extracted from the gel as described in Methods. This was then ligated with 0.5  $\mu\text{g}$  pUC18, previously digested with BamHI and terminally dephosphorylated. After transformation of *E.coli* NM522, transformants were selected on media containing 100  $\mu\text{g ml}^{-1}$  ampicillin, and recombinants identified by their inability to produce a blue pigment in the presence of IPTG and X-Gal. (see methods). Plasmid DNA was prepared from 12 white colonies and following gel electrophoresis, those plasmid which were larger than pUC18 were restricted with SstI to identify those constructs that contained the correct insert and in addition the orientation of that insert. Subsequent analysis showed that some plasmids contained the 5.2 kb BclI fragment, and that this insert had been cloned in the orientation shown (Fig 4.11), resulting in the construct pGJE10. The 4.6 kb BclI fragment from pGJ2 was cloned into pUC18 in both possible orientations to generate pGJE2 and pGJE20. Further restriction analysis was carried out on plasmids pGJE10 and pGJE2 and a more detailed restriction map constructed (Fig. 4.11).

Experiments to determine if the resistance phenotype was expressed in *E.coli* NM522, were not possible since this strain is known to possess intrinsic ribosomal resistance towards lincomycin (Chang, *et al.*, 1966). Analysis of the resistance phenotype conferred by smaller subclones was therefore carried out in *Streptomyces* as described in Section 4.5. However one subclone was constructed in *E.coli* from pGJE10 to enable analysis of the protein(s) in a coupled transcription-translation system.



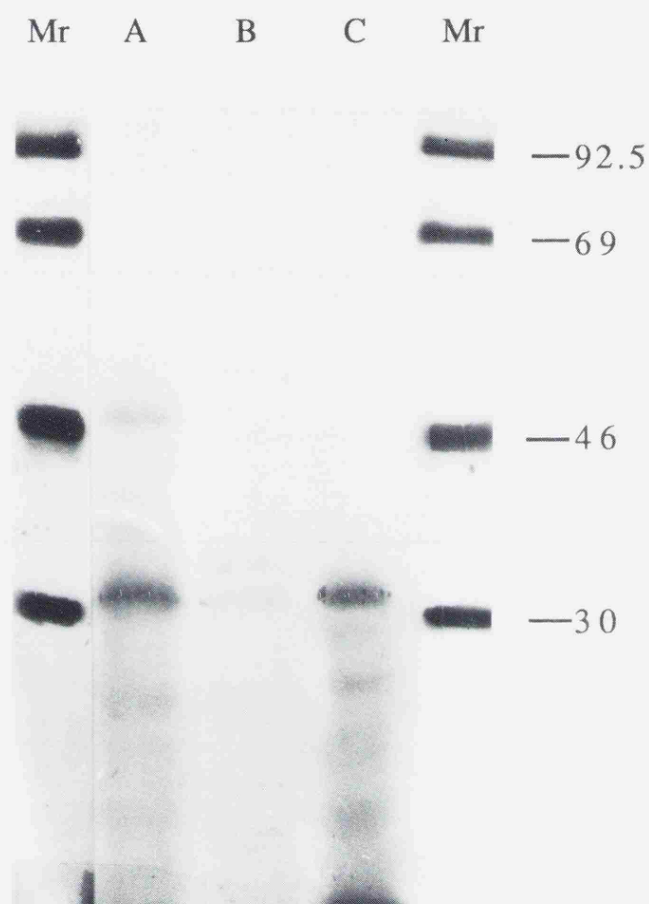
**Fig. 4.11 Restriction maps of *E. coli* plasmids containing *Streptomyces* DNA.** Plasmids GJE10, and pGJE2 were generated by subcloning Bcl I fragments of pGJ1 and pGJ2 respectively into the BglII site of pIJ702

Plasmid pGJE10 was digested with KpnI, the products separated by LMP agarose gel electrophoresis, and the largest fragment isolated. Religation of this fragment, followed by transformation of *E.coli* NM522 resulted in the formation of pGJE11 (Fig. 4.11). Subclones generated from pGJE10 were used in the analysis of protein products, since expression of the methylase was more evident from the mutated fragment than from the wild type DNA.

#### 4.9. *In vitro* Coupled Transcription-Translation

Plasmids pGJE10, pGJE11 and pUC18 were used to direct coupled transcription-translation in cell-free extracts of *S.lividans*, using [<sup>35</sup>S]-methionine to label the protein products. Following separation by SDS-polyacrylamide gel electrophoresis, the products were visualised by fluorography (Fig 4.12). Plasmid pGJE10 gave rise to an extra band running slightly slower than the 46KDa protein marker, when compared with the pUC18 control. Additional bands may have been present in the 30KDa region but due to the high background in this area, any plasmid specific protein product running at this level could not be distinguished. In reactions containing plasmid pGJE11, that contains the entire methylase gene, a smaller (~35KDa) protein product was evident (Fig. 4.12 track B). This smaller protein was presumably a truncated form of the 46 KDa protein. These data indicated that this latter protein, which could not be the product of *lrm*, was likely to be that of the second resistance gene. The methylase gene was not obviously expressed from either plasmid in systems such as those described. However, the possibility remains that the *lrm* product may correspond to a 30 KDa protein, but as indicated earlier its presence may be masked by the  $\beta$ -lactamase pre-protein of *E.coli* and other products of the S30 running in the same region. Alternatively, adequate amounts of methylase enzyme may not be expressed from the plasmids *in vitro* to be





**Fig. 4.12** Coupled transcription-translation *in vitro*.

Electrophoretic analysis of the products of coupled transcription-translation directed by: (A) pGJE10; (B) pGJE11; and (C) pUC18. The products were subjected to electrophoresis on SDS-polyacrylamide gels together with [ $^{14}\text{C}$ ] protein markers whose molecular weights (in kDa) are indicated.

detected in this type of system, without prior induction with erythromycin. Whatever the reason, the size of *lrm* remained unknown.

#### 4.10. Southern Analysis of Two Resistance Determinants From *S.lividans*.

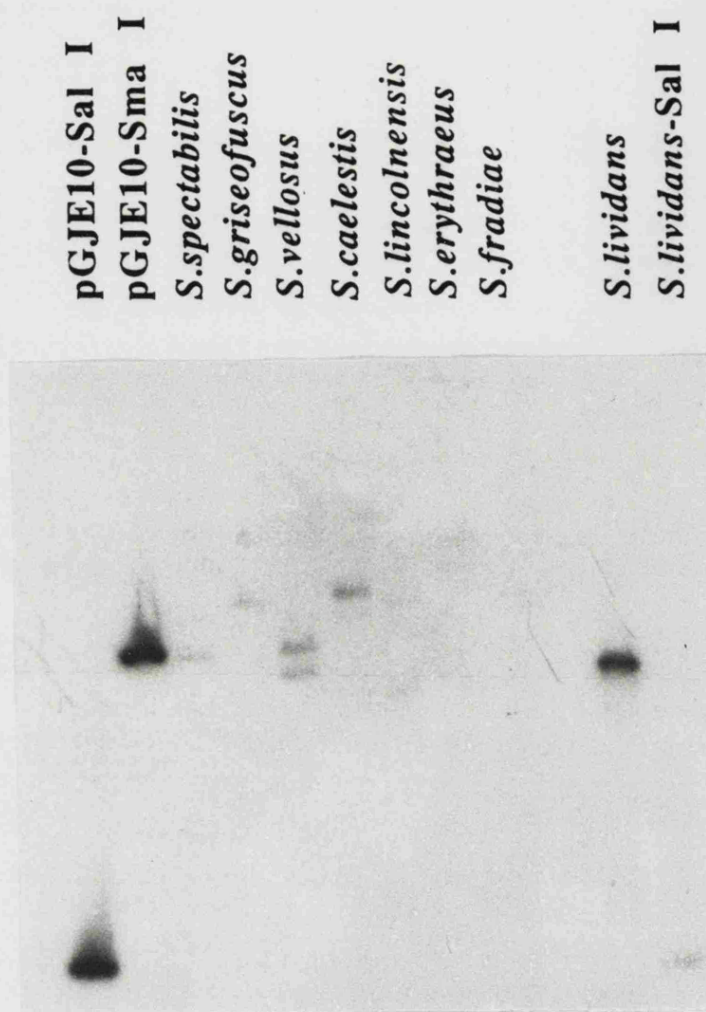
##### a) *lrm*.

In order to confirm that the 3.0 kb fragment in pGJE2 originated from *Streptomyces lividans* and to demonstrate any homology between *lrm* and similar genes encoding rRNA methylases present in other organisms, Southern analysis was carried out.

A radioactive probe was synthesised using the 400 bp. NcoI→XhoI fragment from GJE2 as template, whilst various genomic DNA preparations were subjected to agarose gel electrophoresis and then transferred to Hybond-N membrane. In addition plasmid pGJE10 (digested with SmaI and SalI) was subjected to similar treatment as a positive control. After overnight hybridization of probe DNA with membrane-bound DNA, the membrane was subjected to high stringency wash so that the probe only remained bound to DNA with which it had greater than 90% similarity.

The autoradiogram (Fig 4.13) shows that the probe hybridized to a single fragment in all three digestions of an independent preparation of *S.lividans* DNA, and to the digestions of pGJE10. These data show that the isolated DNA fragment originated in *S.lividans* TK21 and in addition substantiate the evidence that *lrm* does not encode a ribosomal RNA species, since these are present as multiple copies in the chromosome.

The probe was also seen to hybridize to genomic DNA from a number of



**Fig. 4.13.** Southern analysis of chromosomal DNA's from various *streptomyces* spp., using *irm* as probe. Various chromosomal DNA's were digested with Bam H1 and probed with the Nco I-Xho I fragment of pGJE10, in addition *S. lividans* TK21 DNA and pGJE10 were also digested with Sal I. In each case 5 $\mu$ g of chromosomal DNA was loaded and 50 ng of plasmid.

other *Streptomyces* strains. The celesticetin producer *S.caelestis* is known to possess a rRNA monomethylase and this is presumably the gene with which the *lrm* fragment has similarity. Both *S.vellosus* and *S.lincolnensis* are producers of lincomycin, and whilst rRNA methylating enzymes have not yet been detected in these strains, the results indicate that such enzymes are probably present. This would also substantiate the finding of monomethylated 23S rRNA in strains of *S.lincolnensis* grown in lincomycin production media (see Chapter 1). The existence in both of these latter genomic DNA preparations, of two fragments showing a certain identity with *lrm* could be explained by the presence of only one rRNA methylase in both strains which has been cut into two fragments upon digestion with BamH1. Alternatively, two such rRNA methylases exist in both strains.

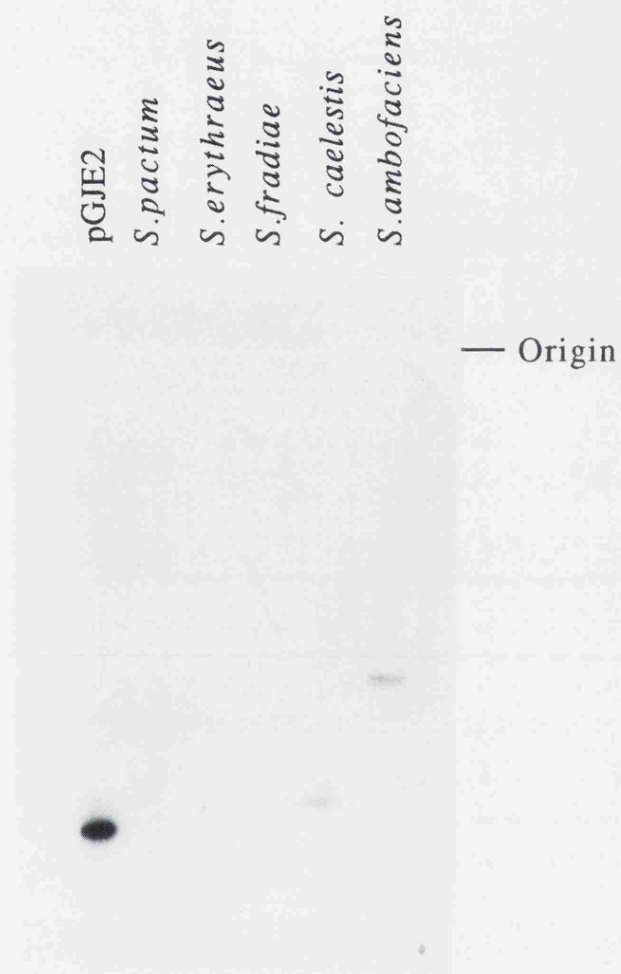
Surprisingly, no homology was detected between *lrm* and either of the dimethylating enzymes known to be present in *S.erythraeus* and *S.fradiae*. Both of these enzymes act at an identical site to the *lrm* methylase, and a high degree of similarity was expected. In addition to the dimethylating enzyme present in *S. fradiae* (*tlrA*), a monomethylating enzyme (*tlrD*) which acts in an identical manner to *lrm* has also been described (Zalacain & Cundliffe; in press). The gene encoding this enzyme however was not sufficiently similar to *lrm* to be detected in this case.

In these studies *S.griseofuscus* and *S.spectabilis* were used as negative controls, however in both cases a DNA fragment showing homology with *lrm* was detected. Subsequently, *S.spectabilis* has been shown to possess resistance to MLS antibiotics, and the present results would suggest that resistance is due to a ribosomal modification system. The identification of an homologous sequence within *S.griseofuscus* remains a mystery.

b) The second resistance determinant.

Since the second resistance gene is found closely associated with *lrm*, Southern analysis was carried out using, as a probe, a fragment of GJE10 that encompasses the majority of this former gene, to determine if other strains possessing a rRNA methylating enzyme whose site of action is A-2058 have a similar resistance gene.

An 800 bp. (SstI→KpnI) fragment from pGJE2 was used as a template to allow synthesis of a radioactive probe, whilst genomic DNA preparations were subjected to agarose gel electrophoresis and transferred to the membrane. Having allowed the probe DNA to hybridize to membrane bound DNA overnight, the membrane was again subjected to a high stringency wash. The probe was found to hybridize to a single BamHI fragment in the genomes of *S.caelestis* and *S.ambofaciens* (Fig.4.14) Subsequent sequence analysis has shown that the 800 bp. fragment used as a probe contains only the second resistance gene, and therefore the positive signals observed were not due to C-terminal sequences of *lrm* being present as part of the probe. In considering the possibility that contaminating fragments of *lrm* may have been present in the probe can be discounted at least in the case of *S.caelestis*, since the DNA fragment showing similarity was of a different size from that showing similarity to *lrm* (Fig. 4.13). Evidently, genes similar in nucleotide sequence to the second resistance gene of *S.lividans* are present in at least two other *Streptomyces* strains which produce rRNA monomethylases. It would be of considerable interest to determine if these apparently similar genes are also physically associated with the rRNA monomethylase genes of *S.caelestis* and *S.ambofaciens* and whether they confer extra macrolide resistance in those strains. No similarity was detected between the probe and any sequences in *S.fradiae* or *S.erythraeus*.



**Fig. 4.14** Southern analysis of genomic DNA's from macrolide /lincosamide producers, using the second resistance gene as a probe. Various chromosomal DNA's were digested with BamHI and probed with the SstI/KpnI fragment of pGJE2. In each case 5 ug of DNA were loaded except that only 50 ng of pGJE2 digested with SstI were used.

## **CHAPTER 5**

### **NUCLEOTIDE ANALYSIS OF TWO RESISTANCE DETERMINANTS FROM *S.LIVIDANS***

### 5.1. Nucleotide sequence of two resistance genes from *S.lividans*.

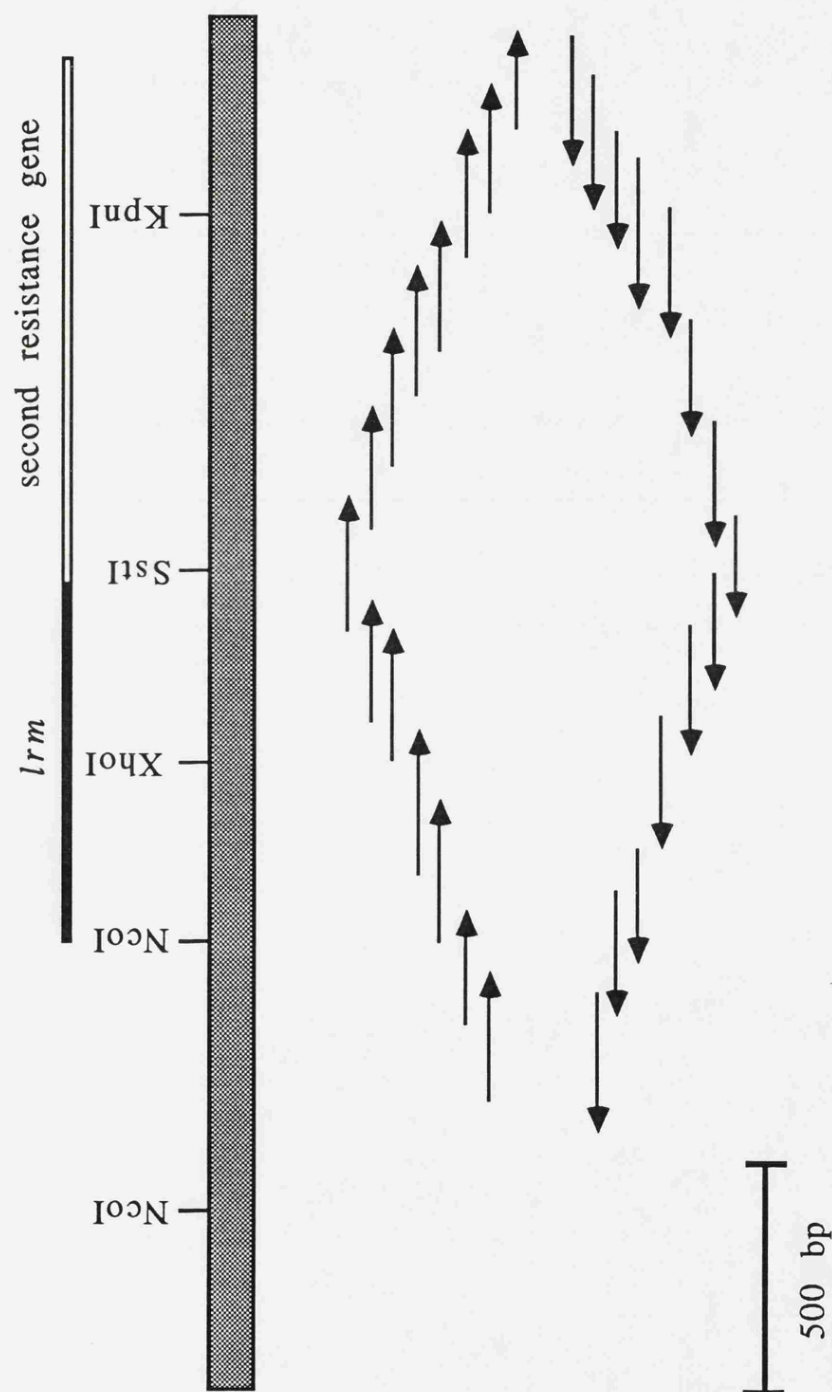
The sequence of the 3.0kb fragment from pGJE2 of *S.lividans* TK21 DNA embracing *lrm* and the second resistance gene was determined using the di-deoxy chain termination method of, Sanger *et al.*, 1977, using T7 polymerase according to the recommendations of the supplier as described in methods. Double stranded DNA was used to minimise the chance of sequencing erroneous subclones, and in addition fewer premature terminations tend to occur during the sequencing reactions when this method is employed.

Sub-clones of pGJE2 and pGJE20 were generated by a series of nested deletions in which the latter plasmids were digested with XbaI and PstI restriction endonucleases, thus generating linear plasmids with 5' and 3' overhanging ends, prior to incubation with ExoIII and S1 nucleases, by sequencing these sub-clones with universal and reverse primers overlapping sequence data was obtained. In addition a series of chemically synthesised 18 to 20 base oligonucleotide primers were also used to obtain overlapping sequence in regions where adequate deletions had not been obtained. To reduce gel compressions, which are particularly prevalent when sequencing *streptomyces* DNA due to its high G+C content, certain areas were resequenced using 7-deaza dGTP or 7-deaza dITP as a substitute for dGTP. The sequencing strategy employed is indicated in Fig 5.1. and the full nucleotide sequence is given in Fig. 5.2

On analysing the sequence data using the UWGCG programme CODON PREFERENCE (Gribskov, et al., 1984) ,two open reading frames were observed (Fig. 5.3) from nucleotide 640 to 1420 and 1420 to 2674 (Fig.5.2). The first open reading frame consisted of 260 codons that could code for a protein with a predicted molecular weight of 26KDa, whilst the second embraced 419

av. mol weight of 41.9 kDa





**Fig. 5.1** Partial restriction map of pGJ2 and corresponding sequencing strategy. The starting points and directions of sequencing are indicated by the arrows, whose lengths correspond to the number of nucleotides determined

CGTCTCCCGGTCTTTCGCGGGCGACGTGCGCGTCAGCGCGCCGTCTTTCGGGGCGCGGCACGGCACGGCC  
-----+-----+-----+-----+-----+-----+-----+ 70  
GCAGAGGGCCAGGAAGCGCCCGCTGCAGCGGCAGTTCGCGCGGCAGGAAGCCCCGCGCCGTGCCGTGCCGG

TCAGCCCGCGGCTGGCCGCAATCCAGGAGCTGCCCGAGGTGGACACCGCCGTTCGGCCTCGGCCGGGGCGT  
-----+-----+-----+-----+-----+-----+-----+ 140  
AGTCGGGCGCCGACCGGCGTTAGGTCTTCGACGGGCTCCACCTGTGGCGGCAGCCGAGCCGGCCCCGCA

CGCTGAAGTGGACGGCCAGGGAAGGGCGTTGACCGTACCCGACCGGCCGCCCTGAACCGCACCTTCGAC  
-----+-----+-----+-----+-----+-----+-----+ 210  
GCGACTTCACCTGCCGGTCCCTTCCCGCAACTGGCAGTGGCTGGGCCGGCGGGACTTGGCGTGGAAGCTG

CTGGGCGCCGTACGGCTCGCTGGACGACCTCGGCAGCCACGGCATCGCGATCGGCATGGACGTACTGCG  
-----+-----+-----+-----+-----+-----+-----+ 280  
GACCCGCGGCAGTGCCGAGCGACCTGCTGGAGCCGTGCTGCCGTAGCGCTAGCCGTACCTGCATGACGC

PvuII <sup>P2</sup> →

TCACGGCTGGAGGAGGACGGCAACCTCGTACTCGCCCGCTACCAGCGCTGACGGCCGCCCGCGGAAAAC  
-----+-----+-----+-----+-----+-----+-----+ 350  
AGTGCCGACCTCCTCCTGCCGTTGGAGCATGAGCGGGCGATGGTCGCGACTGCCGGCGGGCGCGCTTTTG

P1 →

CCGTTGCCCGCGGCCACGCGCCCGCTAACGTCCCTCCGTGGCAGCTTTCAACGCGATCTTCAACTCCG  
-----+-----+-----+-----+-----+-----+-----+ 420  
GGCAACGGGCGCCGGTGC CGGGCGGATTGCAGGGGAGGCACCGTTCGAAAGTTGCGCTAGAAGTTGAGGC

V A A F N A I F N S G

GTCTCGGCCGGGGCTGCACGTACTCCATCAGGATGCGTTCGTGGCCCCGGTGCCCTGACCGGCCCGGGGTC  
-----+-----+-----+-----+-----+-----+-----+ 490  
CAGAGCCGGCCCCGACGTGCATGAGGTAGTCTACGCAGCACCGGGGCCACGGGACTGGCCGGGGCCCCAG

L G R G C T Y S I R M R R G P G A L T G P G S

CCCCGCGCGCTGACGCACTGACGCGTCCGGCACGTCCGACGCGCGCGCGGGGGCTGAGCCTCCGCAT  
-----+-----+-----+-----+-----+-----+-----+ 560  
GGGGGCGGCGACTGCGTGAGCTGCGCAGGCCGTGCAGGCTGCGCGCGCGCGCCCCGACTCGGAGGCCTA

P R R \*

TCCCTTTTGCCTTCGTCTTCCGGTCAGGGCCTTCGGCTGCCCTTTTCCCCTCCTGTCACGGAAGACAAGG  
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 630  
AGGGAAAACGGAAGCAGAAGGCCAGTCCCGAAGCCGACGGGAAAAGGGGAGGACAGTGCCTTCTGTTC

NcoI  
 ACCGCAGGCCATGGCCCCGCCACCCAGCGTGCCCGCACGCTCTCGCAGAACTTCCTCGCCGACCGCGCC  
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 700  
 TGGCGTCCGGTACCGGGCGGGGTGGGTTCGCACGGGCGTGCGAGAGCGTCTTGAAGGAGCGGCTGGCGCGG

M A R P T Q R A R T L S Q N F L A D R A

ACCGCCGAACGTGTGGCGCGCCTCGCCGTCCCCGACCGGGGGCGCCCGCCCCTCCTGCTCGAAGTGGGGCG  
+-----+-----+-----+-----+-----+-----+ 770  
TGGCGGCTTGCACACCGCGCGGAGCGGCAGGGGCTGGCCCCGCGGGCGGGGAGGACGAGCTTCACCCGC  
T A E R V A R L A V P D R G R P P L L L E V G A

CGGGCAACGGCGCCCTCACCGAGCCGCTCGCCCCGCCGAGCCGCGAACTGCACGCCTACGAGATCGACCC  
-----+-----+-----+-----+-----+-----+-----+ 840  
GCCCGTTGCCGCGGGAGTGGCTCGGCCAGCGGGCGGCGTTCGGCGCTTGACGTGCGGATGCTCTAGCTGGG

G N G A L T E P L A R R S R E L H A Y E I D P

CCGGCTCGTCCCGGGCTCCGCGCCCGTTTCGCGCGCAGCCCCACGTCCACGTCTGTCGCCGGTGACTTC  
-----+-----+-----+-----+-----+-----+-----+-----+ 910  
GGCCGAGCAGGGGGCCGAGGCGCGGGCAAGCGCGCGTCTGGGGGTGCAGGTGCAGCAGCGGCCACTGAAG  
  
R L V P G L R A R F A R S P H V H V V A G D F

CTCACC GCCCGCCCCGCGCACCCCGTTCGCCGTCGCCGGGAACGTGCCCTTCTCGCGCACCGCCGACA  
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 980  
GAGTGGCGGGCCGGGGGCGCGTGGGGCAAGCGGCAGCGGCCCTTGCACGGGAAGAGCGCGTGGCGGCTGT  
L T A R P P R T P F A V A G N V P F S R T A D I

TCGTCGACTGGTGCCTGACCGCGCCCGGCCCTACCGACGCCACCCTCCTCACCCAGCTCGAGTACGCCCG  
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1050  
AGCAGCTGACCACGGACTGGCGCGGGCCGGAGTGGCTGCGGTGGGAGGAGTGGGTTCGAGCTCATGCGGGC  
V D W C L T A P G L T D A T L L T Q L E Y A R

CAAACGCACCGGCGACTACGGCCGCTGGACCCCTGCTCACGGTCTGACCTGGCCGCGCCACGAGTGGCGG  
-----+-----+-----+-----+-----+-----+-----+ 1120  
GTTTGCGTGGCCGCTGATGCCGGCGACCTGGGACGAGTGCCAGGACTGGACCGGCGCGGTGCTCACCGCC  
  
K R T G D Y G R W T L L T V L T W P R H E W R

CTGGTGGGCCGGGTGGGCCGCAGCCGCTTCTGCCCCGGCGCCGCGCTCGACGCCGGCATCCTCCGGATCG  
-----+-----+-----+-----+-----+-----+-----+ 1190  
GACCACCCGGCCCAACCGGCGTCTGGCGAAGACGGGCCGCGGCGCGCAGCTGCGGCCGTAGGAGGCCTAGC  
L V G R V G R S R F C P A P R V D A G I L R I E

AACGCCGCCCCACTGCGCTGCTCACCGGCGCCGCCGCCGCCGCGACTGGGCGGACCTGGTTCGAGCTGGG  
-----+-----+-----+-----+-----+-----+-----+ 1260  
TTGCGGCGGGGTGACGCGACGAGTGGCCGCGGCGGCGGGCGGCGCTGACCCGCCTGGACCAGCTCGACCC  
R R P T A L L T G A A A R R D W A D L V E L G

CTTCTCCGGCGTCTGGCGGCTCGCTGCACGCGTCCCTGCGCCGCGCCCACTCCCGGCGCCGGGTGGACGCG  
-----+-----+-----+-----+-----+-----+-----+ 1330  
GAAGAGGCCGCGAGCCGCCGAGCGACGTGCGCAGGGACGCGGCGCGGGTGAGGGCCGCGGCCACCTGCGC  
F S G V G G S L H A S L R R A H S R R R V D A

GCGTTCGCGCGCCGCGGGCTCGATCCCGGTGTCTCTGTCGGCGAGGTGCGCCCCGACCGGTGGCTCCGGC  
-----+-----+-----+-----+-----+-----+-----+ 1400  
CGCAAGGCGCGGCGCGCCGAGCTAGGGCCACAGGAGCAGCCGCTCCAGCGGGGGCTGGCCACCGAGGCCG  
A F R A A R L D P G V L V G E V A P D R W L R L

RBS  
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-----+-----+-----+-----+-----+-----+-----+ 1470  
GCGTGCTCCTCAACTGCCGTACTTCGCTTTTCTCAACGTGCTCTGCAGAGCAGAGCGTATGCCAGCAGCG  
H E E L T A \*  
M K R K E L H E T S R L A Y G R R

ATGACCACTCGCCCCGCGCACATCGCCATGTTCTCCATCGCCCTGCACGGCCACGTGAACCCCAGCCTGG  
-----+-----+-----+-----+-----+-----+-----+ 1540  
TACTGGTGAGCGGGGCGCGTGTAGCGGTACAAGAGGTAGCGGGACGTGCCGGTGCACCTTGGGGTTCGGACC  
M T T R P A H I A M F S I A L H G H V N P S L E

SstI SstII  
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V I R E L V A R G H R C T Y A I P R L L A D K

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V A E A G A E P K L W N S T L P G P D A D P E

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A W G S T L L D N V E P F L A D A I Q S L P Q L

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A Q A Y E G D E P D L V L H D I A S Y T A R V

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L G R R W E V P V I S L S P C M V A W E G Y E

SstII  
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Q E V G E P M W E E P R K T E R G Q A Y Y A R F

TCCACGCCTGGCTGGAGGAGAACGGGATCACCGACCACCCCGACCCGTTTCATCGGCCGCCCGACCGCTC  
-----+-----+-----+-----+-----+-----+-----+ 2030  
AGGTGCGCA^CCGACCTCCTCTTGCCCTAGTGGCTGGTGGGGCTGGGCAAGTAGCCGGCGGGGCTGGCGAG

H A W L E E N G I T D H P D P G I G R P D R S

CCTGGTGCTGATCCCCAAGGCGCTCCAGCCCCACGCCGACCGGGTGGACGAGACGACGTACACCTTCGTC  
-----+-----+-----+-----+-----+-----+-----+ 2100  
GGACCACGACTAGGGGTTCCGCGAGGTGCGGGTGCGGCTGGCCACCTGCTCTGCTGCATGTGGAAGCAG

L V L I P K A L Q P H A D R V D E T T Y T F V

GGCGCCTGCCAGGGGGACCGCACCGCCGAGGGCGACTGGGCCCCTCCCGAGGGCGCGGAGAAGGTCGTCC  
-----+-----+-----+-----+-----+-----+-----+ 2170  
CCGCGGACGGTCCCCCTGGCGTGCGGGTCCCGCTGACCCGGGCAGGGCTCCCGCGCCTCTTCCAGCAGG

G A C Q G D R T A E G D W A R P E G A E K V V L

TGGTCTCGCTCGGTTTCGGCCCTTACCAAGCAGCCCGCGTTCTACCGGGAGTGCGTCCGGGCCTTCGGTGA  
-----+-----+-----+-----+-----+-----+-----+ 2240  
ACCAGAGCGAGCCAAGCCGGAAGTGGTTCGTGCGGGCGCAAGATGGCCCTCACGCAGGCCCGGAAGCCACT

V S L G S A F T K Q P A F Y R E C V R A F G E

GCTGCCCCGCTGGGCACACCGTGCTCCAGGTCGGCCGGGCACGTAGACCCGGCCGAGCTGGGCGACGTACCG  
-----+-----+-----+-----+-----+-----+-----+  
CGACGGGGCGACCGTGTGGCACGAGGTCCAGCCGGCCGTGCATCTGGGCCGGCTCGACCCGCTGCATGGC 2310

L P G W H T V L Q V G R H V D P A E L G D V P

KpnI  
GACAACGTGGAAGTCCGCACGTGGGTACCGCAGTTGGCGATCCTCCAGCAGGCCGACCTGTTTCGTCACCC  
-----+-----+-----+-----+-----+-----+-----+ 2380  
CTGTTGCACCTTCAGGCGTGCACCCATGGCGTCAACCGCTAGGAGTCTGTCGGCTGGACAAGCAGTGGG

D N V E V R T W V P Q L A I L Q Q A D L F V T H

ACGCGGGCGCGGGCGGCAGCCAGGAGGGTCTGGCCACCGCCACGCCGATGATCGCCGTACCGCAGGCCGC  
-----+-----+-----+-----+-----+-----+-----+ 2450  
TGCGCCCCGCGCCCGCCGTCGGTCTCCAGACCGGTGGCGGTGCGGCTACTAGCGGCATGGCGTCCGGCG

A G A G G S Q E G L A T A T P M I A V P Q A A

GGACCAGTTCGGCAACGCCGACATGCTCCAGGGCCTCGGCGTCGCCCCGACCCCTCCCGACCGAGGAGGCC  
-----+-----+-----+-----+-----+-----+-----+ 2520  
CCTGGTCAAGCCGTTGCGGCTGTACGAGGTCCCGGAGCCGCAGCGGGCGTGGGAGGGCTGGCTCCTCCGG

D Q F G N A D M L Q G L G V A R T L P T E E A

ACCGCGAAGGCGCTGCGCACCGCCGCCCTCGCCCTGGTTCGACGACCCGGAGGTGGCGGGCGCGCCTGAAGG  
-----+-----+-----+-----+-----+-----+-----+ 2590  
TGGCGCTTCCGCGACGCGTGGCGGGCGGAGCGGGACCAGTGTCTGGGCCTCCACCGCCGCGCGGACTTCC

T A K A L R T A A L A L V D D P E V A A R L K E

AGATCCAGGCGCGGATGCCCCAGGAGGCCGGCACCCGCGGGCCCCGCCGACCTCATCGAGGCCGAACCTGGC  
-----+-----+-----+-----+-----+-----+-----+ 2660  
TCTAGGTCCGCGCCTACCGGGTCTCCGGCCGTGGGCGCCCCGGCGGCTCCAGTAGCTCCGGCTTGACCG

I Q A R M A Q E A G T R G P A D L I E A E L A

CGCCGCGCGCGGCTGAGCCCGGCACCGCACGCCGAGGGCCCGTGGCGTTCCCCCGGAACCGACGGGGCC  
-----+-----+-----+-----+-----+-----+-----+ 2730  
GCGGCGCGCGCCGACTCGGGCCGTGGCGTGGCGGTCCCGGGCACGCCAAGGGGGCCTTGGCTGCCCCGG

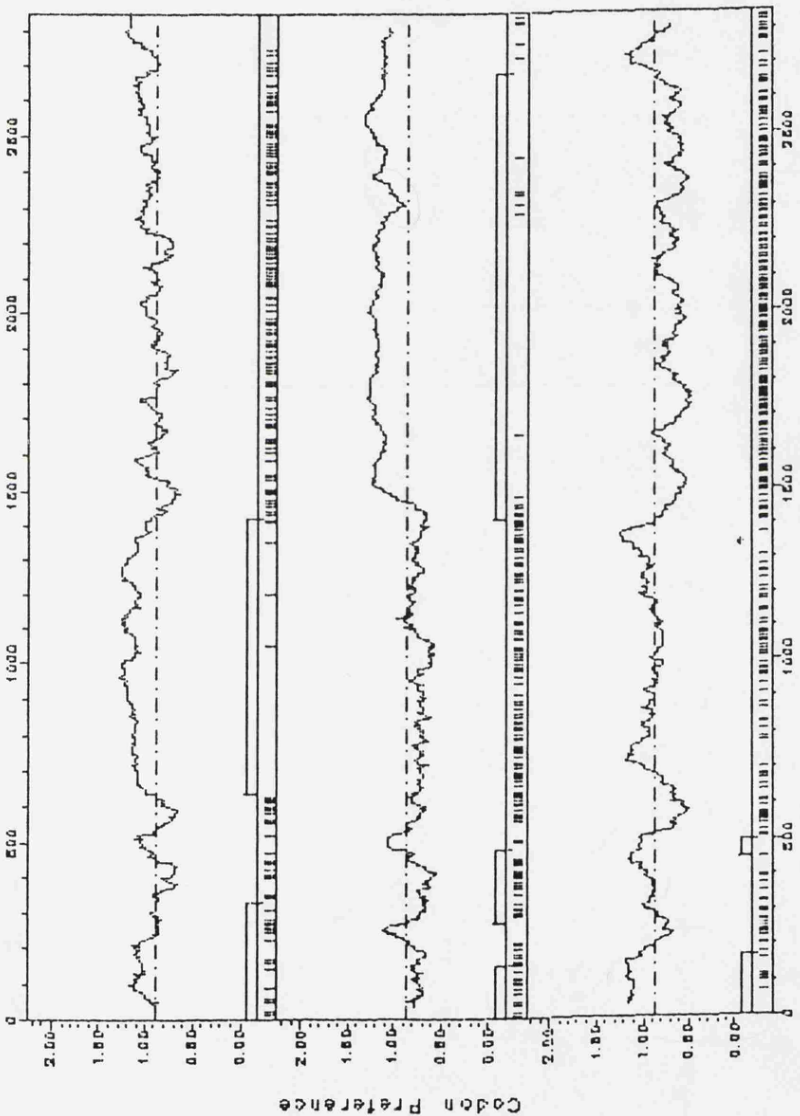
A A R G \*

TGCGGGCGTCTGCGACGGCGTGCCGGTCAGACCCCGACCCGTTGCCCCCGTCCCCGCGCTCGCCCTCG  
-----+-----+-----+-----+-----+-----+-----+ 2800  
ACGGCCGCAGCACGCTGCCGCACGGCCAGTCTGGGGCTGGGCAAGCGGGGCGAGGGGCGCGAGCGGGAGC

```
CCGCCCCGCTCCGGCGACGCGATCGCCTCGGGCGACTCGTCGTGGTCA
-----+-----+-----+-----+-----
GGCGGGGCGAGGCCGCTGCGCTAGCGGAGCCCGCTGAGCAGCACCAGT
```

FIG.5.2. Nucleotide sequence of DNA embracing 1rm and the second resistance gene together with their predicted amino acid sequence (below). The putative ribosome binding sites are underlined (RBS) and the transcription initiation sites for 1rm are indicated (●).

CODONPREFERENCE of: liv.rft Ck: 1084, 1 to 2649 28-SEP-1990 11:12  
 Table: streptomycetes.cod FileCk: 5886 Window: 25 Threshold: 0.10 Density: 93.5



codon frequency table  
 - compares codons to  
 those in frequency  
 programme

Fig 5.3. Analysis of the DNA sequence from *S.lividans* using the UWGCC programme  
 CODONPREFERENCE. The analysis used the default settings and a codon usage table based on a  
 number of *Streptomyces* gene sequences. Open reading frames are shown as boxes below their  
 respective reading frames. "Rare" codons are marked below the reading frame plot.



codons that could code for a predicted 42KDa protein. Both open reading frames exhibited the biased codon usage, normally associated with DNA from *Streptomyces* spp (Table 5.1 a&b) as illustrated using the UWGCG programme CODONFREQUENCY. However, this analysis revealed a small cluster of codons ending with A or T centred around position 2300 (Fig 5.3) within the second open reading frame , and as a result this area of the sequence was examined with particular care. The total G+C content of both coding sequences is 77% and 73% respectively and in addition the G+C content in each of the codon positions conforms with those from other actinomycetes. For the *lrn* coding sequence, the G+C content in positions 1,2 + 3 is 78%, 58%, and 93% respectively, similarly for the second gene the G+C content is 74%, 48%, and 94% for each of the three codon positions.

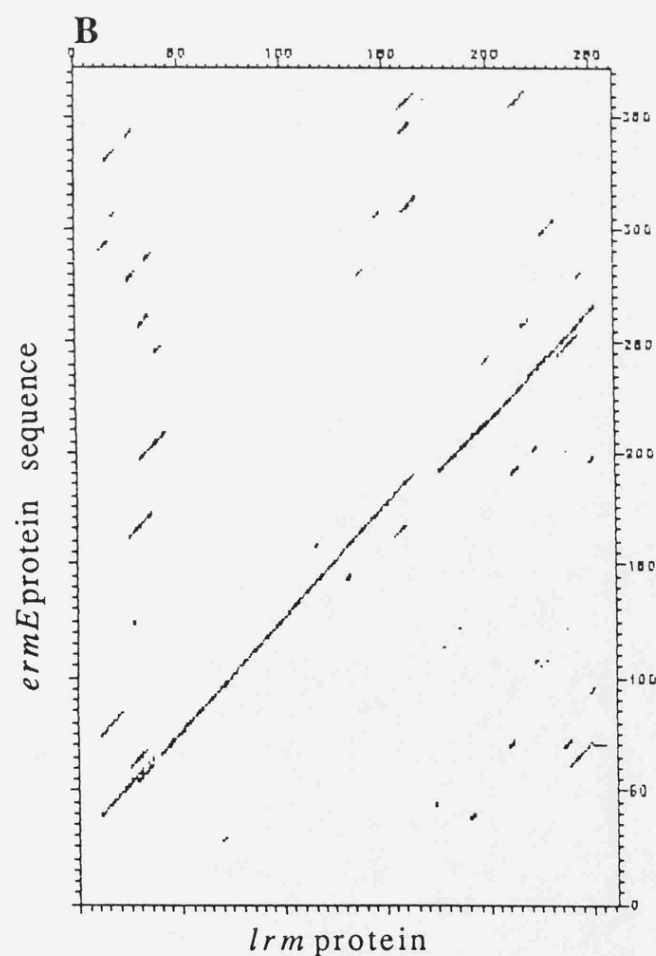
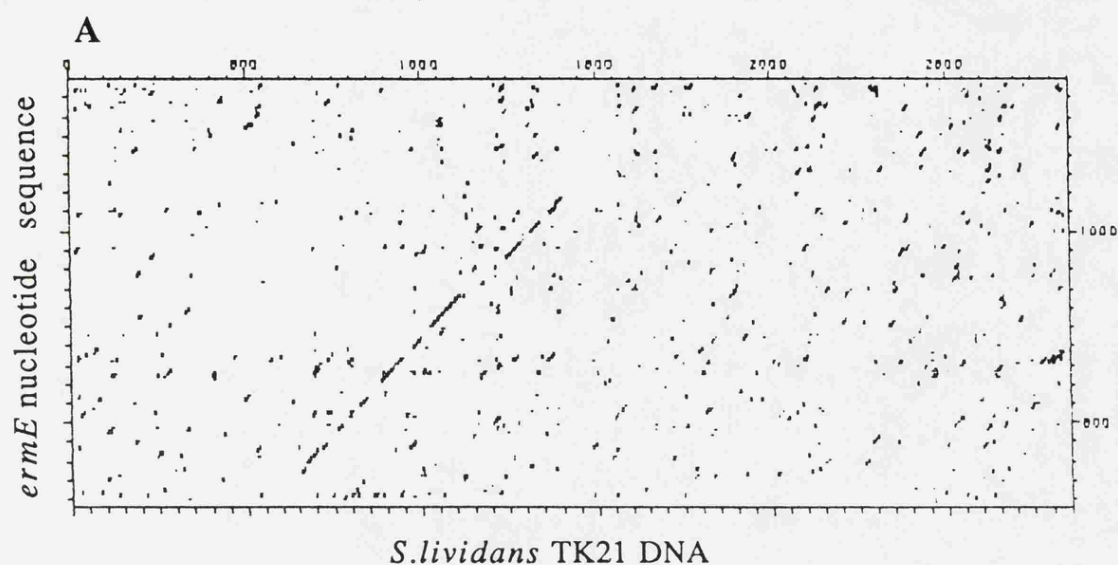
Previously, a significant degree of similarity had been demonstrated between *lrn* and other 23S rRNA methylases that act at the same site, by southern analysis (see section 4.10). However, to determine more accurately the degree of similarity at the nucleotide level, the nucleotide sequence of *lrn* was compared with that of *ermE* and *carB* from *Sacc. erythraea* and *S.thermotolerans* resepctively using the UWGCG programme COMPARE (Maizel Jr. and Lenk, 1981). This analysis revealed a high degree of similarity between the region of *S.lividans* DNA which encodes *lrn* and that of *Sacc. erythraea* which embraces *ermE* (Fig.5.4 A), indeed the similarity ranged from approximately nucleotide 650 to 1400 of the *S.lividans* DNA; an area identical to that covered by the first open reading frame (Fig. 5.3). A similar analysis was subsequently carried out of the predicted protein sequences, and the degree of similarity found to be extremely high (Fig.5.4 B) suggesting that these two genes are perhaps homologous. With the aid of such analysis the ATG at position 641 (Fig.5.2) was identified as being the most likely candidate for the translational initiation codon of *lrn*. In

Table 5.1 Codon usage by *lrm* (a).

| Amino acid |        | Amino acid |        | Amino acid |     | Amino acid |        | Amino acid |  |
|------------|--------|------------|--------|------------|-----|------------|--------|------------|--|
| codon      |        | codon      |        | codon      |     | codon      |        | codon      |  |
| Gly        | GGG 3  | Ser        | AGT 0  | TGC 2      | His | CAT 0      |        |            |  |
|            | GGA 0  |            | AGC 3  |            |     | CAC 7      |        |            |  |
|            | GGT 2  |            |        | TAT 0      | Tyr |            |        |            |  |
|            | GGC 15 | Lys        | AAG 0  | TAC 3      |     | Leu        | CTG 13 |            |  |
|            |        |            | AAA 1  |            |     |            | CTA 0  |            |  |
| Glu        | GAG 8  |            |        | TTG 1      | Leu |            | CTT 0  |            |  |
|            | GAA 4  | Asn        | AAT 0  | TTA 0      |     |            | CTC 19 |            |  |
|            |        |            | AAC 3  |            |     |            |        |            |  |
| Asp        | GAT 1  | Met        | ATG 1  | TTT 0      | Phe | Pro        | CCG 7  |            |  |
|            | GAC 13 |            |        | TTC 8      |     |            | CCA 0  |            |  |
|            |        |            |        |            |     |            | CCT 0  |            |  |
| Val        | GTG 6  | Ile        | ATA 0  | TCG 3      | Ser |            | CCC 12 |            |  |
|            | GTA 0  |            | ATT 0  | TCA 0      |     |            |        |            |  |
|            | GTT 0  |            | ATC 4  | TCT 0      |     | Term       | TGA 1  |            |  |
|            | GTC 14 |            |        | TCC 3      |     |            |        |            |  |
|            |        | Thr        | ACG 3  |            |     |            |        |            |  |
| Ala        | GCG 11 |            | ACA 0  | CGG 11     | Arg |            |        |            |  |
|            | GCA 1  |            | ACT 1  | CGA 0      |     |            |        |            |  |
|            | GCT 0  |            | ACC 14 | CGT 3      |     |            |        |            |  |
|            | GCC 24 |            |        | CGC 27     |     |            |        |            |  |
|            |        | Trp        | TGG 6  |            |     |            |        |            |  |
| Arg        | AGG 0  |            |        | CAG 3      | Gln |            |        |            |  |
|            | AGA 0  | Cys        | TGT 0  | CAA 0      |     |            |        |            |  |

Table 5.1 Codon usage by the second resistance gene (b).

| Amino acid |     | codon |  | Amino acid |     | codon |  | Amino acid |    | codon |     | Amino acid |    | codon |  |
|------------|-----|-------|--|------------|-----|-------|--|------------|----|-------|-----|------------|----|-------|--|
| Gly        | GGG | 8     |  | Ser        | AGT | 0     |  | TGC        | 3  | His   | CAT | 0          |    |       |  |
|            | GGA | 0     |  |            | AGC | 4     |  |            |    |       | CAC | 12         |    |       |  |
|            | GGT | 4     |  |            |     |       |  | TAT        | 0  | Tyr   |     |            |    |       |  |
|            | GGC | 20    |  | Lys        | AAG | 9     |  | TAC        | 9  |       | Leu | CTG        | 20 |       |  |
| Glu        | GAG | 33    |  |            | AAA | 1     |  |            |    |       | CTA | 0          |    |       |  |
|            | GAA | 3     |  | Asn        | AAT | 0     |  | TTG        | 2  | Leu   | CTT | 0          |    |       |  |
| Asp        | GAT | 0     |  |            | AAC | 6     |  | TTA        | 0  |       | CTC | 18         |    |       |  |
|            | GAC | 24    |  | Met        | ATG | 8     |  | TTT        | 0  | Phe   | CCG | 14         |    |       |  |
| Val        |     |       |  |            |     |       |  | TTC        | 10 |       | CCA | 0          |    |       |  |
|            | GTG | 10    |  | Ile        | ATA | 0     |  | TCG        | 4  | Ser   | CCC | 16         |    |       |  |
|            | GTA | 4     |  |            | ATT | 0     |  | TCA        | 0  |       | CCT | 0          |    |       |  |
|            | GTT | 0     |  |            | ATC | 14    |  | TCT        | 1  |       | TGA | 1          |    |       |  |
| Ala        | GTC | 17    |  |            |     |       |  | TCC        | 4  |       |     |            |    |       |  |
|            |     |       |  | Thr        | ACG | 6     |  |            |    |       |     |            |    |       |  |
|            | GCG | 21    |  |            | ACA | 1     |  | CGG        | 9  | Arg   |     |            |    |       |  |
|            | GCA | 1     |  |            | ACT | 1     |  | CGA        | 1  |       |     |            |    |       |  |
| Arg        | GCT | 0     |  |            | ACC | 16    |  | CGT        | 3  |       |     |            |    |       |  |
|            | GCC | 37    |  |            |     |       |  | CGC        | 17 |       |     |            |    |       |  |
|            |     |       |  | Trp        | TGG | 9     |  |            |    |       |     |            |    |       |  |
|            | AGG | 0     |  |            |     |       |  | CAG        | 18 | Gln   |     |            |    |       |  |
|            | AGA | 0     |  | Cys        | TGT | 0     |  | CAA        | 0  |       |     |            |    |       |  |



**Fig. 5.4 Comparison of *lrm* and *ermE* DNA sequences.**

**A.** Comparison of the *S.lividans* DNA sequenced and *ermE*  
**B** Comparison of the *lrm* and *ermE* products

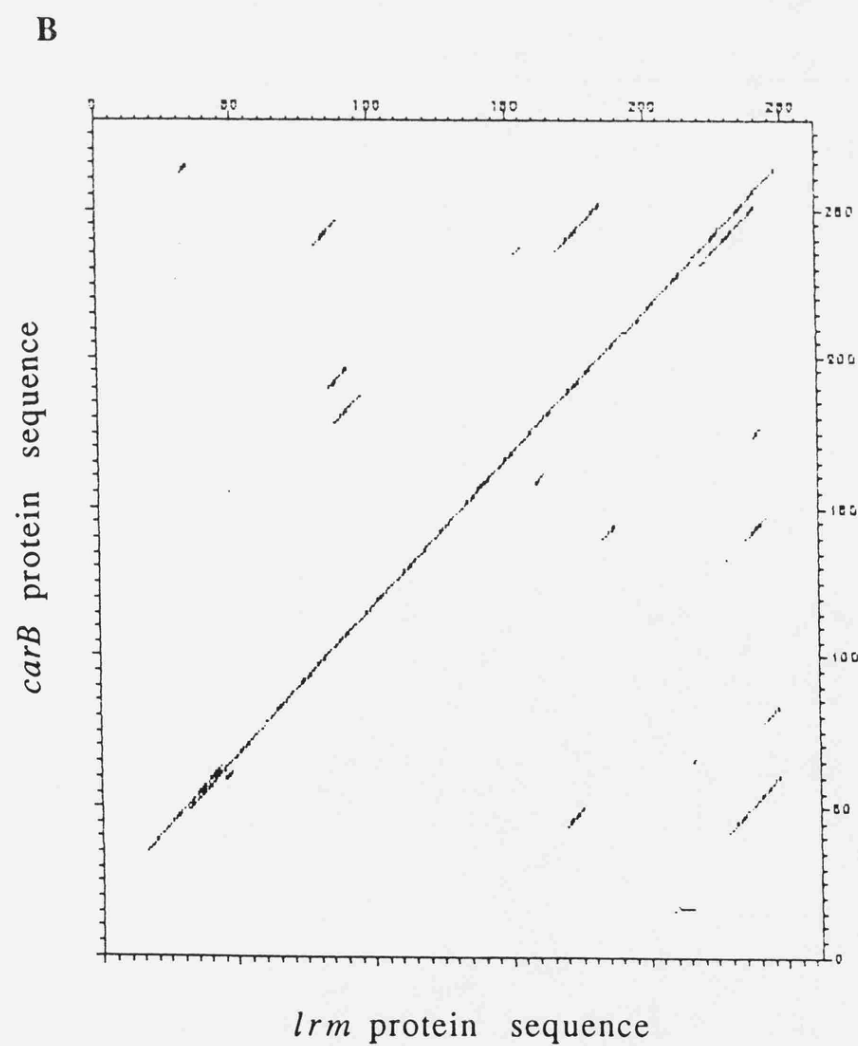
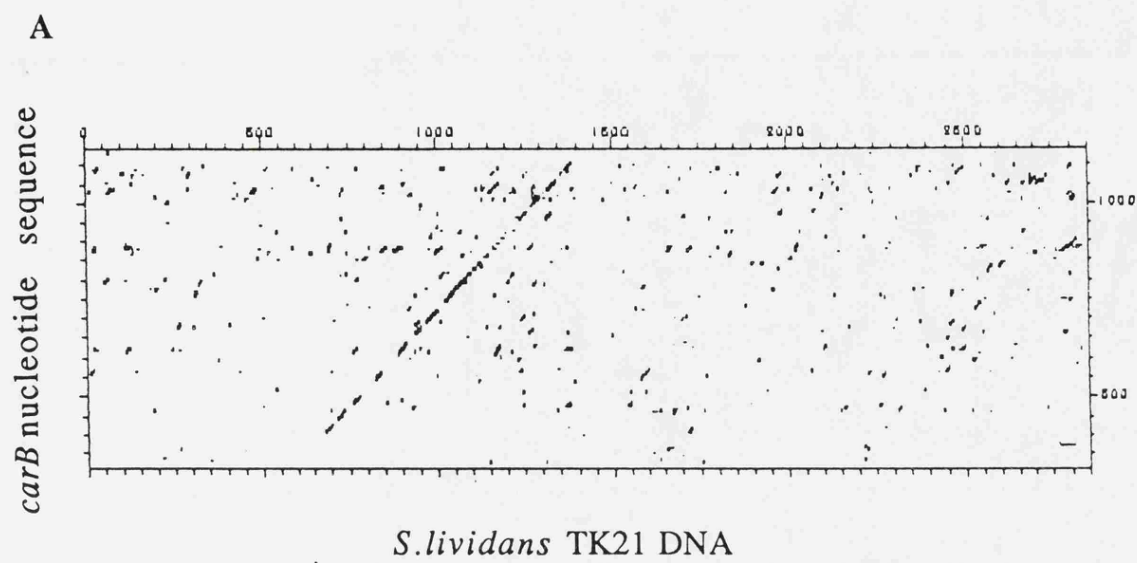
The comparisons were made using the UWGCG programmes COMPARE and DOTPLOT. The default settings were window size= 21 + stringency= 14 for **A.** and window size =30 + stringency= 8 for **B**

addition, the nucleotide and protein sequences of *lrm* were also compared to those of *carB*; the resistance gene isolated from *S. thermotolerans* (Fig.5.5). Whilst the degree of similarity between the two genes had not been identified during southern analysis, there appeared to be a stronger degree of similarity between these two resistance determinants than between *lrm* and *ermE*, this is perhaps to be expected given that *lrm* and *carB* both encode monomethylases whilst the product of *ermE* is a dimethylating enzyme.

Following the first open reading frame which embraces the *lrm* coding sequence, a frame shift occurs giving rise to a second open reading frame, presumably corresponding to the coding region of the second resistance gene. Three possible initiation codons exist for this second gene (see Fig. 5.2), but it would appear that the first ATG codon is the most likely candidate since it is the only possible initiation codon with a putative ribosome binding site occurring some 9-13 bp.s upstream. However the other possibilities cannot be discounted since ribosomes from *Streptomyces* do not demand a strong 'Shino-Delgarno' interaction, indeed a number of streptomycete genes have been described that exhibit no apparent ribosome binding site at all. In such cases transcription has frequently been shown to initiate at, or next to, the first base of the initiation codon (Janssen, *et al.*, 1985). This raises the intriguing question as to what the ribosome actually recognises in such cases.

The second gene would appear to encode a protein with a predicted size of approximately 42KDa, a size which would correspond to the product observed when plasmid pGJE10 was expressed *in vitro* in the coupled transcription-translation system (section 4.9). The precise function of this 40KDa protein is as yet unknown, but when various protein databases were searched, using the LIPMAN -PEARSON programme FASTP, significant similarity was obtained between the predicted amino acid sequences of this

**Fig. 5.5 Comparison of *lrn* and *carB* DNA sequences. A).** Comparison of the *S.lividans* DNA sequenced and *carB*. **B).** Comparison of the *lrn* and *carB* products. The comparisons were made using the UWGCG programmes COMPARE and DOTPLOT. The default settings were as indicated in Fig.5.4



**Fig. 5.5**

resistance determinant and a number of eukaryotic UDP- glucuronosyl transferases. Whilst the overall degree of similarity is not great ( Fig.5.6), several regions exist in which a substantial percentage of amino acids are identical. A region exists within the protein sequences of the UDP-glucuronosyl transferases, that clearly resembles the consensus sequence for nucleotide binding domains (Fig.5.7). Interestingly, the sequence of the resistance determinant from *S.lividans* also contains a similar region to that observed in the eukaryotic enzymes, though the degree of identity towards the consensus sequence is limited in this case. It has also been reported that the glucuronosyl transferases are glycoproteins (Iyanagi, et al., 1986), and in accordance with this, two potential glycosylation sites (Asn-X-Ser/Thr) have been identified in the predicted primary structure of the product of the second resistance gene. Possibly, the second resistance gene encodes an enzyme that catalyses the glucuronidation ( or addition of another sugar derivative) of macrolide antibiotics.

## 5.2. Transcript analysis of *lrm*.

The transcriptional start site of *lrm* was determined by high resolution S1 nuclease mapping, using the PstI-NcoI fragment of pGJE10 (Fig. 4.11) labelled at the NcoI site with [ $\gamma$ - $^{32}$ P]ATP as a probe, together with total RNA isolated from *S.lividans* GJ2. Following hybridisation of RNA and DNA and digestion of the hybrid with S1, the product was subjected to electrophoresis on a 7M urea/6% polyacrylamide gel along with suitable controls. A sample of the labelled probe was also treated according to the chemical sequencing method of Maxam & Gilbert and ran as a size marker. As a result two bands of smaller size than the intact probe were observed corresponding to 253 and 377 nucleotides in length (Fig. 5.8) and from this the transcript start sites were predicted along with possible -10 and -35 regions (Fig.5.2).



**Fig. 5.6. Optimal matched alignment of the deduced amino acid sequences of two eukaryotic glucuronosyl transferases and the second resistance gene.** The predicted amino acid sequence of the *S.lividans* protein (gene2) has been aligned alongside the predicted protein sequences of rat 4-nitrophenol UDP-glucuronosyl transferase (4NP-GT) and rat aldosterone UDP-glucuronosyl transferase (AD-GT) with identical (•) and conserved (+) amino acids indicated above and below the sequences respectively.

|        |   |               |     |
|--------|---|---------------|-----|
|        |   | •++ • ••• +   |     |
| 4NP-GT | M.....EIVEHLSEGRHDIIVL                        |               | 58  |
| Gene2  | M.....EVIRELVARGHRVTYA                        |               | 56  |
| AD-GT  | M.....IILDELVQRGHEVTVL                        |               | 26  |
|        |   | ++ ••• ••• •• |     |
|        | +• ••   |               |     |
| 4NP-GT | VPEVNLLL.....                                 |               | 293 |
| Gene2  | IP---RLL.....                                 |               | 244 |
| AD-GT  | KPSAYFFL.....                                 |               | 271 |
|        | +• •  |               |     |
|        | • •++ •••• + • + • • +• •••                   |               |     |
| 4NP-GT | GEHGIVVFSLGSMVSEIPEKKAMEIAEALGRIP--QTVL---W   |               | 331 |
| Gene2  | GAEKVVLVSLGSAFTKQPAFYRECV-RAFGELPGWHTVLQVGR   |               | 286 |
| AD-GT  | GEHGIVVFSLGSMVSNMTEEKANAIWALAQIP--QKVL---W    |               | 309 |
|        | • •++ •••• ++ + • +++• ••                     |               |     |
|        | + ++ • + ••• +• + •••••• •                    |               |     |
| 4NP-GT | RYTGTRPSNLAKNTILVKWLPQNDLLGHPKARAFITHSGSHGI   |               | 374 |
| Gene2  | HVDPAELGDVPDNVEVRTWVPQLAILQQA--DLFVTHAGAGGS   |               | 327 |
| AD-GT  | KFDGKTPATLGPITRVYKWLPQNDILGHPKTKAFVTHGGANGL   |               | 352 |
|        | • + + • ••• +• + •••••• •                     |               |     |
|        | ••+ + ••+ • +•• •• ++ • •• •• ••              |               |     |
| 4NP-GT | YEGICNGVPMVMPLFGDQMDNAKRMETRGAGV--TLNVLEMT    |               | 415 |
| Gene2  | QEGLATATPMIAVPQAADQFGNADMLQ--GLGVARTLPTEEAT   |               | 368 |
| AD-GT  | YEAIIYHGIPMIGIPLFGDQPDNIAHMAVAKGAAV--SLNIRTMS |               | 393 |
|        | ••+ + •••••• +•• • + • +• +• +                |               |     |
|        | • •••• + • + ••                               |               |     |
| 4NP-GT | ADDLENALKTVINNKSYPKENIMRLSSLHKDRPIE-----PLD   |               | 452 |
| Gene2  | AK----ALRTAALALVDDPEVAARLKEIQARMAQEAGTRGPAD   |               | 407 |
| AD-GT  | KLDFLSALEEVIDNPFYKKNVMLLSTIHHDQPMK-----PLD    |               | 430 |
|        | •• • + ••                                     |               |     |
|        | • +   |               |     |
| 4NP-GT | LAVFWVEYVMRHKGAPHLRPAAHDLTWYQYHSLDVIGFLLAIV   |               | 495 |
| Gene2  | LIEAELAAARG*                                  |               | 418 |
| AD-GT  | RAVFWIEFIMRHKGAKHLRPLGHNLPWYQYHSLDVIGFLLTCF   |               | 473 |
|        | +   |               |     |
| 4NP-GT | LTVVFIVYKSC-AYGCRKCFGGKGRVKKSHKSKTH*          |               | 529 |
| AD-GT  | AVIAALTVK-CLLFMYPF-F-----VKKEKKMKNE*          |               | 500 |

## Amino Acid Sequence

|                              |   |
|------------------------------|---|
| consensus                    | (R)K x x x V x G x G x x G x x x x x x x x x x x x x D        |
| AD-GT <sup>1</sup>           | K A F V T H G - G A N G L Y E A I Y H G I P M I G I P L F G D |
| 4MU-GT <sup>2</sup>          | K A F V A H G - G T N G I Y E A I Y H G I P I V G I P L F A D |
| 4NP-GT <sup>3</sup>          | R A F I T H S - G S H G I Y E G I C N G V P M V M P L F G D   |
| <i>S.lividans</i><br>protein | * * * * + * * * * * * * * * * * * * * * * *                   |
|                              | D L F V T H A - G A G G S Q E G L A T A T P M I A V P Q A A D |

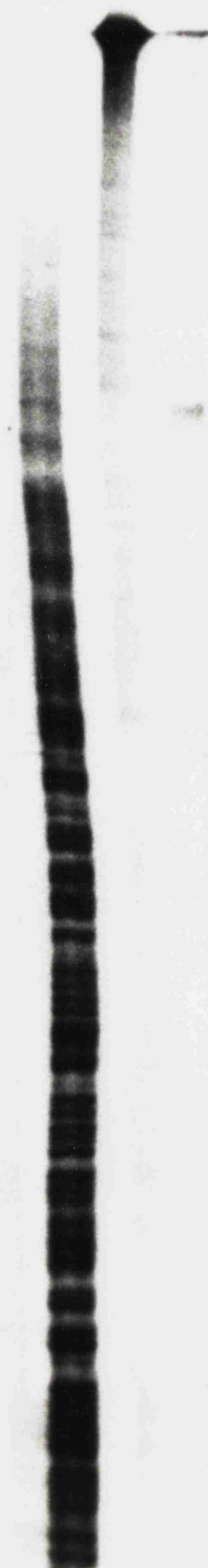
<sup>1</sup> aldosterone glucuronosyl transferase from rat  
<sup>2</sup> 4-methylumbelliferone glucuronosyl transferase from human  
<sup>3</sup> 4-nitrophenol glucuronosyl transferase from rat

**Fig. 5.8. Determination of the 5' end of the *lrn* transcript.**  
The Nco-Pst fragment of DNA from pGJE10 (See Fig. 4.11) labelled at the Nco I site was hybridized with RNA from *S.lividans* TK21 (Lane 3), and *S.albus* (Lane 2). Following digestion with S1 the final products were separated on a 6% urea-polyacrylamide gel together with the intact probe (Lane 1). In addition the labelled fragment was sequenced using the Maxam & Gilbert method and used as a size marker.

Fig.5.8

A+G 1 2 3

G  
 • C  
 \* C  
 • G  
 T  
 A  
 C  
 C  
 T  
 G  
 C  
 T  
 T  
 G  
 A  
 C  
  
 A  
 G  
 G  
 G  
 G  
 A  
 G  
 • G  
 \* C  
 • A  
 C  
 C  
 G  
 A  
 A  
 G



These promoter regions have been compared with the consensus sequence for *Streptomyces* promoters (Fig. 5.9). The two most highly conserved nucleotides of the "-10" prokaryotic consensus sequence, the T and the A at positions 6 and 2 respectively, are both found in the -10 region of the *lrn* promoter P1 along with the third most conserved residue; the T at position 1. However the -10 sequence of this second promoter P2 only contains one of these conserved residues, the T at position 6. Despite the fact that comparisons of this type are limited due to the wide variation of promoters known to exist in *Streptomyces* (see Table 1.1), it is possible that transcription from these two promoters is directed by different forms of RNA polymerase holoenzyme.

Currently, the only other example of an MLS resistance methylase gene transcribed from two promoters is the *ermE* gene of *Sacc.erythraea* (Fig.5.9b). Interestingly, recognition of *ermEP1* by RNA polymerase results in the initiation of transcripts at the first base of the predicted GTG translational start codon. Expression of *ermE* in this case therefore occurs in the absence of a conventional ribosome binding site. Similarly, it is proposed that transcription of *lrn* arising from *lrnP1* also initiates at the predicted translational start point of the leader peptide (see later).

### 5.3. Regulation of *lrn* by translational attenuation?

Prior to the coding sequence of *lrn*, a leader sequence of 251 or 375 nucleotides (depending on the transcriptional start point) exists, which contains a putative ribosome binding site at position 624 to 631 (Fig. 5.2). It would appear that this leader sequence is capable of forming a series of very stable hairpin loops and, in addition, an area exists between nucleotide 390 and 503 that could encode a short leader peptide of 38 amino acids (see

A.

|              |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
|--------------|---|---|---|---|---|---|---|----|----|---|---|---|---|---|---|---|
| <i>lrnP2</i> | T | G | G | A | C | G | - | 13 | bp | - | C | G | G | C | A | T |
|              |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
| consensus    | T | T | G | A | C | A | - | 18 | bp | - | T | A | G | G | A | T |
|              |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
| <i>lrnP1</i> | T | T | G | C | C | C | - | 18 | bp | - | T | A | A | C | G | T |

B.

|               |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
|---------------|---|---|---|---|---|---|---|----|----|---|---|---|---|---|---|---|
| <i>ermEP1</i> | T | G | G | A | C | A | - | 13 | bp | - | T | A | G | G | A | T |
|               |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
| <i>lrnP2</i>  | T | G | G | A | C | G | - | 13 | bp | - | C | G | G | C | A | T |

|               |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
|---------------|---|---|---|---|---|---|---|----|----|---|---|---|---|---|---|---|
| <i>ermEP2</i> | T | T | G | A | C | G | - | 18 | bp | - | G | A | G | G | A | T |
|               |   |   |   |   |   |   |   |    |    |   |   |   |   |   |   |   |
| <i>lrnP1</i>  | T | T | G | C | C | C | - | 18 | bp | - | T | A | A | C | G | T |

**Fig. 5.9** Nucleotide sequences of the promoters directing transcription of *lrnP*. The putative "-10" and "-35" regions of the two promoter sequences of *lrnP* are shown, and compared with the consensus sequence for *Streptomyces* promoters and those of *ermE*.

Fig.5.2). These observations are consistent with the hypothesis that translational attenuation may be responsible for regulating induction of *lrm*, and accordingly two different conformations were postulated for the 5' end of the *lrm* transcript (Fig. 5.10).

Prior to induction, the *lrm* transcript is proposed to adopt the conformation shown in Fig. 5.10A, where base pairing occurs between stems 1 + 2 and 5 + 6. By analogy with the *ermC* leader sequence it is predicted that this conformation would be 'inactive', since the ribosome binding site preceeding the methylase coding sequence is occluded by base pairing between loops 5 + 6. However, since stem 2 is also complementary in sequence to stem 5, the mRNA is also capable of folding into a further 'active' conformation (Fig. 5.10B). It is therefore proposed that in the absence of induction the mRNA exists in its 'inactive' form, such that translation of the methylase gene is unlikely. However, if a ribosome stalls during attempted translation of the leader peptide, due to the presence of an inducing antibiotic such as erythromycin, base pairing between stems 1 + 2 would be prevented. This would simultaneously release stem 2 allowing it to base pair with stem 5, thereby freeing the ribosome binding site allowing for the initiation of translation and expression of the methylase gene.

The predicted model could be tested directly by generating a number of mutants which contained various deletions within the leader peptide sequence. A mutant which lacked stem 5 would be expected to express MLS resistance constitutively, since the ribosome binding site preceeding the methylase coding sequence could no longer be sequestered. In addition, mutants possessing a leader sequence in which stem 2 had been deleted, would be expected to exhibit a sensitive phenotype both in the presence and absence of induction, since base pairing between stems 5 + 6 would, in this



A. Inactive conformation SD-2 is sequestered by the association of stems 4 and 5, preventing the attachment of ribosomes and methylases synthesis

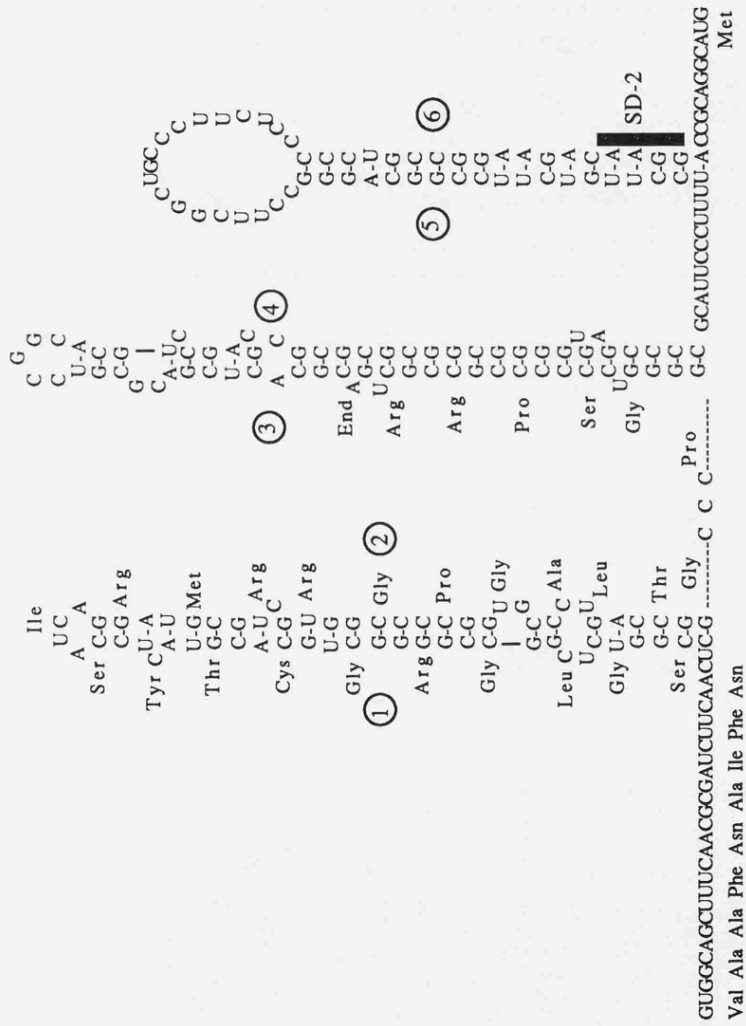


Fig. 5.10 Alternative conformations for *lrm* mRNA. It has been proposed that the *lrm* transcript is capable of folding into two different conformations, an inactive conformation (A) and an inactive conformation (B)



case, not be prevented by pairing between stems 2 + 5. The ribosome binding site would therefore be permanently occluded.

The detection of putative transcripts initiating immediately upstream of the second resistance gene has not been possible due to the absence of convenient restriction sites needed for high resolution S1 mapping. It therefore remains a possibility that both resistance genes are co-transcribed, though this has yet to be demonstrated. If true, it would provide an explanation as to why no resistance phenotype was observed when the second resistance gene was expressed in the absence of *lrn* (i.e. when *lrn* was inactivated as in pGJ15 ; see section 4.5). Examination of the nucleotide sequence indicates that a shift in the reading frame, caused by the inactivation of *lrn*, would have resulted in a translational fusion, since a TGA stop codon would no longer exist in frame between the filled in XhoI site and the proposed translational start point of the second gene. Presumably the resulting fusion protein would be incapable of conferring resistance.

#### 5.4. Analysis of the leader sequence of *lrn* from *S.lividans* MNT13.

The entire sequence of *lrn* from *S.lividans* MNT13 was not determined, since any alteration in DNA sequence resulting in constitutive expression of the resistance phenotype was expected to have occurred within the leader sequence. For this reason, only the nucleotide sequence upstream of the *lrn* coding sequence was determined. To aid this analysis plasmid pGJE10 was digested with NcoI and EcoRI, treated with large fragment polymeraseI to generate blunt ended fragments, and religated to generate pGJE107 following transformation of *E.coli*. Both reverse primer and a chemically synthesised 18 bp oligonucleotide that primed some 4 bp upstream of the BglII site in pIJ702 were then used to generate the required sequence from this plasmid.

Surprisingly, this analysis revealed that the only difference between the two leader sequences was a base change at position 594 (Fig. 5.2) from cytosine in the wild type sequence to adenine. Single base changes in mRNA that result in constitutive expression of the resistance phenotype have previously been detected in the case of *ermC* from *Staph. aureus*. These mutations have been described as having a destabilising effect on the so called 'inactive' mRNA conformation, thus favouring the 'active' form, which in turn results in constitutive methylase production. If the model proposed for translational attenuation of *lrm* is correct then the base at position 594 described above is not involved in base pairing in the inactive conformation, a destabilising effect on mRNA conformation could therefore not account for the constitutive expression of *lrm* observed in strain MNT13. It was noted that the C→A transition within the leader sequence resulted in the generation of a translational stop codon-TAG, but no significance was attributed to this event since a TGA stop codon appears in frame some 36 bp upstream of it and no translational start signal exists between these two putative 'stop' signals.

As yet the reason for the apparently constitutively expressed resistance phenotype in *S.lividans* MNT13 is undetermined. However, two points to be noted include the fact that when isolated from *S.lividans* MNT13, *lrm* was cloned onto a multi-copy plasmid in the absence of the promoter *lrmP2*. The two respective promoter regions from both the wild type and mutant strains could therefore not be compared. The possibility exists that this promoter region has been altered in some way in the mutant strain, which directly or indirectly affects expression of *lrm*, therefore indicating the existence of transcriptional regulation in addition to control at the translational level.

In addition the C→A transition in the mutated DNA results in the sequence TAGGCT, a region almost identical to the canonical sequence described for the -10 region of *Streptomyces* promoters. Indeed, the adenine residue at position 2 has also been described as being one of the most highly conserved residues within the prokaryotic -10 consensus sequence. Possibly in the wild type strain, *S.lividans* TK21, the corresponding region only acts as a weak promoter or is not recognised at all by RNA polymerase. However when this sequence is altered to resemble more closely the typical prokaryotic -10 sequence, as in strain MNT13, transcription from the corresponding promoter may be enhanced and since this transcript would not include the regulating leader sequence constitutive expression of *lrm* would result. Interestingly, a region similar in sequence (TTGCCT) to the -35 consensus sequence proposed for *Streptomyces* (TTGACA) also exists some 18 bp upstream from the observed mutation.

The substituted base described above occurs in a region very close to the NcoI site of pGJE10 used in the generation of the labelled probe for high resolution S1 mapping (see Section 5.2), transcripts initiating in this region would therefore not have been detected in the experiments described here. Possibly further S1 mapping using a different probe may provide some answers.

It is clear that if the idea of translational attenuation as a regulatory mechanism for induction of *lrm* is to be supported or otherwise, further constitutive mutants need to be isolated and the leader sequence preceeding *lrm* in these strains examined. Alternatively, given that *lrm* has already been isolated from *S.lividans* TK21, site directed mutagenesis can be used to alter the leader sequence in a specific manner.

## **CHAPTER 6**

### **DISCUSSION**

## 6.1. Structural Relationships between ribosomal RNA methylases which confer MLS resistance.

The existence of rRNA methylases which confer MLS resistance, presumably by modifying A-2058 in 23S rRNA, is now known to be wide spread among Gram positive organisms. Studies of *in vitro* methylation of 23S rRNA and of *in vivo* expression of the MLS resistance phenotype after transferring various *erm* genes into *B.subtilis* or *E.coli*, has demonstrated that rRNA from a variety of origins can be substrates for the same methylase. The possibility exists that there is an evolutionary relationship between such rRNA methylases which involves divergence from a common ancestor.

The predicted amino acid sequences of four rRNA methylase genes, including *ermC* of *Staph.aureus*, *ermE* of *Sacc.erythraea*, *tlrA* of *S.fradiae*, and *carB* of *S.thermotolerans* have been aligned along with that of *lrm* (Fig 6.1). As expected, the alignment revealed that several clusters of similarity are scattered throughout the amino acid sequences, indicating that these rRNA methylases are closely related at the protein level. However, it is clear that the methylating enzymes from *Streptomyces* spp. show a far greater degree of similarity to each other than to the dimethylase of *Staph. aureus* (the *ermC* product). In addition the protein sequence of *lrm* is seen to conform to these patterns of homology, and in particular the region between residues 40 and 54 of the *lrm* protein (Fig.6.1) appears to correspond to a possible nucleotide binding motif which is highly conserved in proteins that bind nucleotides such as NADH, ATP, or S-adenosyl methionine (Fig. 6.2). If this region does correspond to the S-adenosyl methionine binding domain such an area would be expected to occur in rRNA methylases with differing functions, and indeed a similar domain exists in the *kgmA* methylase of *S.tenebrarius* and the *ksgA* methylase of *E.coli*, which modify 16S and 23S

|      |   |    |
|------|---|----|
| ermE | VSSSDEQPRPRRRNQDRQHPNQ-----                 | 23 |
| tlrA | VARAPRSPHPARSRETSRAHPPYGTRADRAPGRGR-DRDRSPD | 42 |
| carB | MAALLKRILRRRMAEKR-----SGRGRMAAARTTG         | 30 |
| lrn  | MARPT-----                                  | 5  |
| ermC | MNEKNIK-----                                | 7  |

|      |  |    |
|------|--|----|
| ermE | -----RPVLGRTERDNRNRQFGQNFIRDRKTIARIAETAELR   | 60 |
| tlrA | SPNGTSSRDGGRSP-DRARRELSQNFIAARRAVAERVARLVRPA | 84 |
| carB | AQSRKTAQSRSGRSEADRRRRVHQQNFIVDRETVQRFVRFADPD | 73 |
| lrn  | -----QRAE-TLSQNFIAADRATAERVARLAVPD           | 32 |
| ermC | -----HSQNFITSKHNDKIMTNIRLN                   | 29 |

|      |   |     |
|------|---|-----|
| ermE | PDL--PVLEAGPGEGLTRELADRARQVTSYEIDPRLAKSLRE  | 101 |
| tlrA | PGG--LLELVGAGRGVLTALAPYCGRLVAHEIDPRLPALRD   | 125 |
| carB | PGE--VVLEVGAGRGVLTRELARLCRRVVAYEIDRHFADRLRE | 114 |
| lrn  | RGRPPLLELVGAGNGALTEFLARRSRELHAYEIDPRLVPLRA  | 75  |
| ermC | EHDNI--FEIGSGKGHFTLELVKRCNFVTAIEIDHKLCKTTEN | 70  |

|      |   |     |
|------|---|-----|
| ermE | KLSGHPNIEVVNA--DFITAEPEPEFAFV-GAIPYGITSAIV  | 141 |
| tlrA | RFGGPHHAHVRISSGDFIAAPVPEPFALA-GNIPYSRTAGIV  | 167 |
| carB | ATAEDPRIEVVAG--DFIKTSQPKVPFSVV-GNIPFGNTADIV | 154 |
| lrn  | RFARSPHVHVAG--DFITARPEPTEFAVA-GNVEFSRTADIV  | 115 |
| ermC | KLVDHDNFQVLNK--DILQFKFPKNQSYKIYGDIPYNISTDII | 111 |

|      |  |     |
|------|--|-----|
| ermE | DWCLEAPTETATMTVLTQLEFARKRTGDYGRWSRLTVMTWPLFE | 184 |
| tlrA | DWALRARTLTSATFVTQLEYARKRTGDYGRWSLLTVRTWPRHE  | 210 |
| carB | DWCLNARRLRRTTLVTQLEYARKRTGGYRWSRLTVATWPEVE   | 197 |
| lrn  | DWCLTAPGLTDATLLTQLEYARKRTGDYGRWTLLTVLTWPRHE  | 158 |
| ermC | RKIVFDSIANEIIYLIVEYGFA-KRLNNTKRSLLALLMAE--VD | 151 |

|      |   |     |
|------|---|-----|
| ermE | WEFVEK-----VDSAIMRL-RRRAEPLLEGAALE-RY       | 214 |
| tlrA | WRLGRVSRREFRPVPRVDSGIILRIERRER-PLPSAALG-DY  | 251 |
| carB | WRMGERISRWRFPVPAVDSAVIRLERFPV-PLIPPGLMH-DF  | 238 |
| lrn  | WRLVGRVGRSRFCPAPRVDAIILRIERRPT-ALLTGAAARRDW | 199 |
| ermC | ISILSMVPREYFHPKPKVNSSLIRL-SRKKSRI SHKDKQKYN | 192 |



|      |   |     |
|------|---|-----|
| ermE | ESMVELC-----FTGVMGGNIQASLIIRKYPRRRVEAA-FDHA | 249 |
| trlA | HRMVELG-----FSGVGGSLYASLIRAHHRAGPLDAA-FRAA  | 286 |
| carB | RDIVETG-----FTGKGGSLDASLIRRRFPARRV-AAGFRAA  | 273 |
| lrn  | ADIVELG-----FSGVGGSLHASLIRAHSRRRVDAF-FRAA   | 234 |
| ermC | FVMKVVNKEYKKIFTKNQ--FNNSLKHAGIDDLNNIS-F---  | 228 |

|      |   |     |
|------|---|-----|
| ermE | GVGGGAVVAYVFPPEQWLRVFERLDQKNEPRGGQPQRGRRTGG | 291 |
| tlrA | RLDRSVVAYVTPPEQWLTVFRTLRPVRSRPAGR *         | 319 |
| carB | RLEQGVVAYVTPGQWITLFEELHGR *                 | 299 |
| lrn  | RLDPGVIVGEVAPDRWLRRLHEELTA *                | 259 |
| ermC | -----EQFLSLFNSYKLFNK *                      | 243 |

|      |  |     |
|------|--|-----|
| ermE | RDHGDRRTGGQDRGDRRTGGRDHRDRQASGHGDRRSSGRNRD | 333 |
|------|--|-----|

|      |   |     |
|------|---|-----|
| ermE | DGRTGEREQGDQGGRRGPSGGGRTGGRPGRRGGPGQR * | 370 |
|------|---|-----|

**Fig. 6.1 Comparison of rRNA methylases conferring MLS resistance.** The amino acid sequences were deduced from the nucleotide sequences of the genes. The gaps were introduced to ensure optimal similarity between the five sequences. Identical amino acids present in all five sequences are marked with a star, whilst identical amino acids present in the four streptomycete sequences are boxed.

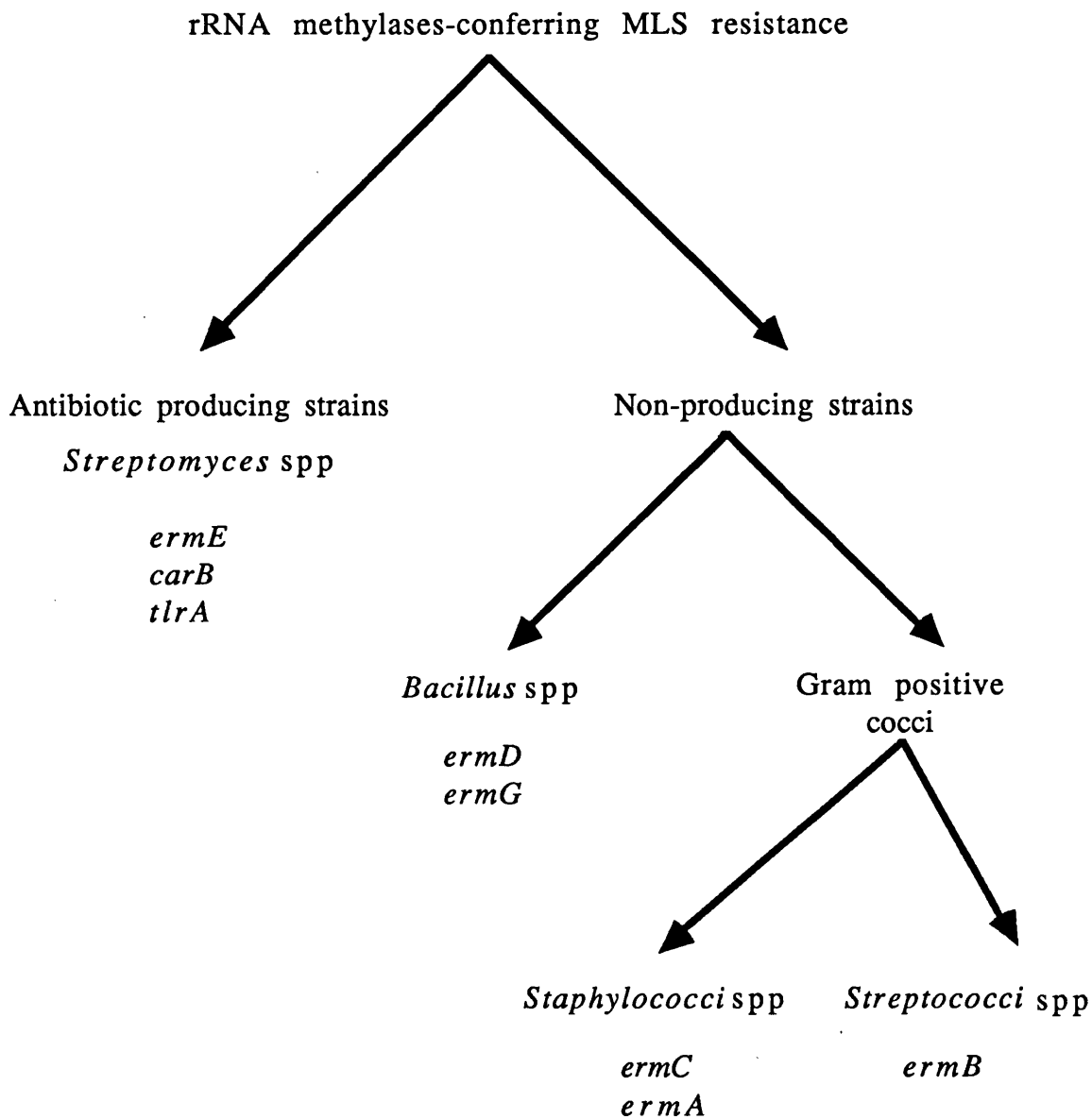
| Gene        | Amino acid sequence <sup>a</sup> | Location |
|-------------|----------------------------------|----------|
| <i>ermE</i> | E A G P G E G L L T R E L A D    | 67-81    |
| <i>tlrA</i> | E V G A G R G V L T E A L A P    | 91-105   |
| <i>carB</i> | E V G A G N G A I T R E L A R    | 80-94    |
| <i>lrm</i>  | E V G A G N G A L T E P L A R    | 41-55    |
| <i>ermC</i> | E I G S G K G H F T L E L V K    | 36-50    |
| <i>ksgA</i> | E I G P G L A A L T E P V G E    |          |
| <i>kgmA</i> | E A G R G Q G W D L I D A I R    |          |

<sup>a</sup>The consensus sequence for a nucleotide binding region is K(R)xxxVxGxGxxGxxxxxxxxxxxxxxxxxxxxD(E).

**Fig. 6.2. Comparison of amino acid sequences around the possible SAM binding region, of the deduced products of bacterial MLS resistance genes. The strongly conserved residues are boxed, and their position relative to the N-terminus of each protein is indicated.**

rRNA respectively (Fig. 6.2). A similar region was not however detected in the *kamA* methylase of *S.tenebrarius*. Two further highly conserved regions exist between the methylases, at residues 13 to 16, and 131 to 142 of *lrn*, presumably one of these areas is involved in recognition of the region surrounding A-2058 in 23S rRNA. Interestingly, the tyrosine residue (Y) at position 134 in the amino acid sequence of the *ermE* protein is highly conserved among other dimethylases conferring MLS resistance. However in the sequences of monomethylating enzymes this tyrosine residue is replaced by another hydrophobic amino acid, phenylalanine. Perhaps the amino acid residue at this site is particularly important in determining whether the enzymes act as monomethylases or dimethylases.

In order to investigate the possible evolutionary relationships between the rRNA methylases, further sequence comparisons were performed, and the analysis indicated that the rRNA methylase genes from producing strains can be clearly differentiated from those of non-producers (Fig.6.3). Whilst the protein sequences of the *Streptomyces* methylases generally show 50-60% identity, each of the sequences only has 20-25% similarity to those of non-producing strains. A further divergence also occurs in this latter group between *Bacillus* spp. and the Gram positive cocci, and finally between *Streptococci* spp. and *Staphylococci* spp. It would therefore appear that the rRNA methylases of producing and non-producing strains have evolved along different biological pathways, and whilst it is evident that these resistance genes belong intrinsically to the genomes of antibiotic producers, the origin of such genes in non-producing strains is unknown. It has been proposed that these MLS resistance methylases were in fact derived from a common ancestor and that the antibiotic producing strains were the original source of antibiotic resistance determinants (Benveniste and Davies, 1973). Following their transfer to other bacterial species the resistance genes may then have



**Fig. 6.3 Relationship between rRNA methylases conferring MLS resistance.** The rRNA methylase genes from non-producing strains can be clearly differentiated from those of producing strains, indicating the existence of different evolutionary pathways.

followed divergent evolutionary pathways in different hosts accounting for the present diversity of rRNA methylase sequences. Indeed, the methylase genes isolated from non-producing organisms have all been plasmid encoded, whilst those of producing organisms are located within the chromosome. Alternatively, the resistance determinants may have been derived from pre-existing methylase genes which are involved in cellular functions other than antibiotic resistance. Such convergent evolution may account for the presence of rRNA methylases in non-producing strains, but given the close association of antibiotic resistance genes with their respective biosynthetic pathways in producing organisms, it would seem unlikely in this case that they have arisen fortuitously from a pre-existing cellular control gene.

One further significant observation is that the G+C content of the rRNA methylase genes is consistent with the average base composition of the bacterial chromosome from which they were isolated. Hence, if these genes were derived from a common ancestor, divergent evolution must have partly involved adaptation of the gene to its new host which in turn suggests that any transfer of genetic information must have occurred a long time ago, and that the existence of these genes in certain non-producers is ancient. One strain of *E.coli* however, has recently been found to possess a rRNA methylase gene with >98% homology at the DNA level to *ermB* of *Strep.sanguis*. Since its codon usage still reflects a strong preference for A+T rich codons, characteristic of genes from Gram positive cocci, the gene must only have recently been acquired (Trieu-Cuot, et al., 1987).

*Ref*

## 6.2. Translational attenuation and induction specificity.

Translational attenuation control, based on the transcriptional attenuators that regulate amino acid biosynthesis (Kolter and Yanofsky,

1982), has now been described for a number of inducible MLS resistance determinants. However these determinants do not all demonstrate the same specificity of induction, it appears that different subsets of MLS antibiotics are capable of inducing the individual systems, which poses the intriguing question as to what determines the relative efficiency with which different MLS antibiotics induce resistance. Given the correlation between amino acid sequences of leader peptides associated with the regulation of amino acid biosynthesis and the operons that they regulate it is possible that a correlation may also exist between leader peptide sequences of MLS resistance genes and the induction specificity. Certainly differences in ribosomes between various species or other cellular differences, such as unequal permeability to different MLS antibiotics do not play a part, since identical patterns of induction are observed for these genes irrespective of the host organism.

The contribution of specific leader peptide amino acid residues within the leader peptides, to induction of *ermC*, has been studied in some detail (Mayford and Weisblum, 1989). Using a model system in which the *ermC* methylase was translationally fused to *E.coli*  $\beta$ -galactosidase as indicator gene, codons of the *ermC* leader peptide were altered systematically by replacement of leader DNA segments with double stranded DNA constructed from chemically synthesised oligonucleotides. Analysis of the leader peptide mutations (Fig. 6.4) revealed that missense mutations that reduced the efficiency of induction by erythromycin involved the codons for amino acid residues 5 to 9, mutations affecting the amino acid sequence upstream of residue 5 and downstream of residue 9 did not affect inducibility. In addition, if STOP codons were introduced at Ser 10 and Val 12, the resulting mutants remained inducible, though the basal level of  $\beta$ -galactosidase was



substantially higher than in the control strain. These findings suggested that the codons for residues 5 to 9 of the leader peptide comprise the critical region in which ribosomes stall in the presence of erythromycin.

Interestingly, when the other three synonymous valine codons were introduced at valine 8, the inducibilities of the resulting mutants were indistinguishable from each other and from the control strain. Since at least two different tRNA's are needed for the translation of four valine codons it would appear that erythromycin is more sensitive to the amino acid side chain than to the tRNA. In addition the sequence changes in codons Phe 7 and Val 8 which did not alter inducibility, encode structurally similar amino acids. Of the codon changes at Val 8 only the substitution of Ile had no effect, and it may therefore be significant that isoleucine and valine have a methyl group on the  $\beta$ -carbon atom. Perhaps this is a feature required for ribosome stall and again indicates that it is the amino acid rather than the tRNA which is the critical element in determining induction specificity.

The proposed inactive conformations of both *ermC* and *lrm* transcripts are indicated in Fig. 6.5 along with the predicted leader peptide sequences. It can be seen from this comparison that the number of amino acid residues prior to the first folded stem (1+2) of *lrm* is similar to that of *ermC* (i.e 10 and 11 residues respectively). Perhaps of greater significance, given the similarity in induction specificity between the two genes, is that of the amino acid substitutions in the *ermC* leader which reduced inducibility by erythromycin, none appear in the corresponding position of the *lrm* leader peptide. Moreover, the residues at position 5 and 9 of the *lrm* leader are both asparagine, and when the serine and isoleucine residues at positions 5 and 9 in *ermC* were replaced by asparagine residues there was no apparent effect on inducibility. In addition both the *lrm* and *ermC* leaders have a

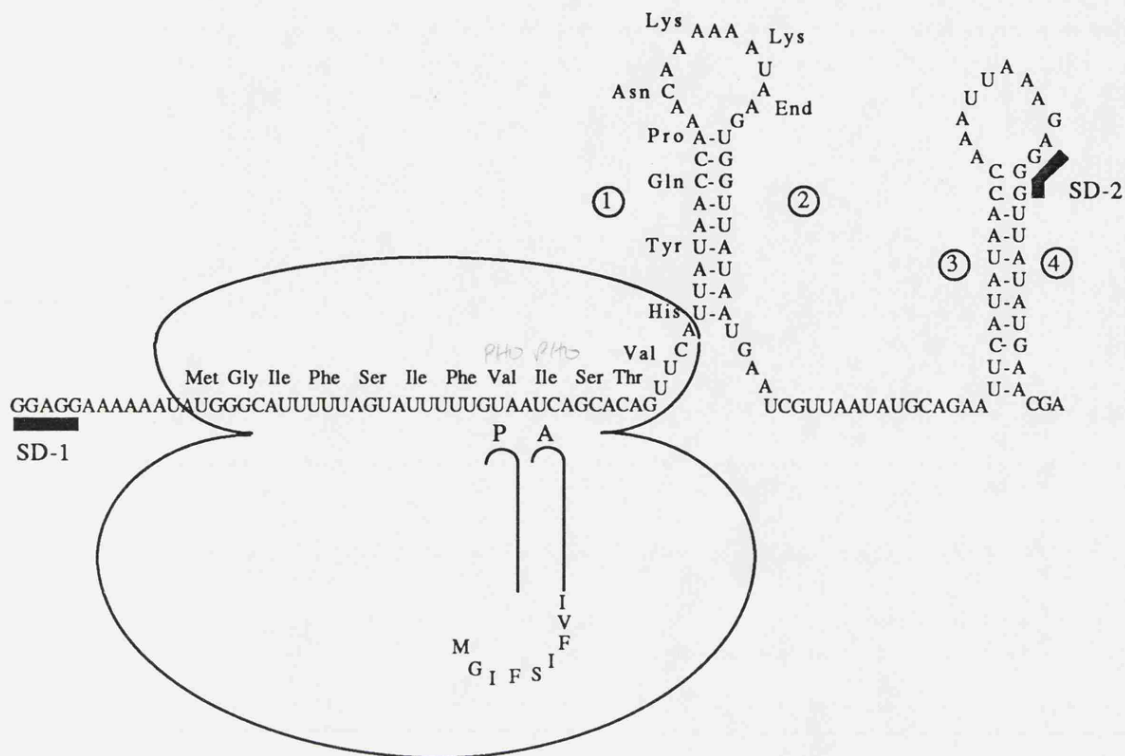


serine residue at position 10. The importance of a valine or isoleucine residue, previously mentioned, at position 8 in *ermC* does not seem to apply to the *lrm* leader peptide. However, an isoleucine residue does exist at the adjacent position 7, and perhaps the importance attributed to position 8 in *ermC* can be applied to position 7 in *lrm*. The similarities between these two leader peptides and their mRNA conformations seem to substantiate the observation that both genes possess the same induction specificity.

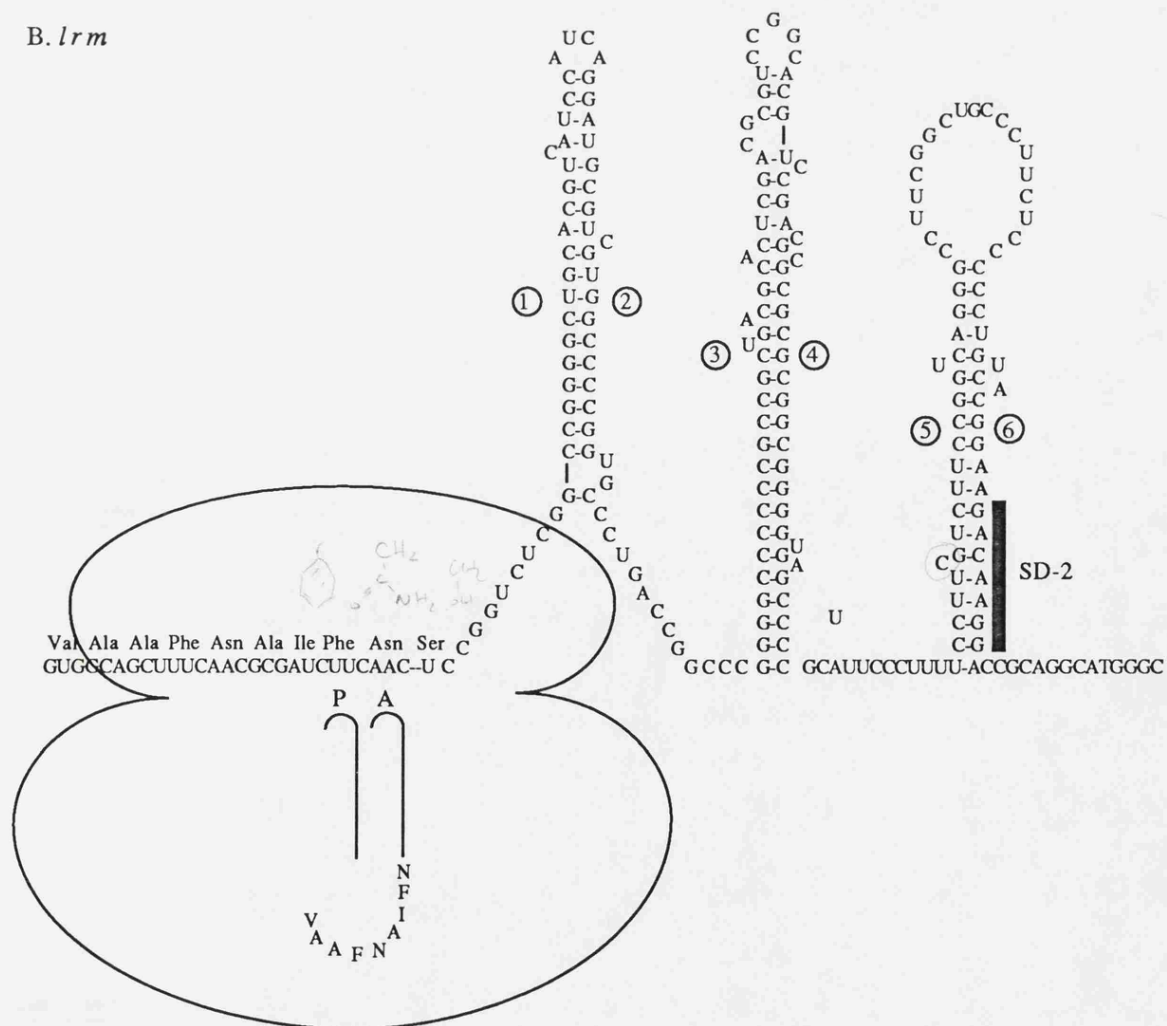
It has been estimated from nuclease protection experiments (Kang and Cantor, 1985) that 10(+/- 1) nucleotides downstream of the A-site and 20(+/- 2) nucleotides upstream of the P site are covered by the translating ribosome. Since mutations that diminish the ability of erythromycin to induce *ermC* were obtained up through leader peptide codon 9, it has been proposed that the ribosome must translate at least as far as residue 9 before stall occurs. The effect of such positioning by the ribosome on the mRNA conformation of *ermC* and *lrm* is indicated in Fig.6.5. In the case of *ermC*, disruption of the four lower base pairs in stem-loop 1+2 has been shown to increase the free energy (Mayford and Weisblum, 1989), indicating that partial disruption of stem-loop 1:2 can result in altered rRNA conformation thereby increasing translation of the methylase. This idea is supported by the single point mutation, described previously, in segment 1 (see chapter 1) which resulted in high level constitutive expression of *ermC*. Similarly in *lrm*, if the ribosome stalls at amino acids 8 or 9, disruption of the four lower base pairs of stem-loop 1:2 is also likely to result in destabilisation of the 'inactive' conformation, thereby favouring the base pairing of stems 2 and 5. In addition, activation of *ermC* mRNA requires all base-pairing (14 base pairs) between stem 1 + 2 to be broken, and whilst the first stem and loop structure of *lrm* is much larger than that of *ermC*, activation still only requires destabilisation of 14 base pairs. Alternatively, with respect to *lrm*,

**Fig. 6.5 Regulation of inducible MLS resistance genes.** The inactive conformations of the, *ermC* message of *Staph. aureus* (A), *lrm* message of *S.lividans* (B), *tlrA* message of *S.fradiae* (C), and *ermB* message of *Strep. sanguis* (D) are shown. The first two conformations (A + B) show an erythromycin bound ribosome stalled at the 9th amino acid residue in each leader peptide.

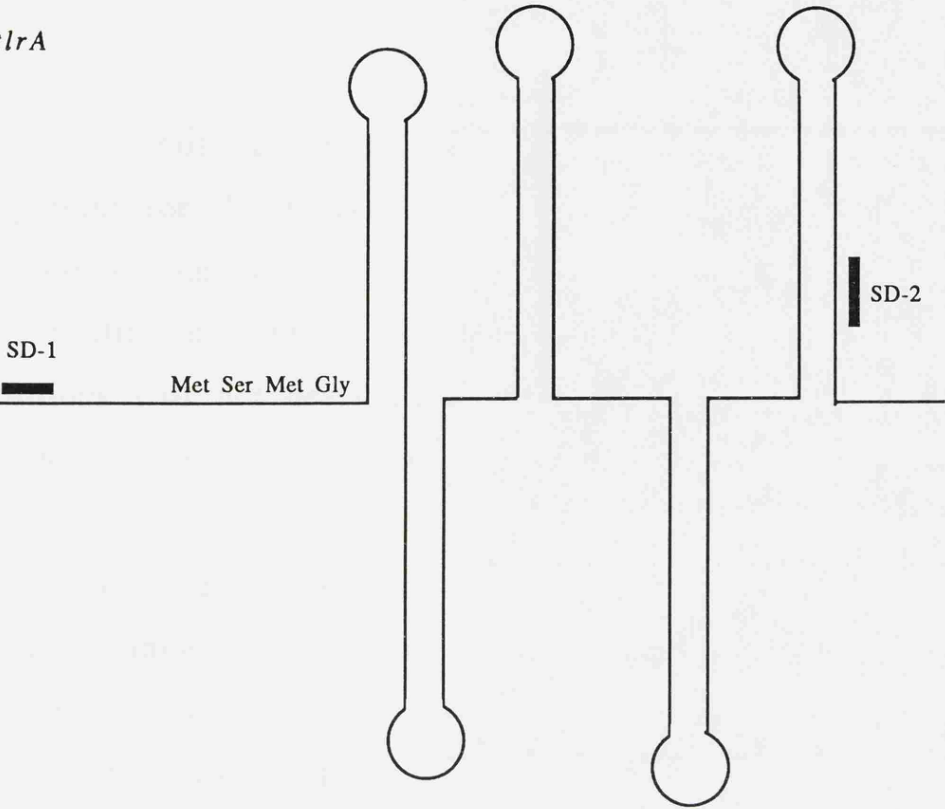
*A. ermC*



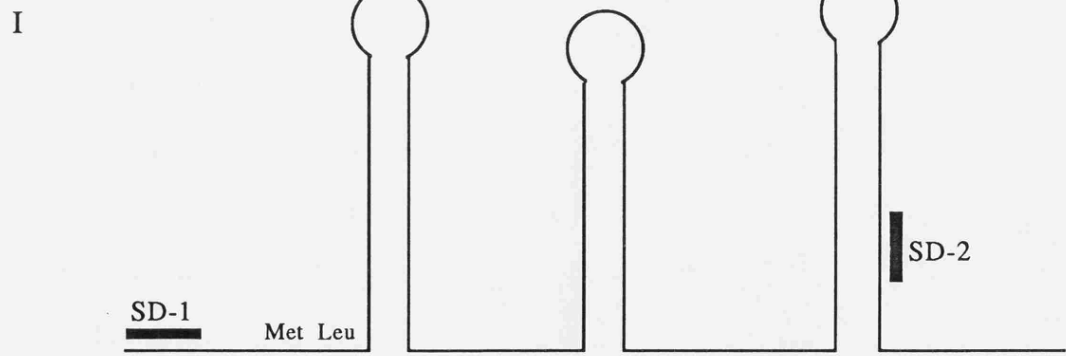
*B. lrm*



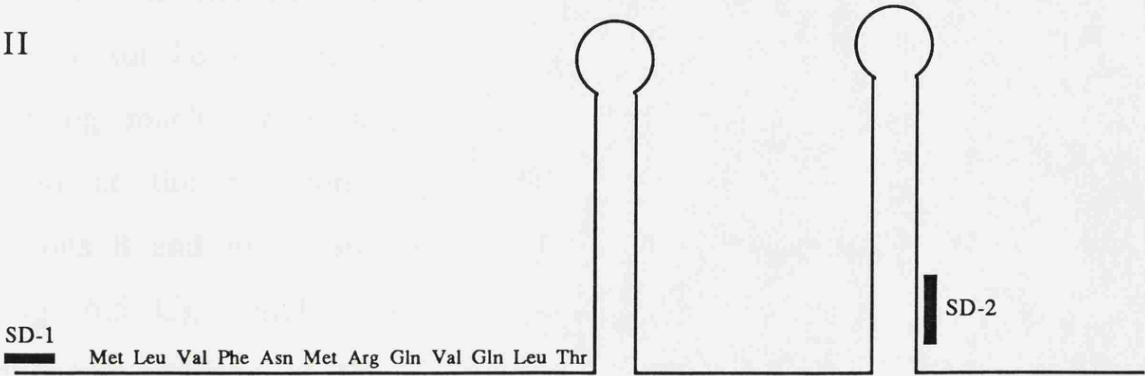
*C.tlrA*



*D.ermB*



II



ribosome stall at amino acids downstream from positions 8 + 9 may be important for the induction of methylase synthesis. Mutations in the codons of amino acids in positions 2 and 4 were reported to have no effect on the inducibility of *ermC*, clearly (Fig. 6.5), any ribosome stalling at these positions will not destabilize the stem loop 1+2 and induction of methylase synthesis will therefore not occur.

Whilst the amino acid composition of the leader peptide does appear to have an important role in determining the induction specificity it is likely that the actual mode of action of the MLS antibiotics is also involved. As described previously (chapter 1) the MLS antibiotics have varying effects on the elongation of the polypeptide chain. Whilst carbomycin, tylosin and lincomycin (among others) were found to inhibit ribosomes bearing short nascent chains (2 or 3 amino acids long), erythromycin was capable of inhibiting ribosomes bearing longer peptides. If this information is applied to ribosome stall during attempted translation of leader peptides it indicates that tylosin, carbomycin, and lincomycin would only be capable of stalling ribosomes involved in the translation of the first three amino acid codons of any peptide. Ribosomes bearing nascent peptides, greater than three amino acid residues in length would be unaffected by the above drugs. With regards *ermC* and *lrm*, stalling of ribosomes during translation of the first three amino acid codons of the leader peptides would have no effect on the stability of the first stem and loop and as a result methylase production would not be induced. In contrast, erythromycin is able to inhibit ribosomes bearing much longer nascent peptides and would therefore be able to stall them at the position required for induction of methylase synthesis (i.e codons 8 and 9). A similar argument can be applied to *ermSF* of *S.fradiae* (Fig. 6.5 C), which is inducible by erythromycin but not tylosin. Again, a ribosome stalling at the first three codons would not disrupt the stem:loop

1:2 sufficiently to enable the active configuration to form.

It has been reported that all the MLS antibiotics are capable of inducing the *ermB* methylase of *Strep. sanguis*. However, the number of inverted complementary repeat sequences in the control region of *ermB* is far greater than for *lrm* or *ermC* and as a consequence a number of inactive conformations for the mRNA are possible (Fig. 6.5). If the leader sequence of *ermB* folds into conformation I, then MLS drugs which inhibit the formation of di- and tri- peptides would be able to disrupt the first stem and loop, thereby inducing the system. However, the formation of configuration II would only allow drugs that can inhibit ribosomes carrying longer nascent peptides to induce, such as erythromycin. Since both conformations are possible *in vivo*, all antibiotics of the MLS group are subsequently able to induce resistance. It has been reported that the MLS resistance rRNA methylase of *S.ambofaciens* is inducible by lincomycin but not by erythromycin (J.L. Pernodet; unpublished data). An attenuation model has not yet been proposed for the control of this gene, but it would be of some interest to see if the secondary structure eventually proposed involves a leader peptide which begins immediately prior to a stem and loop structure, such that stalling of a ribosome during attempted translation of the first two amino acid codons would be sufficient to disrupt the secondary structure of the mRNA leader sequence.

In summarising the factors which determine induction specificity of these inducible MLS resistance genes, it is proposed that the initial determinant is the distance between the putative leader peptide initiation codon and the base of the first stem and loop which needs to be destabilised. A distance of only two or three amino acid codons would enable carbomycin, tylosin, and lincomycin to act as inducers, whilst a greater distance requires stalling of ribosomes by antibiotics such as erythromycin or celesticetin. In

addition, the actual amino acid sequence of the leader peptide affects the efficiency with which these antibiotics can induce, indicating that the inhibitory action of the drug is affected by the amino acid being incorporated at that time.

It has been reported that transposition as well as MLS resistance is induced by erythromycin in various strains of *Streptococci* (Tomich and Clewell, 1980), indicating that regulation by translational attenuation may not be unique to MLS resistance determinants. Possibly inducers of MLS resistance may switch on a wide range of inducible genes in addition to the rRNA methylases. In cases where amino acid biosynthesis is controlled by transcriptional attenuation, the corresponding leader peptide contains multiple codons for the particular amino acid whose synthesis it regulates. These codons serve as sensors of the level of charged tRNA and indirectly the level of amino acid in the cell, and it is therefore possible that a similar metabolic linkage may also occur in *Streptomyces* during the switch from primary to secondary metabolism. Indeed this event has been connected with a reduction in the availability of nutrients to the cell and any temporary starvation of amino acids may therefore serve to trigger induction of genes involved in secondary metabolism which are controlled by such a translational attenuation system.

### 6.3 Autoregulation of *lrm*.

Unlike the situation with antibiotic inactivating enzymes, the levels of resistance conferred by rRNA methylases are not related to the amounts of enzyme within the cell. Clearly, once all the rRNA molecules have been methylated *in vivo*, the strain will become fully resistant and the level of resistance will remain the same regardless of the number of methylase

molecules present in the cell at that time. This contrasts sharply with resistance conferred by antibiotic inactivating enzymes, where the level of resistance is related to the levels of enzyme within the cell. If the amounts of antibiotic inactivating enzyme in the cell are increased, presumably the level of resistance will increase accordingly. High levels of rRNA methylase are thus considered wasteful, and it is therefore not surprising that the level of expression is highly regulated.

According to the model proposed for the control of *ermC*, induction of methylase expression will eventually become self limiting. The gradual increase in methylase expression following induction will lead to a progressive increase in the proportion of resistant ribosomes within the cell, and since these ribosomes will no longer be able to stall during translation of the leader sequence, they will eventually trigger a breakdown in the induction mechanism. Presumably this type of 'negative feedback' would also occur with *lrm* and indeed any other gene encoding a ribosomal RNA methylase whose expression is controlled by translational attenuation.

A further more direct mechanism for shutting down methylase production from *ermC* has also been proposed, namely translational autoregulation by the methylase protein itself (see chapter 1). The sequence around the ribosome binding site of *ermC* is identical to that surrounding and including the site of action of the methylase in 23S rRNA to which it must bind. It has therefore been proposed, that the *ermC* gene product binds to its own mRNA and prevents utilisation of the latter by ribosomes. It is possible that such autoregulation may also operate to regulate *lrm* expression. Within the *lrm* leader sequence close to the ribosome binding site (SD-2), is the sequence ACGGAA, which is also found embracing the site of action (residue A-2058) of the *lrm* product in 23S rRNA (Fig. 6.6); perhaps the *lrm* product



• • AACCAAUU **AAAGAG** GGGUUAUAAUG • • *ermC* mRNA

• • CAGGACGG **A**AGACCCCG • • 23S rRNA  
|                      |  
2050                      2060

• • UGUCA **CGGAAG** GACAAGGACCGCA • • *lrn* mRNA

\* Site of action of the *lrn* methylase

**Fig. 6.6 Autoregulation of *lrn*.** A region exists in the leader sequence of *lrn* , that is identical to the sequence embracing the site of action of that methylase within 23S rRNA. Possibly the, methylase regulates its own production by binding to its own transcript. A similar situation has been described for the *ermC* methylase.

recognises this sequence in its own mRNA and thereby regulates its own production. The sequence similarity observed here is however slightly different to that seen between the *ermC* leader and 23S rRNA, in fact only the residues at A-2058 and A-2059 are common to both the *lrn* and *ermC* leaders. Perhaps this indicates that these rRNA methylases actually recognise a wider sequence within 23S rRNA than originally anticipated, with A-2058 and A-2059 being particularly important residues.

#### 6.4. Evidence for an antibiotic biosynthetic pathway in *S.lividans*

This thesis reports the existence of two macrolide/lincosamide resistance genes in the widely used organism *S.lividans*. Since this organism is not known to produce any antibiotic the existence of one resistance gene seemed surprising, the presence of two is perhaps more than coincidence. The strain TK21 has, as far as can be established, been cured of all plasmids to facilitate its use as a common host in *Streptomyces* cloning experiments, and it is therefore highly likely that the two resistance genes are chromosomally located. This is in contrast with MLS resistance genes isolated from other non-producing strain, which have invariably been located on plasmids.

Further support for the idea of a biosynthetic pathway being present in *S.lividans* was obtained during southern analysis, in which genes homologous to the second resistance gene were detected in both *S.ambofaciens* and *S.caelestis*. Both strains possess monomethylating enzymes which confer MLS resistance and significantly both are antibiotic producers, producing the lincosamide celesticetin and the macrolide spiramycin respectively. Perhaps genes homologous to the second resistance gene isolated from *S.lividans* are also intrinsically linked with the monomethylating enzymes in these producing strains.

The above observations clearly raise the possibility that *S.lividans* contains all or part of a macrolide or lincosamide biosynthetic pathway. If true, such biosynthetic genes are likely to be associated with the two resistance genes already isolated from *S.lividans* thereby enabling the isolation of such genes with relative ease.

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