

**GENTAMICIN RESISTANCE
IN
*MICROMONOSPORA PURPUREA***

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

By

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Gentamicin resistance in *Micromonospora purpurea*.

Ph.D. Thesis by Joanna Bacon.

The gene, *grmA*, from the gentamicin producer *Micromonospora purpurea*, confers high level resistance to gentamicin and kanamycin. The ribosomes of *M. purpurea* are constitutively resistant to these antibiotics by modification of the 16S rRNA in the 30S subunit.

grmA encodes a methyltransferase. In this study, reverse transcriptase assays confirmed that GrmA methylated the 16S rRNA at residue G1389 in *S. lividans*. However these assays also revealed that G1389 was not methylated in *M. purpurea*, indicating that *grmA* is silent in its native host.

grmA, driven by a constitutive promoter (*ermEp**), was introduced into *M. purpurea* by conjugal transfer with the hope that the effects of a putative repressor would be titrated out by the expression of an extra copy of the gene. Changes in the level of *grmA* transcript and methylation of the 16S rRNA were subsequently observed.

RNA hybridisation analysis revealed that *grmA* was transcribed in the wild type strain of *M. purpurea*, and that the transcript was probably full length. There was a higher level of *grmA* transcript in *S. lividans* containing the gene than in *M. purpurea*, suggesting that *grmA* was transcriptionally regulated in *M. purpurea* but not in *S. lividans*.

The extra copy of *grmA* in *M. purpurea* was expressed efficiently from *ermEp**. However, 16S rRNA extracted from this strain, had not been methylated at G1389, although the transcriptional control of *grmA* was apparently alleviated. These data suggested that there may have been more than one control mechanism regulating the expression of *grmA*.

If *grmA* is not expressed in *M. purpurea*, there must be another resistance gene responsible for the constitutive phenotype being expressed. Despite attempts to isolate alternative gentamicin resistance genes from *M. purpurea*, only *grmA* was found.

I dedicate this work to Gran
who was a great source of strength and inspiration

Jean Bacon 27.05.16 - 21.11.97

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List of Abbreviations

A	adenine
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pairs
C	cytosine
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
G	guanine
HEPES	N-[2-Hydroxyethyl] piperazine-N-[2-ethanesulphonic acid]
IPTG	isopropylthio- β -D-galactoside
kb	kilobases
LB	Luria Bertani
LMP	low melting point
MIC	minimum inhibitory concentration
mRNA	messenger RNA
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
psi	pounds per square inch
RBS	ribosome binding site
RNA	ribonucleic acid

rRNA	ribosomal RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSQ water	sterile super Q water
T	thymidine
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TEMED	N, N, N', N'- tetramethylethylenediamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.
Tris	tris (hydroxymethyl)amiomethane
tRNA	transfer RNA
U	uracil
UTR	untranslated region
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside.

Chapter 1

Introduction

Chapter 1

Introduction

1.0 An introduction to antibiotics

Antibiotics are secondary metabolites, which are defined as substances that do not play an essential role in the survival of the organism. Seventy percent of all known antibiotics are produced by the actinomycetes, a family of Gram positive filamentous bacteria. Actinomycetes have a complex life cycle, which initially involves mycelial growth that culminates in sporulation and the biosynthesis of secondary metabolites. They are saprophytic and inhabit soil and aquatic environments.

Antibiotic biosynthetic genes studied in these organisms appear to be clustered together. Such clusters commonly contain other genes involved in the regulation of biosynthesis or resistance to the antibiotic (Stone and Williams 1992). An example of this can be found in the streptomycin biosynthetic cluster in *Streptomyces griseus* (Pissowotzki et al. 1991).

In *Streptomyces* spp, two phases of growth can be defined: the trophophase (exponential phase) in which cell growth and replication occurs, and the idiophase (the stationary phase) when cell growth slows down and secondary metabolites are produced. However, it has become apparent that some antibiotics and other secondary metabolites are synthesised during the trophophase (Martin and Demain 1980).

1.1 The role of antibiotics in nature

Antibiotics are chemical substances produced by one organism that are harmful to others. It has been argued that organisms evolved the ability to biosynthesise such

compounds because they confer a selective advantage over other organisms in nature. However, this function has not been proven to be their primary role. There may be an alternative explanation for the existence of antibiotics.

It has been suggested that antibiotics could have had an important role during biochemical evolution (Davies 1990). The first proteins were formed from amino acids by spontaneous chemical reactions in a 'primordial soup'. Non-protein amino acids, which are similar to those found in peptide antibiotics, have been discovered in meteorites. This suggests that the antibiotics may be as old as some amino acids, formed by chemical condensation in the absence of enzymes. Protein synthesis inhibitors interact very specifically with ribosomal sites. They could have been precursors to ribosomal proteins and may have played an important part in the evolution of translation apparatus.

1.2 The genus *Micromonospora*

The *Micromonospora* spp. belong to the family of actinomycetes. By chemotaxonomic analysis and comparisons of their 16S rRNA with other actinomycetes, the *Micromonospora* spp. have been placed in the suprageneric group, actinoplanetes (Goodfellow 1990) along with the genera, *Actinoplanes*, *Dactylosporangium*, *Amorphosporangium*, *Ampulariela*, and *Pilimelia*.

Micromonospora spp. are the predominant actinomycetes in sea bed sediments and are particularly prevalent in deep mud layers. Under these conditions, their spores remain more viable than those produced by their companions, *Streptomyces* and *Nocardioforms*. *Micromonospora* spp. also inhabit fresh water. Strains isolated from lakes possess enzymes that decompose the chitin, lignin, and cellulose present in the sediments. These microorganisms have an important function in the lacustrine ecosystem by recycling useful carbon and nitrogen sources.

The morphology of *Micromonospora* spp. consists of branched, septated, mycelia (Kawamoto 1989). Growth on solid media is only at the level of the substrate. Colonies appear raised and folded. Young colonies are pale yellow to orange becoming

deep orange, red, purple, brown, or blue/green with age. Some strains produce short, sterile aerial hyphae whereas in other strains the upper mycelial layer can become covered by a mass of spores, usually black in colour. *Micromonospora melanospora* is a good example of a strain that sporulates in this way. The spores appear as a black, waxy layer. On the other hand, *M. purpurea* produces spores that are buried and accumulate in certain areas within the colony. As the name suggests, single spores are produced terminally on short hyphal branches and can appear clustered together (Kawamoto 1989).

Much is known about the genetics of differentiation and secondary metabolism in *Streptomyces*. The same cannot be said for *Micromonospora* spp. despite the fact that they synthesise a wide range of secondary metabolites including aminoglycosides, macrolides, quinones, and antitumour drugs. The first useful antibiotic to be discovered in *Micromonospora* was gentamicin, which is synthesised by *M. purpurea*. During fermentation, a mixture of three gentamicins are synthesised (C1, C1a and C2) known as the gentamicin complex (Figure 1.0). This complex is biologically active.

Gentamicin is a broad-spectrum antibiotic that has been used in the treatment of serious Gram negative infections. It has proved invaluable in the hospital environment against opportunistic pathogens such as *Pseudomonas aeruginosa*. It has been used synergistically with penicillins to treat patients, post appendectomy.

1.3 Fortimicin antibiotics

Fortimicin A (astromicin) is an aminoglycoside, synthesised by *Micromonospora olivasterospora*. Although many of the antibiotics produced by *Micromonospora* spp. are well characterised chemically, only the fortimicin A biosynthetic cluster has been studied extensively. This has been partly due to the lack of suitable systems for studying biosynthetic genes in *Micromonospora* spp. and to the difficulty with which some strains could be transformed. However, protoplasts from three strains have been efficiently transformed with broad host range *Streptomyces* plasmid pIJ702: *M. purpurea* (Kelemen

et al. 1991), *Micromonospora melanospora* (Kojic et al. 1991) and *Micromonospora rosaria* (Matsushima and Baltz 1988). Host-vector systems have been developed for cloning biosynthetic genes from *Micromonospora griseorubida* (Takada et al. 1994) and *M. olivasterospora* (Hasegawa et al. 1991) in order to study the biosynthesis of mycinamicin (a macrolide antibiotic) and fortimicin A respectively.

The fortimicin antibiotics are synthesised by actinomycetes belonging to various genera: *Micromonospora olivasterospora* (fortimicin A), *Micromonospora* sp SF-2098 (SF 2052 compounds), *Dactylosporangium matsuzakiense* (dactamicins), *Streptomyces tenjimariensis* (istamycins), *Streptomyces sannanensis* (sannamycins), and *Saccharopolyspora hirsuta* (sporaricins). The fortimicin A biosynthetic genes in *M. olivasterospora* seem to be organised in the same manner as the biosynthetic clusters studied in *Streptomyces*. Ten biosynthetic genes and one fortimicin A resistance gene, *fmrO*, have been located to a region of about 27 kb (Dairi et al. 1992). There appears to be conservation between the biosynthetic genes in this diverse group of fortimicin producers, however these strains contain fortimicin resistance genes that are divided into 2 types (Ohta et al. 1993, Ohta and Hasegawa 1993). All strains are resistant to fortimicin A and kanamycin. The *Streptomyces* spp. and *Saccharopolyspora* spp. are resistant to neomycin but not gentamicin, whereas the *Micromonospora* spp. and *Dactylosporangium* spp. are resistant to gentamicin but not neomycin. It was discovered that these two types of *fmr* genes did not cross hybridise during Southern analysis (Ohta et al. 1993).

1.4 Antibiotic resistance

Mechanisms of resistance are dictated by the mode of action of the antibiotic in question. For example, actinomycetes that produce inhibitors of protein synthesis are often resistant by modification of their ribosomes. There are several resistance mechanisms employed by antibiotic-producing organisms (reviewed by Cundliffe 1989). These include: modification of the target site, inactivation or sequestration of

intracellular drug molecules, and the presence of permeability barriers, which are sometimes accompanied by efflux mechanisms.

1.4.1 Target site modification

Antibiotics bind to specific receptors within the cell. Many inhibitors of protein synthesis function by interacting either with the 30S or the 50S subunit of the 70S ribosome. Resistance occurs when the binding site is modified or replaced by an insensitive target site. Total resistance to an antibiotic is usually achieved in organisms that are resistant to a protein synthesis inhibitor by methylation of ribosomal RNA. A very good example of this is the thiostrepton producer, *Streptomyces azureus* (Thompson and Cundliffe 1981).

Thiostrepton is a modified sulphur-containing peptide antibiotic consisting of thiazole rings with heterocyclic residues, and is very active against Gram positive bacteria. It inhibits protein synthesis by binding to a domain in the 50S subunit that contains part of the aminoacyl tRNA binding site. Resistance to thiostrepton is achieved by methylation of residue A1067 within the 23S rRNA (Thompson et al. 1982). Ribosomal modification is quite widespread among producers of protein inhibitors and can be found in actinomycetes that synthesize tylosin, carbomycin, spiramycin, kanamycin and gentamicin. Table 1 lists some of the ribosomal methylases that have been characterised in actinomycetes.

Some *Streptomyces* spp. achieve resistance by synthesising alternative regions of the antibiotic target enzyme. For example *S. sphaeroides* and *S. niveus* produce two types of DNA gyrase B subunit, one of which is resistant to novobiocin. The expression of the resistance gene *gyrB* is induced by the presence of novobiocin. The promoter concerned with expression of *gyrB* is controlled by responses to changes in DNA topology.

Organism of origin	Resistance gene	Resistance phenotype	Site of methylation	Reference
<i>Sacc. erythraea</i>	<i>ermE</i>	MLS	23S rRNA A2058	Skinner et al. 1983
<i>Streptomyces fradiae</i>	<i>tlrD/tlrA</i>	MLS	23S rRNA A2058	Zalacain & Cundliffe 1989 & 1991
<i>Streptomyces lividans</i>	<i>lrm</i>	Lincosamides	23S rRNA A2058	Jenkins and Cundliffe 1991
<i>Streptomyces tenjimariensis</i>	<i>kam A</i>	Kanamycin Apramycin	16S rRNA A1408	Skeggs et al. 1985
<i>Streptomyces tenebrarius</i>	<i>kgmB</i>	Kanamycin Gentamicin	16S rRNA A1405	Skeggs et al. 1987
<i>Streptomyces tenebrarius</i>	<i>kamB</i>	Kanamycin Apramycin	16S rRNA A1408	Skeggs et al. 1987
<i>Sacc. hirsuta</i>	<i>kamC</i>	Kanamycin Apramycin	16S rRNA A1408	Holmes et al. 1991
<i>Streptomyces pactum</i>	<i>pct</i>	Pactamycin	16S rRNA A964	Ballesta and Cundliffe 1991

Table 1

Presented here, are some of the ribosomal methylases that have been characterised for *Streptomyces* spp. and *Saccharoployspora* spp. (*Sacc.*). MLS is an abbreviation for macrolides lincomides and streptogramin B antibiotics.

1.4.2 Inactivation of antibiotics

Some of the best examples of antibiotic inactivation are found in aminoglycoside producing actinomycetes, discussed in section 1.8.2. However, there are other examples: *Streptomyces capreolus* produces capreomycin, which is a cyclic peptide consisting of four components: IA, IIA, IIB and IIIB. Two genes, *cac* and *cph*, which encode capreomycin modifying enzymes, have been isolated from *S. capreolus*, (Thiara and Cundliffe 1995). The product of *cac* is an acetyltransferase that can modify all four components of the antibiotic. The product of *cph*, on the other hand, will only phosphorylate IA and IIA (Thiara and Cundliffe 1995). Ribosomes from this organism are not modified.

1.4.3 Efflux mechanisms

Streptomyces rimosus, producer of tetracycline possesses a gene, *tetB*, which encodes a membrane-bound protein, involved in energy-dependent efflux of the drug. Like *tetB*, most efflux systems typically deal with a narrow range of structurally related substrates. However, there are export systems that can handle a wide range of structurally dissimilar compounds. These are termed multi-efflux systems, and could present an alarming clinical problem, since the acquisition of such a system by a cell could increase its resistance to a wide range of antibiotics (reviewed by Paulsen et al. 1996). One such group of multidrug efflux pumps is the ATP-binding cassette (ABC) family. Members of this family have been found to be responsible for ATP-dependent export-mediated multidrug resistance to malarial agents in *Plasmodium falciparum* (Foote et al. 1989) and cytotoxic drugs in *Schizosaccharomyces pombe*. ABC exporters are believed to exist in a diverse number of organisms, ranging from *Arabidopsis thaliana* to *Staphylococcus aureus*.

1.5 The acquisition of resistance genes by clinical isolates

The pharmaceutical industry has been remarkably successful over the last 30 years at creating antibiotics that keep potentially fatal bacterial disease at a negligible level. Society has become complacent. There now exists a situation where clinical isolates are multiresistant to antibiotics and can, once again, cause potentially untreatable diseases. An example of this is multiresistant *Staphylococcus aureus* (MRSA).

New populations of antibiotic-resistant cells emerge due to the selection pressure applied by the presence of the antibiotics. In clinical isolates, resistance can occur by spontaneous mutation or the acquisition of resistance genes by conjugation, transformation, transposition or transduction. Conjugation and transposition are the two main mechanisms by which antibiotic resistance genes are transmitted between bacteria.

1.5.1 Conjugation

Shigella, resistant to sulphonamides streptomycin, chloramphenicol and tetracycline, were isolated from patients with dysentery in Japan in the 1950's. Most patients harbouring multiresistant shigella in their intestinal tract also harboured multiresistant *E. coli*. It was therefore suspected that resistance determinants could be transferred from one Gram negative organism to another. It is now clear that conjugation can also occur between Gram positive and Gram negative bacteria and vice versa.

The genetic elements associated with the introduction of resistance genes by conjugation are known as R plasmids (Thomas et al. 1987). These plasmids are autosomal and belong to the IncP incompatibility group, which have a broad host range. They consist of a distinct set of genes that initiate and control conjugation and a series of one or more linked genes that confer resistance to antibacterial agents. They also have the ability to transfer non-conjugative plasmids by mobilisation and retrotransfer (Sia et al. 1996).

Transfer functions in IncP plasmids are not expressed constitutively. Conjugation is quite an energetic process. A complex regulatory system has evolved which allows their low level expression. The cell is constantly ready for transfer of the plasmid, without using up precious energy (Zatyka and Thomas 1998, Zatyka et al. 1997).

1.5.2 Transfer elements

Some R plasmids carry complex transposons, which can insert themselves into a variety of genomic sites. In the simplest type of transposon, the genetic information is only concerned with insertion functions. More complex elements, known as composite transposons, contain additional DNA including resistance genes. For example, the Tn5 transposon carries a gene conferring resistance to kanamycin.

Composite transposons owe their mobility to the presence of flanking of direct inverted repeat sequences, enabling them to transfer between genomic DNA and plasmid DNA. Sequences that are homologous to insertion element IS256, are present in many strains of *Enterococcus*. These strains also possess multiple resistance markers, raising the possibility that new composite transposons could develop (Rice et al. 1995). An example of a composite transposon in *Enterococcus faecalis* is Tn5384, which confers resistance to erythromycin and gentamicin (Rice et al. 1995).

Conjugative transposons are found in both Gram positive and Gram negative bacteria. They can excise from, and integrate into, DNA like transposons. However, unlike non-conjugative transposons they transpose through covalently closed circular (CCC) intermediates (Salysers et al. 1995).

Tn916 is the best studied conjugative transposon. It was originally found in *E. faecalis*. It carries a tetracycline resistance determinant, *tetM*. Transfer of Tn916 is stimulated by the presence of tetracycline. Genes *int* and *xis* are involved in the excision and integration of the transposon, and are located downstream of *tetM*. Tetracycline may cause increased transcription of these genes and subsequently stimulate transposition (Showsh et al. 1992). This is an interesting observation, because it indicates that

antibiotics not only select for resistant strains, but they also stimulate the spread of resistance genes. It appears that use of antibiotics could be contributing further to a situation in which strains will no longer be susceptible to treatment with antimicrobial agents.

Integrations are mobile elements located on plasmids and transposons. Large numbers of resistance genes have been found in clusters in these transfer elements. Each gene is contained in an individual cassette that can be inserted into, or excised from, an integron by site-specific recombination (reviewed by Hall and Collis 1995)

A combination of such a wide variety of transfer elements has provided an efficient system for the spread of antibiotic resistance genes, in clinical isolates. Some strains of MRSA are now resistant to all the available antibiotics including vancomycin, which is considered to be the last line of defence against these strains.

1.6 Control of the emergence of antibiotic resistance in clinical strains.

Resistant bacteria will continue to appear, due to the selection pressure applied by the use of antibiotics. The rise in the number of resistant bacteria can be attributed in many cases to the continual misuse of antibiotics. There are several practices that should be subject to close scrutiny as they seem to be contributing to the rising problem. In many countries apart from Britain, antibiotics are dispensed without a prescription. Also, doctors prescribe antibiotics for viral diseases such as influenza, whilst being fully aware that these drugs are only effective against bacterial infections.

Antibiotics are also used unnecessarily by farmers as growth promoters in feedstuff. At present, vancomycin is the only antibiotic that is still effective against some multiresistant strains of *Enterococcus faecium* and MRSA. Studies carried out in Britain and Europe (Schouten et al. 1997, Thal et al. 1996, Wise 1996) have identified a possible link between vancomycin resistant enterococcus (VRE) in the community and the VRE isolated from farm animals fed with a related glycopeptide antibiotic,

avoparcin. The use of avoparcin has been suspended by the European Union until May 1999, whilst researchers find more evidence for the link between the two.

One aspect of gene transfer that has not been discussed here, is that antibiotic resistance genes acquired by clinical strains are extremely stable even in the absence of the antibiotic. The acquisition of foreign DNA can affect the growth of a microorganism. If selection for a resistance gene is removed, the organism would be expected to lose the resistance determinant after a few rounds of cell division. Bacteria can apparently acquire secondary mutations in their genome which will ensure "healthy" growth, without losing the resistance determinants, even in the absence of antibiotics (Schrage et al. 1997). This finding could have broad implications. Removal of antibiotics from agricultural use and medicine may not be effective at combating the rise in bacterial resistance.

Since sensitive bacteria can have an advantage over resistant ones in that they grow more healthily, a decrease in the level of antibiotic in the environment would result in a larger proportion of sensitive bacteria. However, a small number of resistant bacteria would remain which would predominate if antibiotic selection were reapplied.

Plasmids carry resistance determinants other than those conferring resistance to antibiotics. Heavy metal (mercury) resistance genes are often clustered with antibiotic resistance genes. The presence of the heavy metal provides co-selection and maintenance of the antibiotic resistance genes. An example of this is a tooth filling containing mercury. This may select for antibiotic resistant flora in the human gut. (Summers et al. 1993).

1.7 The aminoglycoside antibiotics.

Aminoglycosides are characterised by the presence of two or more amino sugars linked by glycosidic bonds to a central aminocyclitol ring known as 2-deoxystreptamine. Most aminoglycosides belong to the 4, 6-disubstituted 2-deoxystreptamine group of antibiotics, which includes kanamycins and gentamicins. The gentamicin complex is characterised by the presence of a branched sugar (garosamine), linked to the 6 position of 2-deoxystreptamine (in the place of 3-amino-3-deoxyglucose in kanamycin) and the presence of α -2, 6-diaminoglucosyl derivative at position 4 (Figure 1.0).

1.7.1 Mode of action of aminoglycosides.

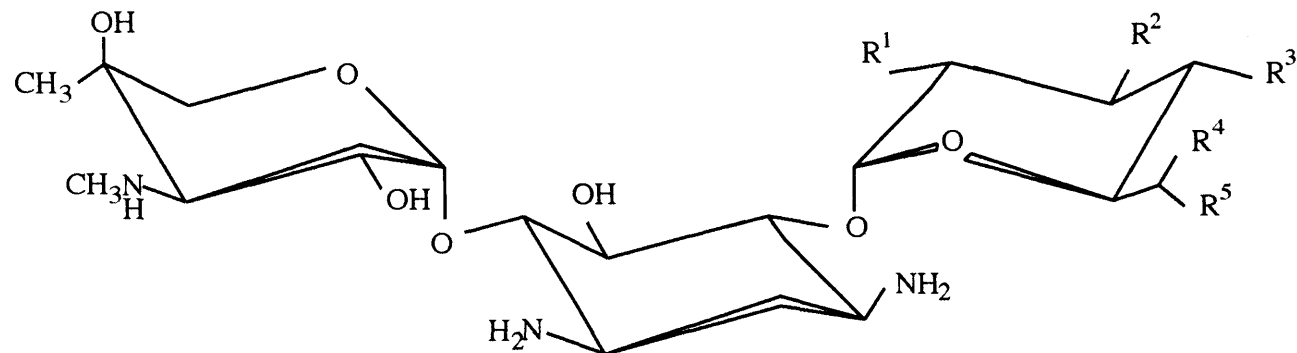
Aminoglycosides: kanamycins, gentamicins, and neomycins bind to the 16S rRNA in the 30S subunit of bacterial ribosomes. They inhibit protein synthesis by interfering with translocation and cause misreading of mRNA codons (Davies and Davis 1968).

During elongation an aminoacyl tRNA binds to the complementary anticodon in the A site of the ribosome. This requires elongation factors EF-Tu and EF-Ts and hydrolysis of GTP. The A site is next to the P site in which lies the terminal acyl group of the growing peptide chain. Breakage of the acyl group then occurs and the peptide is transferred to the amino acyl tRNA in the A site. The peptide bearing tRNA in this site is then transferred to the P site. This is referred to as translocation and requires EF-G and hydrolysis of GTP. Neomycins, kanamycins and gentamicins do not affect the ribosome bound amino acyl-tRNA. However, they inhibit the stimulation of this reaction by factor EF-G and GTP.

Streptomycin (sometimes considered to be an aminoglycoside) binds a single site whereas neomycins, kanamycins, and gentamicins, are thought to bind multiple sites. They have a multiphasic effect on the ribosomes at varying concentrations of antibiotic (Tai and Davis 1979). At a low level of gentamicin, ribosomes are inhibited whereas at a

higher level, increased misreading of mRNA codons is observed and inhibition is reversed. At even higher concentrations, inhibition increases and misreading is reduced.

Figure 1.0 The structure of gentamicin



Gentamicin is synthesised as a complex of three structurally similar compounds C1, C2 and C1a. The table below shows the variation between them.

	R ¹	R ²	R ³	R ⁴	R ⁵
Gentamicin C1	NH ₂	H	H	CH ₃	NHCH ₃
Gentamicin C2	NH ₂	H	H	CH ₃	NH ₂
Gentamicin C1a	NH ₂	H	H	H	NH ₂

1.8 Resistance to aminoglycosides

1.8.1 Resistance mechanisms used by clinical isolates

Inactivation by enzyme modification is one of the principal mechanisms of resistance to aminoglycosides in clinical isolates. There are three main types of antibiotic modification: *N*-acetylation of amino groups, *O*-phosphorylation of hydroxyl groups and *O*-adenylylation. Acetyltransferases that are very prevalent can inactivate useful aminoglycosides such as gentamicin, tobramycin and amikacin. Many are carried by the transfer elements described in section 1.5.2 (Bunny et al. 1995, Rice et al. 1995, Horaud et al. 1996, Schowcho et al. 1995). Acetylation of the 3*N* position (AAC(3)) of gentamicin, was first described in *Pseudomonas aeruginosa*. It is now apparent that members of the *Enterobacteriaceae*, and *Acinetobacter* families also possess AAC(3).

Resistance to aminoglycosides can also occur due to reduced binding of the drug to the cell or reduced diffusion through the outer membrane. *Pseudomonas aeruginosa* exhibits the former. Aminoglycosides are believed initially to interact with the cell by binding to the lipopolysaccharides (LPS) on the cell surface. The antibiotics are taken up through the outer membrane. Active transport is then required for their movement across the cytoplasmic membrane. In *P. aeruginosa* overproduction of outer membrane protein H1, is thought to protect LPS from the antibiotic binding (Nica 1983).

1.8.2 Resistance to aminoglycosides in producing actinomycetes

Aminoglycoside-producing actinomycetes are a good example of organisms that have more than one type of resistance mechanism, possessing antibiotic modifying enzymes and alteration of the antibiotic target site. There are two main types of antibiotic modification: *N*-acetylation of amino groups; and *O*-phosphorylation of hydroxyl groups. The cofactors for these enzymes are acetyl CoA and ATP, respectively.

It seems that the role of antibiotic modifying enzymes is not always restricted to inactivation of an antibiotic. They may also be necessary in the modification of intermediates in its biosynthetic pathway. *Streptomyces kanamyceticus* possesses an acetyltransferase that acts upon the 6' moiety (AAC(6')). When cloned in *S. lividans*, AAC(6') functions as a resistance determinant. However, this does not appear to be its only function in *S. kanamyceticus*. It was discovered that the introduction of multiple copies of AAC(6') into *S. kanamyceticus* resulted in the increased levels of kanamycin resistance and antibiotic yield (Crameri and Davies 1986). This data suggests that AAC(6') has a direct role in the biosynthesis of kanamycin.

The ribosomes of some aminoglycoside producers are resistant by methylation of the 16S rRNA in the 30S subunit (see Table 1 for some examples). Aminoglycosides act by interacting with the decoding region on the 30S subunit, inhibiting protein synthesis. Methylation of the rRNA also occurs in the decoding region and subsequently protects this area from the aminoglycoside binding. The close association between the interaction of antibiotics, the decoding region (Moazed and Noller 1987, Purohit and Stern 1994), and clusters of modified residues is described in more detail in Chapter 4, sections 4.1.3 and 4.1.4.

1.8.3 Identification of modified residues in the 16S rRNA

A resistance gene denoted *kgmA* was first thought to have been isolated from *M. purpurea*. This gene conferred resistance to kanamycin and gentamicin and was shown to encode a 16S rRNA methylase (Thompson et al. 1985). It was later realised that *kgmA* originated via contamination from *S. tenebrarius* and not from *M. purpurea*. At that time two resistance determinants were isolated from *S. tenebrarius*, *kgmB* which is the same as *kgmA*, having an identical sequence over 650 bp (Holmes and Cundliffe 1991) and *kamB* which confers resistance to kanamycin and apramycin (but not gentamicin) also by methylation of the 16S rRNA.

Further biochemical analysis was performed, to study the site of methylation by *kgmB* (Beauclerk and Cundliffe 1987). At this stage, *kgmB* was still assumed to be *kgmA*, originating from *M. purpurea*. Investigations were carried out on *S. lividans* clones containing *kamA* from *S. tenjimariensis* and *kgmB* to ascertain the type of residue that the gene product was methylating. rRNA was treated *in vitro* with methylase extracted from *S. lividans* clones containing the two genes using tritiated S-adenosyl-methionine as the cofactor. The rRNA was rendered down to purine bases, ribose-phosphate and pyrimidine-5'-mononucleotides. These were then separated by paper chromatography. The methylated base was found to be 1-methyladenosine for *kamA* and 7-methylguanine for *kgmB*.

Reverse transcriptase assays were used to locate the exact position of the modified base in the 16S rRNA (Beauclerk and Cundliffe 1987). The methodology behind this procedure is described in detail in Chapter 4 section 4.1.4. *kamA* was found to act at residue A1392 (1408 in *E. coli*) and *kgmB* to act at residue G1389 (1405 in *E. coli*) (Figure 4.0, Chapter 4).

Some time after these data had been published, an authentic gentamicin resistance gene, *grmA*, was cloned (Kelemen et al. 1991). This was also found to encode a methyltransferase. The site of action of *grmA* has never been published, however data from preliminary primer extension experiments (personal communication G. H. Kelemen) suggested that the gene product acted on the 16S rRNA at residue G1389 (*E. coli* 1405) when *grmA* was cloned in *S. lividans*.

1.9 The gene, *grmA*

grmA (gentamicin resistance methylase) confers resistance to kanamycin and gentamicin. It was originally cloned in *S. lividans* on a 1.9 kb *Bam*HI fragment (Kelemen et al. 1991). Two open reading frames, transcribed in the same direction, are present on the cloned DNA fragment. One open reading frame, *grmA* has been sequenced and nucleotides 12 to 836 encode a protein of 274 amino acids. The G+C

content of *grmA* is 64%, which is lower than the 73% average for actinomycetes. The second open reading frame starts at nucleotide 954 and remains open to the end of the *Bam*HI fragment.

Figure 1.1 Restriction map of *grmA*

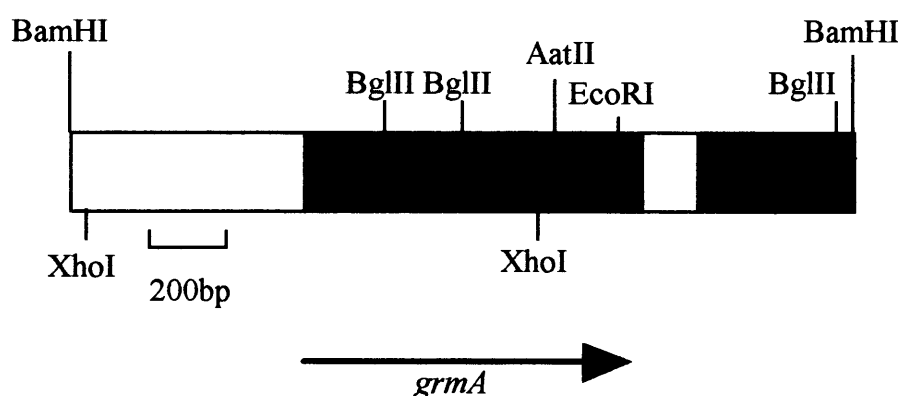


Figure 1.1

Shows the restriction map of the 1.9 kb *Bam*HI fragment containing *grmA* and part of the downstream gene.

The *grmA* gene of *M. purpurea* hybridises to DNA restriction fragments found in other aminoglycoside producing *Micromonospora* spp. For example it hybridizes strongly with an apparently homologous gene (*grmB*) present in *Micromonospora rosea* (Kelemen et al. 1991) producer of sisomicin. This indicates that resistance determinants in aminoglycoside producing *Micromonospora* spp. have similar sequences and function (Kelemen et al. 1991). The extent of the similarity between *grmA* of *M. purpurea* and *grmB* of *M. rosea* is 90% identity. The ORF downstream of *grmA* closely resembles the ORF downstream of *grmB* with 92% identity. Transcription of *grmA* and *grmB* in *S. lividans* initiated about 11 nucleotides upstream of the translational start site. In both

cases transcription appears to proceed into the downstream ORF, suggesting that the *grm* genes and their downstream genes are cotranscribed (Kelemen et al. 1991).

Sisomicin and gentamicin are closely related antibiotics. The similarities between *grmA* and *grmB* and their downstream ORFs indicate that biosynthetic or regulatory genes encoding products of similar function may be adjacent to the resistance genes in these *Micromonospora* spp. Biosynthesis of gentamicin has only been partially characterised in *M. purpurea* (Gonzalez et al. 1995) and it is not known whether the production of the antibiotic is organised and controlled in the same way as examples studied in *Streptomyces* spp. and other *Micromonospora* spp.

Primer extension analysis revealed that 16S rRNA extracted from *M. purpurea* was not methylated at G1389, although it had been previously demonstrated that ribosomes from this strain were constitutively resistant to gentamicin (Piendl and Böck 1982, Piendl et al. 1984). It therefore appears possible that *grmA* may be silent in its native host, in which case there must be another determinant responsible for the constitutively resistant phenotype being expressed.

There is evidence to suggest that another resistance gene does indeed exist in *M. purpurea* (Ohta et al. 1993, Ohta and Hasegawa 1993). Fortimicin A resistance gene, *fmrO*, is similar to *grmA* in that it confers resistance to kanamycin and gentamicin but not neomycin. The deduced amino acid sequence of FmrO showed an identity of 30.8% to GrmA of *M. purpurea* and 35.8% to GrmB of *M. rosea*, suggesting that *fmrO* encodes a 16S rRNA methyltransferase. The *M. purpurea* genome has been probed with *fmrO* (Ohta et al. 1993). The gene hybridised with a 6.1kb *Bam*HI fragment rather than a 1.9kb *Bam*HI fragment, as would have been expected for *grmA* from the work of Kelemen et al. (1991). There is further, stronger, evidence to suggest the presence of another resistance gene in *M. purpurea*: a novel gene distinct from *grmA*, and highly similar to *fmrO*, has apparently been cloned (Ohta and Hasegawa 1993). *fmrO* and *grmA* confer resistance to similar antibiotics. However, it has not been made clear whether this putative gene is the same as that found on the 6.1kb *Bam*HI fragment, described in the previous paragraph.

1.10 The regulation of gene expression during differentiation and secondary metabolism in actinomycetes.

Actinomycetes undergo a complex life cycle of morphological differentiation. *Streptomyces* spp. initially grow as substrate mycelium consisting of branched hyphae. The next phase of growth may be triggered by nutrient limitation. Aerial hyphae are synthesised from the products of substrate mycelial hydrolysis. The final step involves the septation of the aerial mycelium, to form spores. Development is often accompanied by the biosynthesis of secondary metabolites. Sporulation and antibiotic biosynthesis are dependent on differential gene activity. Some aspects of the two processes are coregulated and others seem to be controlled individually by distinctly separate genes.

Genes responsible for the formation of aerial hyphae and spores have been defined by mutations *bld* (Merrick 1976) and *whi* (Chater 1972) respectively and are not essential for vegetative growth. Unlike antibiotic-biosynthetic genes, they are not clustered but are scattered in the genome. Each stage in the morphological differentiation of *S. coelicolor* has been defined by *bld* or *whi* mutations. Eight *whi* genes (*whiA* to *whiL*) have been identified that are involved in the development of aerial hyphae into spores.

The *bld* regulatory cascade controls many aspects of aerial hyphae formation. One of the roles of the *bld* genes is their involvement in the extracellular signals that control SapB, a protein that coats the aerial filaments allowing them to overcome surface tension and disperse (Willey et al. 1991).

Differentiation can be suppressed by catabolite repression in the presence of a rich carbon source. It has been suggested that the *bld* cascade may also have a role in the control of cell signalling during differentiation in response to the nutritional state of the substrate mycelium (Pope et al. 1996).

The gene *bldA* is interesting because it encodes the leucyl tRNA that recognises the rare codon UUA (Leskiw 1991). Deletions in *bldA* interfere with aerial mycelium formation and antibiotic production, but do not have an effect on vegetative growth. Most TTA containing genes encode functions associated with the regulation of the onset

of morphological differentiation, antibiotic production, or antibiotic resistance; leading to the prediction that genes required for primary metabolism may be devoid of this rare codon (Leskiw et al. 1991a).

1.10.1 Differentiation and streptomycin biosynthesis in *Streptomyces griseus*

The genetics of differentiation and streptomycin biosynthesis have been extensively studied. The streptomycin biosynthetic cluster is a mixture of resistance, regulatory and biosynthetic genes. The regulation and expression of these genes involves a complex cascade of events.

The onset of differentiation and antibiotic production is controlled by a hormone known as A-factor (2-S isocapryloyl-3-R hydroxymethyl γ -butyrolactone) which is synthesised by gene *afsA*. Several A-factor homologues have been found to act as autoregulators in the control of antibiotic production and/or cell differentiation. An example are the virginiae butanolides that are involved in the control of virginamycin in *Streptomyces virginiae* (reviewed by Horinouchi and Beppu 1994). A regulatory gene, *afsR*, found in *S. coelicolor*, globally regulates the synthesis of A-factor and appears to be part of a two component sensor regulator in the cytoplasmic membrane (Horinouchi and Beppu 1992). The AfsR protein is phosphorylated by a eukaryotic type kinase encoded for by *afsK*. (Matsumoto et al. 1994). *afsR* and *afsK* are very similar to the *afsQ1/afsQ2* pair in *S. lividans* (Ishizuka et al. 1992).

A regulatory gene, known as *strR*, located upstream of the biosynthetic cluster is positively activated in the presence of A-factor (Vujaklija et al. 1991). Upon activation, A-factor binds to an A-factor-receptor-protein, ArpA (Onaka et al. 1995), which normally acts as a repressor for an unknown gene(s) (called X for the purposes of this introduction). Repression of X is alleviated and the protein product positively activates the expression of the A factor dependent protein which in turn binds to and activates expression from the *strR* promoter (Vujaklija et al. 1993). This results in the biosynthesis of streptomycin, sporulation and streptomycin resistance.

The resistance determinant, *aphD*, encodes a streptomycin phosphotransferase and is located adjacent to and downstream of *strR*. StrR positively activates the biosynthetic genes in the cluster by binding to multiple promoters within the region (Retzlaff and Distler 1995) (Figure 1.2). The *aphD* gene is not controlled by the StrR protein but by readthrough from the *strR* promoter. The purpose of this may be to ensure that the strain is resistant to the antibiotic, and therefore protected, before streptomycin is biosynthesised.

1.10.1.1 Quorum sensing-mediated control of antibiotic biosynthesis

Some Gram negative bacteria produce signalling molecules that trigger antibiotic biosynthesis. *Erwinia cartovora* is a plant pathogen that causes soft rot in green vegetables such as cabbage and broccoli. This bacterium produces a beta lactam antibiotic, 1-carbapen 2-em-3-carboxylic acid. The genes responsible for the biosynthesis of this antibiotic are found in an operon, providing another example of antibiotic biosynthetic genes clustered in the genome (Holden et al. 1998).

The release of plant tissue degrading enzymes and coordinated production of carbapenem occurs in response to an accumulation of a small diffusible pheromone, N-(3-oxohexanoyl)-L-homoserine lactone (OHHL). This response is also cell density dependent. Individual cells can sense how many neighbouring cells there are by the concentration of the pheromone being excreted. In this way, the bacteria can determine whether there are enough cells to initiate the expression of virulence genes and antibiotic biosynthetic genes. This is known as quorum sensing. Another example of quorum sensing is N-acyl-homoserine lactone (HSL) control of phenazine biosynthesis in *Pseudomonas aureofaciens* (Pierson et al. 1998).

N-acylhomoserine lactone-based quorum sensing systems are ubiquitous amongst Gram-negative bacteria. Scientists are now realising the great potential of blocking quorum sensing in the control of bacterial infection.

Figure 1.2

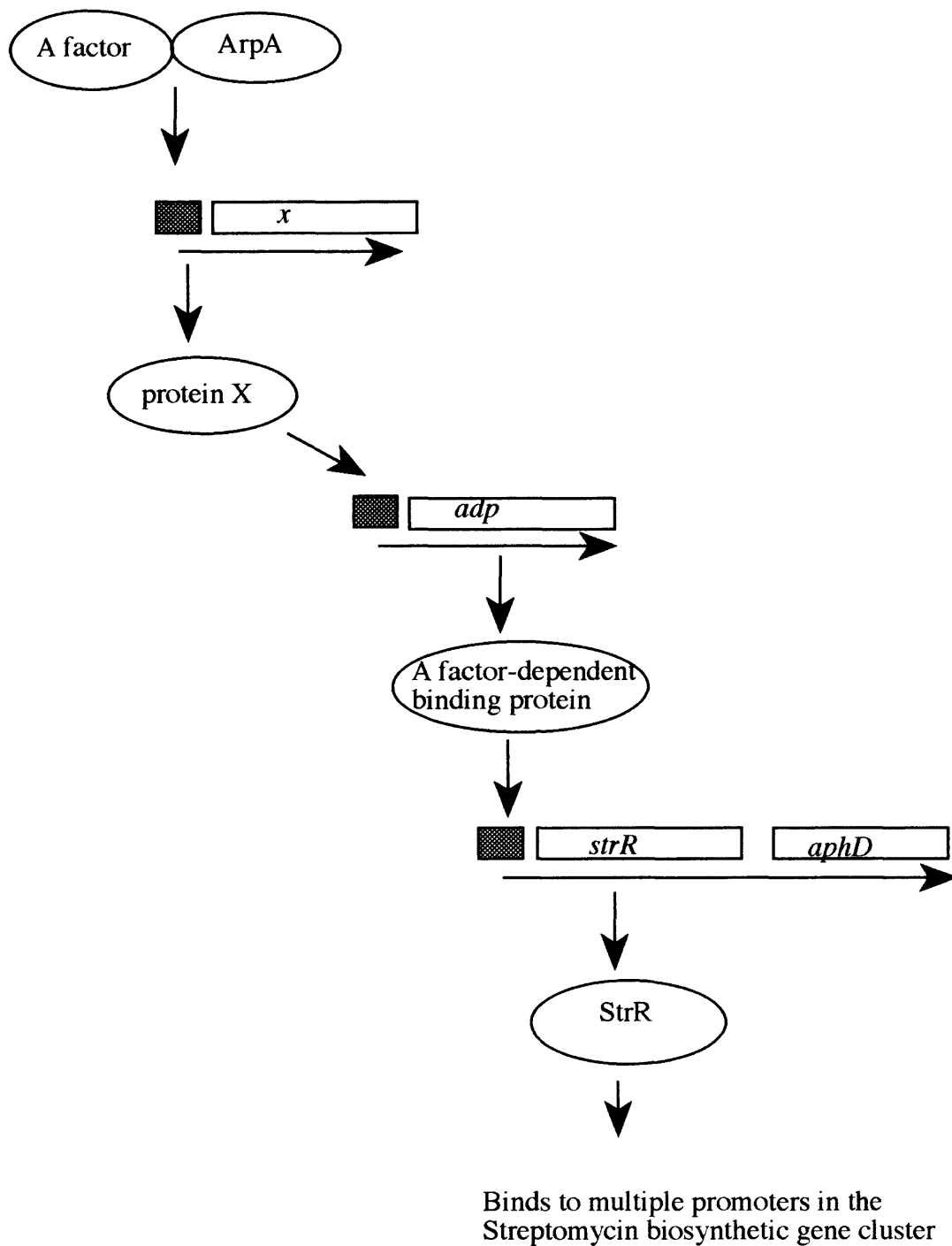


Figure 1.2 A model for regulation of streptomycin production in *S. griseus*
Details of the pathway are given in section 1.10.1

1.10.2 Actinomycete promoters

Gene expression in actinomycetes is controlled by a vast array of regulatory mechanisms. *E. coli* promoters generally fall into two categories; those that are recognised by the $E\sigma^{70}$ and those that fall into the $E\sigma^{54}$ category. However, in actinomycetes the situation is different. There is a wide variety of promoter sequences. Numerous, diverse regulatory mechanisms control transcription from these promoters.

There are two main groups of promoter sequences in *Streptomyces*: those that are similar to promoters recognised by *E. coli* $E\sigma^{70}$ like RNA polymerases and those that do not display typical -10 and -35 regions characteristic of *E. coli* promoters and are very diverse in their sequence (Strohl 1992).

1.10.3 RNA polymerase heterogeneity in *Streptomyces* spp

Sigma factors have an important function in the regulation of developmental processes in both Gram positive and Gram negative eubacteria. It has been established for some time that multiple sigma factors are required for the regulation of endospore formation in *Bacillus subtilis*.

The RNA polymerase holoenzyme of eubacteria consists of five subunits $\alpha\beta\beta'\sigma$. The sigma factor (σ) is required for the recognition of the promoter region by the RNA polymerase.

Holoenzymes have alternative sigma factors, which are required for the expression of specific genes. The genes in question are often involved in physiological change in response to an environmental stimulus. Four sigma factors are synthesised specifically during sporulation in *B. subtilis*.

E. coli has two main categories of sigma factor σ^{70} and σ^{54} and although specific sigma factors are required for the expression of genes involved in processes such as the heat shock response and chemotaxis, there are very few promoters that differ significantly from the σ^{70} consensus.

In actinomycetes, alternative sigma factors govern the the temporal and spatial changes in gene expression that occur during differentiation and sporulation. Six *whi* genes regulate the early stages of sporulation in *Streptomyces coelicolor* (reviewed by Chater 1993). In white colony mutants, the aerial mycelium remains white, instead of exhibiting the typical grey spore pigment. *whiG* encodes a sigma factor which is specifically involved in spore formation, and is essential for the initiation of spore septation. σ^{whiG} directs the transcription of at least one other early *whi* gene, *whiH*.

Another sigma factor, σ^{F} , found in *Streptomyces aureofaciens*, is involved in the late stages of spore maturation (Potuckova et al. 1995). There may be a cascade of events controlled by σ^{whiG} , σ^{F} and further sigma factors that govern the steps in sporulation between initiation and the formation of the mature spore.

It has been observed in *E. coli* and *B. subtilis* that alternative sigma factors are employed for specialist processes such as nitrogen metabolism and motility. This is also important in *Streptomyces* spp, when considering the role of σ^{whiG} in triggering the onset of sporulation. However, a variety of sigma factors have a pivotal role in the transcription of housekeeping genes. Examples of this are the galactose operon and the agarase gene *dagA* (Buttner et al. 1988) in *Streptomyces coelicolor*. The *dagA* gene is transcribed from four different promoters by at least three different RNA polymerase holoenzymes containing sigma factors, σ^{49} , σ^{28} and σ^{35} . Multiple promoters and sigma factors may allow the differential expression of *dagA* under changing growth conditions.

Tandem, temporally regulated promoters have also been discovered in *Micromonospora* species. A gene isolated from the calichemin producer, *Micromonospora echinospora*, has five promoters. The promoter P2 is maximally active during the exponential phase. The P1 cluster consists of four promoters, which are approximately 80 bp upstream of P2. This cluster is active during stationary phase and coincides with the biosynthesis of calchemicin (Baum et al. 1988).

1.10.4 Leaderless mRNA

In several genes, there is no leader sequence upstream of the translational start site so that transcription and translation occur from the same point in the mRNA. Some mRNAs do not possess an obvious Shine-Dalgarno site. Examples of this are the aminoglycoside phosphotransferase (*aph*) in *Streptomyces fradiae* (Janssen et al. 1989, Jones et al. 1992), and the chloramphenicol acetyltransferase (*cat*) in *Escherichia coli* (Wu and Janssen 1997). Eukaryotic organelles also possess genes of this type (Montoya et al. 1981).

The *aph* gene from *S. fradiae* confers resistance to neomycin. Translation of this gene occurs in the absence of a conventional Shine-Dalgarno site. Mutation of the mRNA by the addition of a small leader sequence and/or an alternative initiation site at the 5' end results in differences in the level of translation. This suggests that the position of the start codon with respect to the 5' end is important in determining the efficiency of translation from the gene (Jones et al. 1992). The *aph* gene is expressed in *S. lividans* indicating that *S. lividans* and presumably other *Streptomyces* spp. can translate mRNA with this structure.

1.11 The regulation of genes that encode ribosomal methylases

When considering the expression of genes, whether they are housekeeping genes or those involved in secondary metabolism, it is important to understand the significance of the DNA surrounding the transcriptional and translational start sites. This can apply to the length of the DNA and secondary structure of the mRNA.

The distance between the transcription start site and the initiation site for translation can vary considerably between approximately 10 to 300 nucleotides in length (except for those discussed in section 1.10.4). Long 5' untranslated regions (UTR) can contain extensive secondary structure that is involved in regulation either at the transcriptional or translational level. Some of these mechanisms are described in sections 1.11.1 to 1.11.4

1.11.1 Translational attenuation

A group of ribosomal methylases known as the *erm*-type genes confer resistance to macrolide, lincosamide and streptogramin type B antibiotics (MLS antibiotics). They are found in a range of bacteria, both Gram positives and Gram negatives.

The MLS antibiotics act by binding to the 50S subunit of the bacterial ribosome. Resistance to these antibiotics due to *erm* genes is mediated by the methylation of the 23S rRNA, which alters the binding specificity of the antibiotic to the ribosome.

MLS resistance can be either constitutive or inducible. The gene, *ermC*, originating from *Staphylococcus aureus* and being an example of the latter, is regulated by translational attenuation.

Elements essential for induction have been identified (Weisblum 1984, Mayford and Weisblum 1989) (Figure 1.3). There are two Shine Dalgarno sequences, one of which precedes the transcriptional start site (SD-1). A few bases downstream of this, is the start of an open reading frame that extends over 19 amino acids and terminates at

UAA. The other Shine-Dalgarno site (SD-2) is approximately 8 nucleotides upstream of the translational start site for the methylase. The mRNA encoding the methylase is synthesised constitutively in an inactive form. The 5' end of the transcript contains 4 sets of inverted repeats (regions 1, 2, 3, and 4) downstream of SD-1. Regions 1+2, 3+4, and 2+3 are complementary. The message remains inactive in the uninduced state due to the pairing of regions 3+4 in a hairpin loop structure, which sequesters both SD-2 and the translational start site for the methylase. Regions 1+2 are also paired to form a similar structure. The 19 amino acid leader is translated by the ribosome. However in the induced state, erythromycin binds to the ribosome, causing it to stall. This in turn frees regions 1 and 2 from each other and regions 2 and 3 pair up. Subsequently region 4 is no longer paired to region 3 making SD-2 accessible to ribosomes for methylase synthesis.

Although erythromycin is the inducer in this case, the antibiotic is also an inhibitor of protein synthesis. The two do not seem compatible because after initiation of methylase synthesis, erythromycin would be expected to bind the ribosome and inhibit elongation. However, this does not happen. At low concentrations of erythromycin the ribosomes are not saturated, allowing some ribosomes to be free to bind the ribosomal binding site. Eventually the level of erythromycin-resistant ribosomes would reach a critical level and there would not be enough erythromycin-bound, sensitive ribosomes to stall at region 1. Therefore, the synthesis of the methylase would become self-limiting.

There are other factors involved in the translational regulation of *ermC* other than attenuation (Breidt and Dubnau 1990). For an account of this see "translational autoregulation" in section 1.11.4.

1.11.2 Transcriptional attenuation

The gene, *tlrA*, from *Streptomyces fradiae* was once thought to be regulated by translational attenuation (Kamimya and Weisblum 1988). However, a different mechanism of regulation has been found to control *tlrA*, termed transcriptional attenuation (Kelemen et al. 1994). Many of the features described for translational attenuation are present upstream of *tlrA*, including a large leader region, containing extensive secondary structure and a preceding putative ribosome binding site.

Resistance to tylosin in the producer, *Streptomyces fradiae* is conferred by two resistance genes, *tlrA* and *tlrD*. The gene products of both of these resistance determinants methylate residue A-2058 in the 23S rRNA. The TlrD enzyme is expressed constitutively and monomethylates the adenine residue whereas the expression of TlrA is induced and it will either dimethylate ribosomes that are un-methylated at this site or increase the level of tylosin resistance, by adding a second methyl group to previously monomethylated ribosomal RNA (Kelemen et al. 1994).

Tylosin and all intermediates of the tylosin biosynthetic pathway apart from tylactone cause induction of *tlrA* in *S. fradiae*. However in the absence of *tlrD* the compounds that cause induction are different. Only those intermediates that were modified with one sugar can induce *tlrA*, suggesting that *tlrD* affects the induction specificity of *tlrA*. In the uninduced state a truncated transcript is made. However when *tlrA* is induced transcription proceeds through the entire open reading frame.

1.11.3 Complex regulatory cascades

The streptomycin resistance determinant, *aphD*, (encodes streptomycin-6-phosphotransferase) is transcribed by read-through from the *strR* promoter. This seems to be an efficient way of conferring resistance upon the cell before streptomycin is synthesized. The regulation and expression of these genes involves a complicated cascade of events described in section 1.10.1.

1.11.4 Translational autoregulation

The induction of *ermC* is further controlled by a methylase-mediated feedback loop (Denoya et al. 1986, Breidt and Dubnau 1990). As with translational attenuation and transcriptional attenuation, features can be identified in the DNA surrounding the initiation site for translation and the transcriptional start site, that are common to genes regulated in this way. However, there are also structural similarities between the site on the 23S rRNA, where the ErmC methylase binds, and the mRNA sequestered by the second ribosome binding site. They share the same sequence AAAGA, which is folded in a similar conformation.

Mutants with lesions in the structural gene produce a truncated inactive methylase. In these mutants, overproduction of the truncated methylase indicates that synthesis of active form of the enzyme is required for autogenous control of translation of *ermC* (Denoya et al. 1986)

The gene, *ksgA*, found in *E. coli* is a methyltransferase that methylates two adjacent adenosine residues at the 3' end of the 16S rRNA. This methylation confers kasugamycin sensitivity. The gene has a weak promoter and lacks an obvious ribosome binding site and leader peptide. However, the gene exhibits some characteristics of translational autoregulation in that the enzyme shows affinity for binding its own message (van Gemen et al. 1989). Around 90 nucleotides upstream of the translational start codon is a sequence that is identical to a sequence around the sites of action of KsgA. This property is consistent with other genes known to be regulated in this way such as *sgm* (Kojic et al. 1996) and *kgmB* (Holmes and Cundliffe 1991) discussed in Chapter 3, section 3.1.2.

1.12 Project aims

The first aim of this project was to determine why 16S rRNA in *M. purpurea* was not methylated at residue G1389 and thereby learn more about the expression of *grmA*. One of the objectives of the project was to overexpress *grmA* in *M. purpurea* and titrate out a putative repressor of the gene. Reverse transcriptase assays could subsequently be used to analyse changes in the methylation of the RNA.

The second aim of the project was to determine if there is another gene encoding gentamicin resistance in *M. purpurea* and whether the gene product is a methylase with a different site of action from the methylase encoded by *grmA*.

1.13 Thesis Layout

The results chapters presented in this work are not written in the chronological order in which the work was done. However, they are laid out in the most logical and interesting, manner for the reader.

The work carried out to investigate the expression of *grmA* spans two chapters. The primer extension analysis described in Chapter 4 follows on from the conjugal transfer of *grmA* into *M. purpurea* in Chapter 3. Experiments to look for alternative resistance genes were carried out concurrently with the experiments to optimise the conjugation procedure in Chapter 3. This explains why *grmA* introduced into *M. purpurea* by conjugal transfer (Chapter 3) was isolated from the original shotgun cloning experiment.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

Most of the methods used are described here. Those that were developed or adapted from previously published methods are described in the relevant chapters.

2.0 Media

YEME/PEG broth (supplemented with MgCl₂ and glycine)

(Hopwood et al 1985)

The solution was made up as follows:

Polyethylene glycol 6000	5 g
Difco yeast extract	3 g
Difco peptone	5 g
Oxoid malt extract	3 g
Glucose	10 g
MgCl ₂	1 g
Glycine	5 g

The ingredients were dissolved in 1 litre of super Q water (SQ water)

NE Agar

The solution was made up as follows:

Glucose	10 g
Difco yeast extract	2 g

Beef extract	1 g
Casamino acid	2 g

The ingredients and 20 g of agar were dissolved in 1 litre of SQ water. The pH was then adjusted to 7.2 using 1M KOH, prior to autoclaving. NEF agar was prepared in the same way, but it contained half the concentration of the ingredients for NE, apart from the agar.

YEME/Sucrose (supplemented with MgCl₂ and glycine)

(Hopwood et al. 1985)

The solution was made up as follows:

Difco yeast extract	3 g
Difco peptone	5 g
Oxoid malt extract	3 g
Glucose	10 g
Sucrose	340 g
MgCl ₂	1 g
Glycine	5 g

The ingredients were dissolved in 1 litre of SQ water.

LB broth and agar

The solution was made up in the following way:

NaCl	10 g
Difco yeast extract	5 g
Bacto tryptone	10 g

The ingredients were dissolved in 1 litre of SQ water. To make solid LB, 20g of agar was added.

All media was autoclaved for 20 minutes at a of pressure of 15 psi.

2.1 Bacterial strains

The strain of *Micromonospora purpurea* used was DSM43036 obtained from the German collection of microorganisms in Göttingen. It was grown at 30°C in YEME/PEG broth or on NE agar. Stocks of *M.purpurea* were maintained as hyphal fragments in 5% DMSO and stored at -70°C. *Streptomyces lividans* (*S. lividans*) strains 0S456 and TK21 were grown at 30°C in YEME/sucrose broth or on NEF agar. Stocks were maintained as spore suspensions (Hopwood et al. 1985) in 20% glycerol and stored at -20°C. *Escherichia coli* (*E. coli*) strains were grown in LB broth or on LB agar. Stocks were maintained in 20% glycerol (v/v) and stored at -20°C.

2.2 Antibiotics used

Spectinomycin dihydrochloride, gentamicin sulphate, kanamycin sulphate, and ampicillin (sodium salt) were all supplied by Sigma. Thiostrepton was supplied by Squibb and Sons. Apramycin was supplied by Eli Lilly. All these antibiotics were dissolved in SSQ water and filter sterilised apart from thiostrepton which was dissolved in DMSO.

2.3 Methods for DNA preparation and transformation.

2.3.1 Isolation of *Streptomyces* plasmid

Reagents

Lysozyme solution.

0.3 M sucrose

25 mM Tris-HCL (pH 8.0)

25 mM Na₂EDTA (pH 8.0)

2 mg/ml Lysozyme

Alkaline SDS solution

0.3 M NaOH

2% SDS

This was made on the day it was required.

Procedure

Plasmids were isolated from *Streptomyces* strains according to Hopwood et al. (1985). Spores were grown in 50 ml of YEME/sucrose for about 48 hours at 30°C, shaking at 150 rpm. The cells were harvested by centrifugation for 10 minutes at 3, 500 rpm. Cells were washed twice with 10% sucrose and then resuspended in 5 ml of lysozyme solution. After incubation at 30°C for 30 minutes, 2.5 ml of alkaline SDS solution was added, mixed immediately, and incubated at 70°C for 20 minutes. Once cool, 2.5 ml of acid phenol/chloroform was added, mixed by vortexing for 5 minutes and spun down at 3, 500 rpm for 15 minutes. Two further phenol/chloroform (neutral pH) extractions were performed in the same manner. To precipitate the plasmid, 1/10 of the volume of 3M sodium acetate and 2 volumes of 100% ethanol were added to the supernatant and it was incubated at -20°C for 1 hour. The DNA was collected by spinning it at 3, 500 rpm for 15 minutes, followed by a wash in 2 ml of 70% (v/v) ethanol. The plasmid was vacuum dried and dissolved in SSQ water.

2.3.2 Transformation of *Streptomyces* spp

Hopwood et al. 1985

Media used

R2YE agar (Regeneration media)

The following were added together:

Sucrose	124 g
K ₂ SO ₄	0.3 g
MgCl ₂ .6H ₂ O	12.14 g
Glucose	12 g
Casamino acids	0.12 g

The ingredients were dissolved in 1 litre of SSQ. 6.6 g of agar was added to a 400 ml bottle. To this was added 270 ml of the above solution. The medium was then autoclaved. Before use the medium was remelted, cooled to 45°C and the following sterile solutions were added to each 400 ml bottle:

Trace elements	0.6 ml
0.5% KH ₂ PO ₄	3 ml
20% Proline	4.5 ml
1 M NaOH	1.5 ml
22.92% TES- NaOH pH7.2	6.54 ml
1 M CaCl ₂	5.7 ml
20% Difco yeast extract	7.5 ml

Trace elements solution

The following were added together:

ZnCl ₂	40 mg
FeCl ₃ .6H ₂ O	200 mg
CuCl ₂ .2H ₂ O	10 mg

MnCl ₂ .4H ₂ O	10 mg
Na ₂ B ₄ O ₇ .10H ₂ O	10 mg
(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	10 mg

The ingredients were dissolved in 1 litre of SQ water

Reagents

L buffer

10.3% sucrose	10 ml
22.92% TES	250 µl
2.5% K ₂ SO ₄	100 µl
trace elements	20 µl
0.5% KH ₂ PO ₄	100 µl
50% MgCl ₂	10 µl
1 M CaCl ₂	25 µl

P buffer

Sucrose	10 g
2.5% K ₂ SO ₄	1ml
50% MgCl ₂	400 µl
trace elements solution	200 µl

90 ml of water was added to this and then it was autoclaved. Before use the following were added:

0.5% KH ₂ PO ₄	1 ml
1 M CaCl ₂	2.5 ml
22.92% TES-NaOH (pH 7.2)	2.5 ml

Leicester *Streptomyces* transformation buffer

The following sterile solutions were added together:

SSQ	7.5 ml
10% Sucrose	2.5 ml
Trace elements	20 µl
2.5% K ₂ SO ₄	100 µl

The ingredients were mixed together and 700 µl was removed and discarded. The 700 µl volume removed was replaced by 200 µl of 5M CaCl₂ and 500 µl of 1M Tris malic acid. 3 g of PEG 1000 was autoclaved and whilst molten was added to 9 ml of the above. The transformation buffer was then aliquoted into 200 µl volumes and frozen at -20°C.

Procedure

2.3.2.1 Preparation of protoplasts

Cells were grown in the same way as those used to isolate plasmid. They were harvested after approximately 36 hours of growth by spinning them at 3, 500 rpm for 10 minutes.

The mycelium was washed twice in 10% sucrose and resuspended in 5 ml of L buffer containing lysozyme (1 mg/ml final concentration). This mixture was then incubated for 30 minutes at 37°C.

The development of protoplasts was monitored by looking at the lysing culture under the microscope at 15 minute intervals. After 30 minutes of incubation, cells were triturated using a 5 ml pipette and incubated for a further 15 minutes.

The protoplasts were filtered through a sterile plastic tip (used for Gilson P5000) plugged loosely with sterile cotton wool to remove undigested mycelium. Approximately 10 ml of P buffer was added to the protoplasts and they were sedimented gently by centrifugation at 2000 rpm for 5 minutes. The protoplasts were washed in 10 ml of P buffer and spun down again as before. They were gently resuspended in 400 µl of P

buffer. The protoplasts were aliquoted into 50 µl volumes. The aliquots were frozen rapidly in dry ice/IMS and then transferred to -70°C.

2.3.2.2 Transformation

5 µg of DNA (in a volume of 5 µl), was added to 50 µl of protoplasts (5×10^9) that had been allowed to thaw out slowly on ice. The tube was tapped gently, immediately after the DNA had been added. 200 µl of transformation buffer was added and mixed in by pipetting up and down.

The protoplasts were then spread on to 2 R2YE plates (120 µl on each) which were incubated at 30°C. After 20 hours of incubation, the plates were overlayed with 1 ml of SSQ water containing the appropriate antibiotic selection. In the case of pIJ702 derived vectors the antibiotic used was thiostrepton (20 µg/ml). The protoplasts were incubated at 30°C, for 4-6 days to allow them to regenerate.

2.3.3 Preparation of *E. coli* plasmid

Plasmid DNA used for restriction mapping, transformations and conjugation experiments was isolated from *E. coli* by adaptation of the alkaline lysis method according to Sambrook et al. (1989).

Reagents

TEG

100 mM Na ₂ EDTA (pH 8.0)	8 ml
1M TRIS-HCl (pH 8.0)	2 ml
1.4 M glucose	2.8 ml

SSQ water	67.2 ml
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NaOH/SDS

200 mM NaOH

1% SDS

Potassium acetate solution

5 M KAc	60 ml
Acetic acid	11.5 ml
SSQ water	28.5 ml

Procedure

A flask containing 50 ml of LB broth was inoculated with *E. coli* colonies from an LB plate. The culture was grown for 24 hours at 37°C, shaking at 150 rpm, in presence of antibiotic to select for the plasmid.

The cells were harvested by spinning them down at 3,500 rpm. They were resuspended in 1 ml of TEG. 2 ml of NaOH/SDS was added and the cells were inverted to mix them and placed on ice.

After lysis was evident, which took only a couple of minutes, 1.5 ml of potassium acetate solution was added. This was mixed in by vortexing and left on ice for 15 minutes. The precipitate was spun down and the supernatant saved.

To this was added RNase A (Sigma) to give a final concentration of 30 µg/ml. the supernatant was incubated for 1 hour at 30°C. An equal volume of phenol/chloroform (pH 7.0) was mixed in by vortexing and spun at 3, 500 rpm for 10 minutes. The aqueous layer was removed and added to 1/10 of the volume of 3 M sodium acetate (pH 4.8) and 2 volumes of 100% ethanol. This was incubated at -20°C

for 20 minutes and then spun down. The pellet was washed in 70% ethanol, vacuum dried, and dissolved in 200 µl of SSQ water.

Plasmid was "miniprep'd" from transformed colonies in the same way but the volumes of reagents used were scaled down by a factor of 10.

2.3.4 Transformation of *E. coli*

An adaptation of the protocol according to Sambrook et al. (1989) was followed.

Reagents

Buffer A (prepared on the day)

The following were added together then placed on ice

100 mM MOPS (pH 7.0)	1 ml
100 mM RbCl ₂	1 ml
SSQ water	8 ml

Buffer B (prepared on the day)

The following were added together and placed on ice.

1 M MOPS (pH 6.5)	1 ml
100 mM RbCl ₂	1 ml
100 mM CaCl ₂	1 ml
SSQ water	7 ml

Procedure

Cells were grown overnight in LB broth as previously described for the method of plasmid isolation. 0.5 ml of this culture was used to inoculate 50 ml of LB broth. This was incubated for approximately 2 hours until the optical density at 600 nm reached 0.4-0.5. For *E. coli* S17-1 a longer incubation period was required.

Cells were harvested and resuspended in 4 ml of cold A buffer, and spun down by centrifugation briefly at 12,500 rpm. The supernatant was removed and the pellet was further resuspended in 4 ml of cold B buffer and incubated on ice for 45 minutes. Cells were spun down again briefly and resuspended in 2 ml of B buffer. Plasmid DNA (1-5 µg) was added to 100 µl of the competent cells, mixed in gently and incubated on ice for 30 minutes

Cells were heat shocked for 30 seconds at 50°C and placed on ice. 1 ml of LB broth was added and they were incubated for 1 hour at 37°C. After this time 120 µl was plated out on LB agar containing antibiotic selection for the plasmid and incubated at 37°C overnight. Cells containing vectors derived from pUC18, pHJL401, and pCR2.1 were grown in the presence of ampicillin (100 µg/ml), whereas cells containing pSET152 or pOJ260 derived vectors were grown in apramycin (25 µg/ml).

2.4 Preparation of genomic DNA from *M.purpurea*

The method used according to Chater et al. (1982) with a few adaptations

Total DNA from *M. purpurea* was prepared from a 100 ml culture of YEME/PEG previously inoculated with 200 µl of hyphal fragments and incubated for 3 days at 30°C.

Modifications were made to the cited protocol as problems were encountered with attempts to lyse *M. purpurea* cells. A lysate was prepared by first harvesting the cells and washing these twice in 10% sucrose.

Then 10 ml of lysozyme solution was added containing lysozyme at a concentration of 10 mg/ml; five times the recommended amount of lysozyme used for the preparation of genomic DNA from *Streptomyces*. Cells were incubated at 37°C for 90 minutes in lysozyme solution. 2.4 ml of 0.5 M Na₂EDTA was then added and the lysate was incubated further at 37°C.

After 15 minutes 1.4 ml of 10 % SDS was added and incubated at 50°C for 90 minutes. A further incubation at 60°C for 10 minutes was then performed.

The clear lysate was cooled and an equal volume of phenol/chloroform was added. This was mixed gently on a magnetic stirrer for approximately 10 minutes until the mixture was entirely homogenous. This mixture was transferred to 30 ml glass corex tubes and spun at 10 000 rpm for 15 minutes. The aqueous layer was removed and a further chloroform wash was performed in the same way to remove traces of phenol.

The chromosomal DNA was precipitated by the addition of 1/10 of the volume of 3 M sodium acetate (pH 4.8) and 2.5 x volume of ice cold ethanol. The DNA was then carefully spooled out using a glass rod that had been flamed in ethanol. The DNA was washed in 70% ethanol and then with 100% ethanol and allowed to dry.

The DNA was dissolved in SSQ water overnight. Once the DNA had dissolved it was treated with RNase A at a final concentration of 30µg/ml and incubated at 37°C for 1 hour. Phenol/chloroform extractions and precipitation were repeated as before. The DNA was spooled, washed and redissolved in 0.5 ml of SSQ water. Due to the success of this method, it was also applied to *Streptomyces* spp

The concentration of the genomic DNA isolated was measured by ultraviolet absorbance spectrophotometry. 50 µg/ml of double stranded genomic DNA is equivalent to 1 unit at wavelength 260 nm. The purity was measured by dividing the measurement taken at a wavelength of 260 nm through by a measurement taken at 280 nm. A pure sample of DNA had a value of 1.8. A value less than this indicates that the preparation was contaminated, either with protein or with phenol.

This method was also applied to determine plasmid DNA concentration and purity.

2.5 Isolation of total RNA

2.5.1 Precautions used to prevent RNase contamination

This was an important consideration when preparing and carrying out experiments with RNA. The introduction of RNases which are present on glassware and in solutions, can degrade the RNA. A few simple precautions were taken in the following experiments. All glassware used was baked at 180°C for a minimum of 16 hours. Where ever possible plastic was substituted for glassware as this is often sterilized by irradiation which destroys RNases. All solutions and water were filter sterilized using Sartorius filter flasks (pore size 0.2µM) unless otherwise stated. The reagents used in the solutions were restricted to RNA work. Latex gloves were always worn when they were handled and the use of spatulas was avoided. These precautions were also applied to experiments in Chapters 3 and 4.

2.5.2 The method of isolation of total RNA

Reagents

P buffer

The P buffer used for RNA isolation was made in the same way as the P buffer used for transformation of *Streptomyces* protoplasts (section 2.3.2). The only difference was that all the precautions described in section 2.5.1 were applied.

Double strength modified Kirby mix

2% SDS

100 mM Na₂EDTA (pH 8.3)

12% phenol

100mM Tris HCl (pH8.3)

Neutral phenol/chloroform

Phenol and chloroform were supplied individually by Sigma. Both were RNase free. A bottle of equilibration buffer was provided with the phenol. The two were mixed together which adjusted the pH of the phenol to pH 6.7 to 7.9. The phenol was left to equilibrate for 3 hours at 4°C. After this time an equal volume of chloroform was mixed in.

Procedure

The method according to Hopwood et al. (1985) was used. The procedure was carried out quickly and sterilely (near a bunsen flame), wearing latex gloves throughout. All the apparatus and solutions were chilled before hand. 120µl of *M. purpurea* hyphal fragments or 120µl of *S. lividans* spores were used to inoculate 100ml of YEME/PEG broth. The cultures were grown up for 3 days.

Cells were spun down briefly for 3 minutes at 3500 rpm at 4°C in 50 ml plastic tubes. The supernatant was discarded. The pellet was resuspended in 3 ml of P buffer containing 20 mg/ml of lysozyme. This was incubated for 2 minutes at 37°C.

After this time 3ml of double strength modified Kirby mix was added. This was vortexed for 2 minutes with glass balls to break up the mycelium. To this was added 5ml of phenol/chloroform. The mixture was vortexed again until homogenous and spun for a further 5 minutes at 4,000 rpm at 4°C.

The upper phase was removed and further 5ml of phenol/chloroform added. The mixture was again vortexed, until homogenous and spun at 4,000 rpm for 5 minutes at 4°C. This step was repeated.

The supernatant from the final extraction was transferred to a glass corex tube. To this, 1/10 volume of 3 M sodium acetate, and an equal volume of isopropanol were added. The mixture was left at room temperature to precipitate nucleic acids. After 15

minutes nucleic acid was recovered by spinning it down at 8000 rpm for 10 minutes, rinsed with absolute ethanol, and vacuum dried for 10 minutes.

Once dry, the pellet was dissolved in 1ml of water. To this was added 3 volumes of 4 M sodium acetate. The RNA was left to precipitate at -20°C overnight. After this time, the precipitated RNA was spun down at 8000 rpm for 10 minutes. The supernatant was poured off and the pellet was rinsed in 70% ethanol, vacuum dried, and resuspended in 500 µl of water. The RNA was then quantified and checked for signs of degradation. This was achieved, firstly, by measuring the optical density, and secondly, by separating the RNA by electrophoresis under denaturing conditions in 1% agarose containing 2.2 M formaldehyde.

The optical density was measured as follows: 10 µl of RNA solution was placed in 990 µl of water in a 1ml quartz cuvette. An A₂₆₀ nm reading was taken. 1 unit at 260 nm is the equivalent to 44.1 µg of total RNA. To check how pure and un-degraded the RNA was, a further reading at A₂₈₀ nm was taken. The A₂₆₀ nm reading was divided through by a reading at A₂₈₀ nm to give a ratio. A ratio higher than 2 indicated that the RNA had degraded and a ratio lower than 2 showed the presence of an unacceptably high level of salt or DNA. Preparations containing too much salt were washed in 70% ethanol, vacuum dried, and redissolved in water.

2.5.3 Denaturing RNA gel electrophoresis

Another method employed to confirm whether the message was intact was to visualize the RNA on a 1% agarose gel containing ethidium bromide. The RNA was run under denaturing conditions. Two bands could be identified. The bands of RNA corresponded to ribosomal RNA. 16S rRNA could be seen at approximately 1.6 kb and 23S rRNA at 3.0 kb.

Reagents

10X MOPS buffer

0.2 M MOPS (pH 7.0)

0.05 M sodium acetate

0.01M Na₂EDTA (pH8.3)

Loading buffer

50% glycerol

1 mM EDTA

0.4% Bromophenol Blue

0.4% Xylene Cyanol

20X SSC

Same recipe used for Southern analysis (section 2.8)

2.5.3.1 Preparation of deionised formamide

Formamide freezes at 2°C, therefore any aliquot stored at -20°C which was still liquid, had perished. 30ml of formamide was poured into a glass beaker with a magnetic stirrer. The beaker was put on ice and stirred very gently overnight. The next day, the liquid was discarded, leaving pure solid formamide. This was allowed to thaw at room temperature and deionized by stirring gently with Biorad 20-50 mesh AG501-X8(D) mixed bed resin. After 2 hours the formamide was aliquoted into 500 µl volumes and frozen at -20°C.

2.5.3.2 Preparation of sample buffer and denaturation of the RNA.

4.5 µl of RNA was added to 2 µl of 10x MOPS gel buffer; 3.5 µl of 12.3 M formaldehyde and 10 µl of deionized formamide. The mixture was incubated at 55°C for 10 minutes to denature the RNA. 2 µl of loading dye and 1 µl of ethidium bromide (10 mg/ml) were added to the RNA before it was loaded. The gel was run at 100-150 volts for about 3 hours.

2.5.3.3 Methods for gel preparation

Agarose was melted in SSQ water to give a final concentration of 1%. This was then cooled to 60°C in a water bath. 1x MOPS buffer was added. Formaldehyde was added (37% 12.3 M pH 4.0 supplied by Fisons) to give a final concentration of 2.2 M. The gel was poured in the fume hood. Once set, the gel was allowed to soak in 1 X MOPS buffer for at least 1 hour, otherwise surface tension would result in the loss of the samples during loading.

2.6 RNA dot blot analysis

Dot blot analysis provided an efficient way of determining if a specific message was present. The technique would also allow the rapid detection of the relative amount of the specific transcript of interest.

Reagents

Denaturing solution

10 mM NaOH

1 mM Na₂EDTA (pH 8.3)

Phosphate buffer pH 7.2

1 M Na₂HPO₄ 72 ml

1 M NaH₂PO₄ 28 ml

Hybridisation buffer

1 mM Na₂EDTA

0.25 M Na₂HPO₄ pH 7.2

7% SDS

Procedures

2.6.1 Blotting

For the purpose of these experiments a Biorad Dot blot manifold was used and set up according to the manufacturers guidelines using nylon Zetaprobe membrane (supplied by Biorad). Prior to blotting the RNA was treated with DNase 1 (RNAse free supplied by Beohringer Mannheim). The RNA was incubated a 37°C for 1 hour in the presence of DNase 1 at a concentration of 1 unit per µg of RNA.

The RNA was dissolved in 200 µl of denaturing buffer and loaded into individual wells. The vacuum was applied until the membrane was dry. A further 200 µl of denaturing solution was added to wash the membrane and again the vacuum was reapplied. The dot blot apparatus was dismantled. The membrane was removed and baked for one hour at 80°C. It was stored between two sheets of filter paper at room temperature until required.

2.6.2 Hybridisation

The membrane was incubated in 25 ml of hybridisation buffer containing 100 µl denatured salmon sperm DNA (10 mg/ml) in a shaking hybridisation chamber for 1 hour at 65°C. The labelled probe was denatured by boiling for 3 minutes. It was snap-cooled on ice. The probe was added to the chamber and the membrane incubated for 16 to 18 hours at 65°C.

2.6.3 Washing and autoradiography

Unincorporated radioactivity was removed by washing the membrane with 2 x SSC + 0.1% SDS, for 7 minutes at 65°C. Excess liquid was removed from the membrane by blotting it onto filter paper. The membrane was wrapped in Saran wrap and laid down next to X ray film and incubated at -70°C for 48 hours.

2.7 Preparation of ribosomal RNA

The preparation of rRNA involved several steps. Firstly a 30 000 x g supernatant was prepared from which high salt washed ribosomes were isolated. The rRNA was then extracted from these. Precautions to prevent RNase contamination (section 2.5.1) were applied at every stage.

2.7.1 Preparation of S30 (30,000 x g supernatant)

(Skeggs et al. 1985, personal communication Dr. S. A. Fish)

Reagents

Washing buffer

10 mM HEPES-KOH (pH7.5)

10 mM MgCl₂

1M NH₄Cl

5 mM β -mercaptoethanol

S30 buffer

50 mM HEPES-KOH (pH 7.5)

10 mM MgCl₂

50 mM NH₄Cl

5 mM β -mercaptoethanol

Stock solutions of 1 M HEPES-KOH (pH 7.5), 1 M MgCl₂ and 4 M NH₄Cl were made with SSQ water, treated with DEPC (Sigma) according to manufacturers instructions, and then autoclaved. HEPES-KOH is unstable in the presence of DEPC, therefore it was made with SSQ water that had been previously DEPC treated and autoclaved.

A starter culture of *M. purpurea* was set up by inoculating 50 ml of YEME/PEG with 600 μ l of *M. purpurea* stock (section 2.1). Following incubation for 3 days at 30°C with shaking at 150 rpm, 10 ml of the starter culture was used to inoculate three baffled flasks, each containing 1 litre of YEME/PEG.

The *Streptomyces lividans* inoculum was prepared differently. Plates of NEF (section 2.0) were spread with 50 μ l of spore suspension (section 2.0). These were incubated at 30°C for 4 days. Once they had sporulated, 5 ml of SSQ water containing 0.1% Triton X100 was applied to the surface of each plate. The spores were brought

into solution by gently scraping the surface with a sterile loop. They were removed, spun down at 3,000 rpm for 5 minutes and resuspended in TES buffer (0.05 M pH 8.0) They were then heat shocked at 50°C for 10 minutes. 3 flasks containing 1 litre of YEME/PEG (section 2.0) were inoculated with spores. One plate of spores was used to inoculate each flask.

The 1 litre cultures were incubated at 30°C for 18 hours whilst shaking at 250 rpm. The standard protocol according to Skeggs et al. (1985) was then followed apart from a few adaptations (personal communication Dr. S. A. Fish).

The mycelium were harvested by centrifugation at 7×10^3 rpm for 10 minutes at 4°C (using Beckman JA10 rotor). The cells were then washed twice in washing buffer (1 litre of washing buffer per litre of cells) and once in S30 buffer (same quantities as the washing buffer). The cells were spun in between each wash at 7×10^3 rpm

The washed cells were resuspended in S30 buffer and filtered through Whatman No.1 paper in a Buchner funnel, until they were as dry. The mycelium were weighed and resuspended in S30 buffer +10% glycerol (2.5ml/g of cells). Clumps were removed with a sterile spatula to give a smooth suspension.

The french press cell was autoclaved and rinsed through with SSQ water and S30 buffer before use. The smooth cell suspension was pressed at 10-12 x 10^3 psi. The pressing was repeated with DNase I added (5 µg/ml mycelial suspension, RNase free, supplied by Boehringer Mannheim).

This was then centrifuged at 15×10^3 rpm for 30 minutes at 4°C (using a Beckman JA21 rotor). The supernatant was removed and recentrifuged at 15×10^3 rpm for 30 minutes at 4°C. The resulting S30 extract was aliquoted into 100 µl portions, quick frozen in dry ice/IMS and transferred to -70°C.

2.7.2 Preparation of high salt washed ribosomes

(Skeggs et al. 1985, personal communication Dr. S. A. Fish)

Reagents

HRS Buffer

10 mM HEPES-KOH (pH 7.5)

10 mM MgCl₂

50 mM NH₄Cl

5 mM β mercaptoethanol

High salt buffer

10 mM HEPES-KOH (pH 7.5)

30 mM MgCl₂

1 M NH₄Cl

5 mM β-mercaptoethanol.

Procedure

Ribosomes were made from the S30 extract which was layered over an equal volume of HRS buffer containing 40% sucrose and spun in an ultracentrifuge (Sorvall OTD55B, Rotor Ti70) at 45,000 rpm for 12 hours at 4°C. The supernatant was removed and discarded. The pellet although looking clean, had brown cell debris loosely attached to its surface. A small amount of HRS buffer was added and a round ended glass rod was used to remove the debris from the pellet by gently teasing it. The ribosomal pellet was resuspended in high salt buffer and layered over an equal volume of high salt buffer containing 20% sucrose and spun at 50,000 rpm for 3 hours at 4°C. The pellet contained ribosomes of sufficient purity suitable for the preparation of ribosomal RNA. The

ribosomes were resuspended in HRS buffer containing 10% glycerol. The concentration of the ribosomes was read at 260 nm (1 A₂₆₀ unit is equivalent to 29.4 pmol ribosomes). They were aliquoted into volumes of 100µl, quick frozen in dry ice/IMS and then transferred to -70°C.

2.7.3 Extraction of ribosomal RNA from ribosomes

(Beauclerk and Cundliffe 1987)

High salt washed ribosomes were thawed gently on ice. To each 100 µl aliquot was added 400 µl of SSQ (sterilized by filtration through a 0.2 µM pore). An equal volume of phenol/chloroform (pH 7.0) was added. The mixture was inverted gently until homogenous and spun for 10 minutes at 13,000 g. The supernatant was removed and extracted again with phenol/chloroform. The RNA was precipitated using 1/10 of the volume of 3 M Sodium acetate (pH 4.8) and 2 volumes of 100% ethanol and left at -70°C for 1 hour. After this time the RNA was spun down and washed in 70% ethanol. The RNA was vacuum dried and dissolved in 50 µl SSQ water. The concentration of the RNA was determined and 10 µl aliquots were stored at -70°C until required.

2.8 Southern analysis

(Southern 1975, Sambrook et al. 1989).

Reagents

20x SSC

3 M NaCl

0.3 M tri sodium citrate

Depurination solution

0.25 M HCl

Denaturation solution

0.5 M NaOH

1.5 M NaCl

Neutralisation solution

3 M NaCl

0.5 M Tris-HCl (pH 7.4)

20 x SSPE

The following were added together then dissolved in 1 litre of water

174 g NaCl

27.6 g NaH₂PO₄·H₂O

7.4 g Na₂EDTA

The pH was adjusted to pH7.4 using NaOH (10 M).

Hybridisation buffer

This was prepared fresh. 0.5 g of Marvel was dissolved in 30 ml of SSQ. To this 3.0 g of PEG 8000, 3.75 ml of 20 x SSPE and 5 ml of 10% SDS were added. When all the ingredients were mixed properly, the volume was made up to 50 ml.

Procedure

Chromosomal DNA (5 µg) was electrophoresed overnight in 0.7% agarose at 15 volts (section 2.11.2). The gel was then photographed with a ruler in the picture. Excess gel was cut away leaving the tracks to be blotted. The gel was washed in 250 ml depurination solution for 7 minutes with gentle shaking. The gel was then rinsed briefly in SSQ water. It was denatured for 30 minutes with gentle shaking in 250 ml of denaturation solution. After another rinse in SSQ water, the gel was shaken gently for 30 minutes in 250 ml neutralisation solution.

The blotting apparatus was then prepared. A glass plate was covered in Saran wrap. Three sheets of Whatman No. 3 filter paper immersed in 20 x SSC were placed over the top. A glass pipette was used to roll out any air bubbles. The gel was placed on the Whatman paper DNA side up. Hybond-N membrane was cut to the same size as the gel and soaked in 3 x SSC for 30 minutes. The margin around the gel was overlayed with Saran wrap so that no SSC could pass through it. This would ensure that the capillary action through the gel was as efficient as possible. The membrane was then placed carefully over the gel. Three more pieces of Whatman No. 3 were dipped in 3 x SSC and laid over the top so that the gel and membrane were sandwiched between the 2 layers of Whatman. Paper towels (10 cm high) were stacked on top followed by another glass plate. A mass of 250 g was placed on top of the glass plate to weight it down. This was left overnight. The membrane was rinsed in 3 x SSC and left to dry at room temperature for 3 hours. The membrane was then wrapped in Saran wrap. The DNA was crosslinked to the membrane by placing the membrane under ultraviolet light for 30 seconds.

The membrane was pre-hybridised at 65°C in 50 ml of hybridisation buffer containing denatured salmon sperm DNA (100 µg/ml). The probe was boiled for 5 minutes and added to the hybridisation reaction. This was left to hybridise at 65°C overnight whilst gently shaking. The membrane was then rinsed briefly in 3 x SSC. A series of further, more stringent washes were then applied. Generally, membranes were subjected to two washes in 2 x SSC + 0.1% SDS for 10 minutes at 65°C, followed by 3

washes in 0.5% SSC + 0.1% SDS for 10 minutes at 65°C. The membrane was then laid down next to Xray film at -70°C and exposed for 24 hours.

2.9 Preparing radioactively labelled probes

A variety of probes and labelling techniques were used during the project. For convenience, the labelling techniques are described here.

2.9.1 Labelling of double stranded DNA using random hexamer priming and [α -³²P]dCTP

Reagents

Oligonucleotide labelling buffer

50 mM Tris-HCL (pH 8.0)

5 mM MgCl₂

10 mM 2-mercaptoethanol

20 mM dATP, dTTP, dGTP

200 mM HEPES (pH 6.6)

1.35 A260 units of hexadeoxynucleotides.

Procedure

This method describes the labelling of both strands of a given DNA fragment which was then used as a probe for Southern analysis. The DNA (10 µg) to be used as the probe was restricted and separated by electrophoresis (section 2.11). Then the required fragment was purified from the agarose using jetsorb (AMS Biotechnology). 25 ng of DNA was boiled for 5 minutes and placed on ice. Then 0.6 µl of BSA (10 mg/ml),

4 µl of oligonucleotide labelling buffer 0.6 µl of klenow enzyme (Gibco BRL), and 1.5 µl of [α - 32 P]dCTP (10 µCi/ml) were added to the DNA on ice. The DNA was then incubated at 37°C for 5 hours.

Unincorporated [α - 32 P]dCTP was removed by spinning the probe through sephadex G50. The plunger was removed from a 1 ml syringe and the empty tube was packed loosely at the bottom with glass wool. 1 ml of sephadex G50 slurry was added to pack the column, which was then spun at 2,000 rpm for 1 minute to pack the sephadex properly. SSQ was added to the probe to make the volume up to 100 µl. This was applied to the column. The labelled probe was spun through briefly and collected in an eppendorf.

The efficiency with which [α - 32 P]dCTP had been incorporated into the probe was checked by measuring 1 µl of it in a scintillation counter. If the probe measured approximately 10^6 counts per second then the probe had labelled very efficiently.

2.9.2 End labelling of single stranded probes using T4 polynucleotide kinase and [γ - 32 P]ATP

Reagents

10 x Calf intestinal alkaline phosphatase buffer (Gibco BRL)

0.5 mM Tris-HCl (pH 9.0)

10 mM MgCl₂

1 mM ZnCl₂

10 mM spermidine

5x Forward reaction buffer (Boehringer Mannheim)

(for labelling reaction using T4 polynucleotide kinase)

350 mM Tris-HCl (pH 7.6)

50 mM MgCl₂

500 mM KCl

5 mM β -mercaptoethanol

Procedure

2.9.2.1 Preparation of the DNA

The plasmid DNA was digested with a suitable restriction enzyme to produce a 5' overhang. The DNA (approximately 30 μ g) was digested in a total volume of 50 μ l (section 2.11).

Calf intestinal alkaline phosphatase (CIAP) was added to remove the terminal phosphate group, as follows: The volume was increased to 100 μ l with the following: 5 μ l of 10x CIAP buffer (Boehringer Mannheim), 1 μ l of CIAP (0.1 unit), 46 μ l of SSQ water containing RNase (1 mg/ml). The reaction was allowed to progress for 30 minutes at 37°C.

The reaction was stopped by extraction with phenol/chloroform. The DNA was then precipitated using 1/10 of the volume of 3 M sodium acetate (pH 4.8) and 2 volumes of 100% ethanol. The DNA was collected by centrifugation, washed in 70% ethanol, vacuum dried, and dissolved in 10 μ l SSQ water.

A further digestion was set up with the second restriction enzyme to release the DNA fragment to be labelled (section 2.11). The products were run in 0.7% agarose. The fragment of DNA was excised and purified using Jetsorb according to the manufacturers guidelines (AMS Biotechnology).

2.9.2.2 Labelling

The DNA fragment was then labelled. The following were added together

DNA fragment	34 μ l
--------------	------------

10 X T4 polynucleotide buffer	5 µl
10 units T4 polynucleotide kinase	1 µl
67 pmol [γ - ³² P]ATP	10 µl

The labelling mix was incubated for 30 minutes at 37°C. After this time, a further 10 units of T4 polynucleotide kinase were added and the labelling was allowed to progress for a further 30 minutes.

Unincorporated ³²P was removed by the purification method described for double stranded probes.

2.10 Manual sequencing

2.10.1 Denaturation of the template

DNA used for the template was extracted and purified using a Qiagen midi column. The template (2 µg) was dissolved in SSQ water to give a total volume of 36 µl. To this 4 µl of 2 M NaOH was added. The mixture was vortexed briefly and left at room temperature for 5 minutes. After this time, 4 µl of 3 M sodium acetate (pH 4.8) and 90 µl of 100% ethanol were added. The denatured DNA was precipitated at -70°C for 30 minutes. The DNA was spun down for 10 minutes at 12,500 rpm. The pellet was washed in 75% ethanol v/v, spun down again at 12,500 rpm for 10 minutes, vacuum dried, and dissolved in 10 µl of SSQ water.

2.10.2 Sequencing

The sequencing method used, was a modification of the original method by Sanger and Coulson (1975). The procedure was performed using a commercial single stranded sequencing kit (Pharmacia), according to the suppliers instructions. One of the deoxyribonucleotide triphosphates (dATP) used was radioactively labelled with ³⁵S, to

ensure that the synthesised strand became radioactively labelled. The sequence could subsequently be interpreted by autoradiography.

2.10.3 Gel preparation

Reagents

10x TBE buffer

Trisma Base 88 g

Boric acid 40 g

Na₂EDTA 5.56 g (pH 8.3)

All the ingredients were dissolved in a total volume of 750 ml with SSQ.

Formamide loading buffer

80% formamide

10 mM Na₂EDTA (pH 8.0)

1 mg/ml Xylene cyanol

1 mg/ml bromophenol blue.

Procedure

Sequence and primer extension reactions were analysed by gel electrophoresis on a 6% acrylamide/bisacrylamide gel.

The gel was made from : 16 g of urea dissolved in 30 ml of SSQ water, 4 ml of 10x TBE and 6 ml of acrylamide solution (40% stock stored in the dark at 4°C). This mixture was degased for 5 minutes. Prior to pouring the gel, 640 µl of freshly prepared

1.6% ammonium persulphate and 40 µl of TEMED were added to the solution. Once poured, the gel was left to polymerise for about two hours

The gel was washed with distilled water to remove the excess urea and sealed into the gel tank with 2% agarose. 1x and 0.5x TBE buffers were prepared, and the bottom and top troughs of the tank were filled with the two buffers respectively.

The gel was warmed for 30 minutes at 40 watts before the samples were loaded. Formamide loading buffer was added to the samples. They were subsequently boiled for 5 minutes and placed on ice before they were loaded onto the gel. They were separated by electrophoresis for about 1 hour at 40 watts.

2.10.4 Fixing and drying the gel

The DNA was fixed in the gel by soaking the gel in a bath of 10% acetic acid and 10% methanol for 20 minutes. The gel was then blotted onto a piece of whatman No.3 and vacuum dried. The X-ray film was exposed for 48 hours at room temperature.

2.11 Restriction and gel electrophoresis of DNA

2.11.1 Restriction of DNA using endonucleases

The following were added together:

DNA	10 µg (in 10 µl of SSQ water)
10x buffer supplied	
with the restriction enzyme	2 µl
Restriction enzyme	2 µl
SSQ water	
(10 mg/ml RNase)	6 µl

If more or less than 10 µg of DNA was restricted, the buffer, enzyme and water used were scaled up or down, proportionally.

2.11.2 Separation of DNA by electrophoresis

Reagents

Blue gel dye

The following were added together:

50 x TAE	200 µl
glycerol	5 ml
SSQ water	4 ml
Xylene Cynol FF	0.01%
Bromophenol blue	0.01%

50 x TAE buffer

The following were added together:

Tris base	242 g
Glacial acetic acid	56.8 ml
EDTA (sodium salt)	17.2 g
Glycerol	5 ml

To this was added SSQ water to make the volume up to 1litre.

Procedure

Unless specified otherwise, digested DNA separated on 0.7% agarose (cast in 1 x TAE), at 100 volts for 1 hour or 15 volts for 16 hours. 5 µl of blue gel dye was added to

the samples before they were loaded. The gel was run in a tank containing 1 x TAE buffer. DNA was sized using " kb ladder" (Gibco BRL) For cloning purposes (section 2.14) or for the preparation of probes (section 2.9), DNA was purified from the agarose using Jetsorb, according to manufacturers guidelines (AMS biotechnology).

2.12 The Isolation of specific DNA molecules from agarose gels, using CTAB/butanol

Langridge et al 1980

Reagents

Preparation of CTAB/butanol and CTAB/water

1 g of CTAB was dissolved in 100 ml of butanol. 100 ml of water was added. This was mixed well and allowed to separate at room temperature for 16 to 24 hours. The upper butanolic layer was removed and both solutions were tightly capped and stored at 37°C.

Procedure

Restricted DNA was separated in 1% low melting point agarose. The DNA required, was excised from the gel and incubated at 70°C for 30 minutes, to melt the agarose. The following manipulations were then performed at 37°C, in 1.5 ml eppendorf tubes. An equal volume of water/CTAB and an equal volume of butanol/CTAB were added, in that order. The DNA was agitated for 30 seconds using a vortex mixer. The phases were separated by centrifugation at 12,500 rpm for 1 minute. The upper butanolic layer was removed and saved. The lower aqueous phase was further extracted with half the volume of butanol/CTAB. This was centrifuged as before. The two butanolic phases were pooled.

At this stage the work was continued at room temperature. DNA was extracted by the addition of 0.25 X volume of 0.3 M sodium acetate (pH 7). The DNA was agitated for 30 seconds. The phases were separated by centrifugation for 1 minute. The lower, aqueous phase was extracted with an equal volume of chloroform. The upper phase was removed. 3 volumes of absolute ethanol were added to this and the DNA was precipitated in an IMS/dry ice bath. The precipitated DNA was spun down by centrifugation at 12,500 rpm, vacuum dried and resuspended in 10 µl of SSQ water.

2.13 Partial digestion of plasmid DNA

(personal communication Dr V. Wilson)

Partial digestion was used to excise DNA with restriction enzymes that also recognised sites internal to the fragment. There were two variables to consider in order to generate a partial digest: either the incubation period of the digestion or the quantity of enzyme in the reaction. The following were added together:

30 µg of DNA	30 µl
10 x reaction buffer	10 µl
SSQ water	60 µl

This was divided into volumes of 30 µl, 20 µl, 20 µl 20 µl and 10 µl and transferred to 5 eppendorfs, labelled A-E, respectively. The 5 eppendorfs were placed on ice. To tube A was added 1 or 2 units of the enzyme. This was mixed in. 10 µl was transferred from tube A to tube B. This was repeated through to tube E. The tubes were incubated at 37°C of 15 minutes. Further reaction times of 20 and 30 minutes were also tested in this way. The reactions were stopped by the addition of 5 µl of blue gel dye. The digested products were analysed by gel electrophoresis (section 2.11.2). The desired fragment was excised and purified (section 2.11.2).

2.14 DNA cloning

The term "cloning" is used here to describe ligation of an insert into a vector and the introduction of the ligation mixture into *E.coli* or *S. lividans* to generate a bigger population of cells containing the new plasmid.

2.14.1 Ligations

The quantities of vector and insert used for the ligations described in this work, were calculated using the following equation:

$$\frac{\text{amount of vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{molar ratio} = \text{amount of insert (ng)}$$

The concentration of plasmid DNA was measured spectrophotometrically, as described in section 2.4. The molar ratio of vector to insert, routinely used for ligations, was 1: 3, unless they were of a similar size. Then a ratio of 1:1 was adopted.

The following components were added together:

Insert	10 µl
vector	5 µl
5x T4 ligase buffer	
(Gibco BRL)	4 µl
T4 DNA ligase	1 µl

The vector and insert, which had previously been restricted and purified (section 2.11), were added together first. They were heated for 30 seconds at 60°C, and placed on ice. The T4 DNA ligase buffer and T4 DNA ligase were then added. The ligation mixture was incubated at 16°C for 16 hours and then stored at 4°C until required.

2.14.2 Transformation of the ligation mixture

Most ligation mixtures were introduced into *E. coli* NM522 Gough and Murray 1982) by transformation (section 2.3.4). Many of the plasmids (section 2.17) (pHJL401, pCR2.1, pSET152, and pOJ260) used for cloning, possess the pUC18 multicloning site (Yanish-Perron et al. 1985). These vectors contain the amino terminal fragment of the *lacZ* gene (*lacZ'*) in the appropriate host because β -galactosidase is made. *E. coli* producing β -galactosidase could be identified because they formed blue colonies in the presence of the chromogenic substrate X-gal. Ligation of DNA in the multicloning site caused insertional inactivation of *lacZ* gene and β galactosidase was not made. This resulted in the absence of pigmentation and colonies were white instead of blue (Sambrook et al 1989). Plasmid isolated from recombinant strains

DNA was also cloned using *Streptomyces* plasmid pIJ702 (Figure 2.17.1). The vector and insert were ligated using the same method in section 2.14.1. *S. lividans* protoplasts were transformed with the ligation mixture (section 2.3.2). Plasmid was isolated (section 2.3.1) and confirmed by restriction analysis (section 2.11).

2.15 Minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) of an antibiotic was established for *M. purpurea* and *S. lividans* strains using different methods.

Approximately 100 μ l of *M. purpurea* cells were removed from a hyphal stock and inoculated into a universal containing 10 ml of YEME/PEG. These cells were homogenised and incubated at 30°C for 24 hours. During incubation, the universal was shaken horizontally at 150 rpm. A range of 10 ml NE agar plates containing different concentrations of the antibiotic in question, were divided into equal areas. The number of areas depended on the number of strains being tested. The 10 ml culture was spun down at 3,500 rpm for 5 minutes. The pellet was resuspended in 1 ml of YEME/PEG. A

volume of 5 µl was removed and streaked onto one area of each plate. This was repeated for each strain. Mutants were tested for growth in the absence of antibiotic.

The inoculum for *Streptomyces lividans* was prepared differently. Plates of NEF (section 2.0) were spread with 50 µl of spore suspension. These were incubated at 30°C for 4 days. Once they had sporulated, 5 ml of SSQ water containing 0.1% Triton X100 was applied to the surface of the each plate. The spores were brought into solution by gently scrapping the surface with a sterile loop. They were removed, spun down at 3000 rpm for 5 minutes and resuspended in 1 ml of TES buffer (0.05M pH 8.0) They were then heat shocked at 50°C for 10 minutes. As described for *M. purpurea*, 5 µl of each strain was tested.

2.16 Amplification of genes from actinomycetes by PCR

The method used was originally for the amplification of genes from *Streptomyces fradiae* developed from an Invitrogen PCR optimizer kit by Dr A. Gandecha.

Reagents

5x PCR buffer

300 mM Tris-HCL (pH 8.5)

75 mM (NH₄)₂SO₄

17.5 mM MgCl₂

Procedure

The following were added to a 0.5ml eppendorf:

Template (50ng)	1.0 µl
-----------------	--------

Primers (250ng/μl)	1.0 μl
5x PCR buffer	10 μl
DMSO	5.0 μl
dNTP's (10mM)	5.0 μl
Taq polymerase (Gibco BRL) 1 Unit	
H ₂ O	to 50 μl

The 50μl sample was overlayed with 50μl of mineral oil and placed in the thermo-cycler (Perkin Elmer). The thermal cycle used was:

80°C for 5 minutes for 1 cycle

94°C for 2 minutes for 1 cycle

94°C for 1 minute

60°C* for 2 minutes

72°C for 3 minutes for 35 cycles

72°C for 7 minutes for 1 cycle.

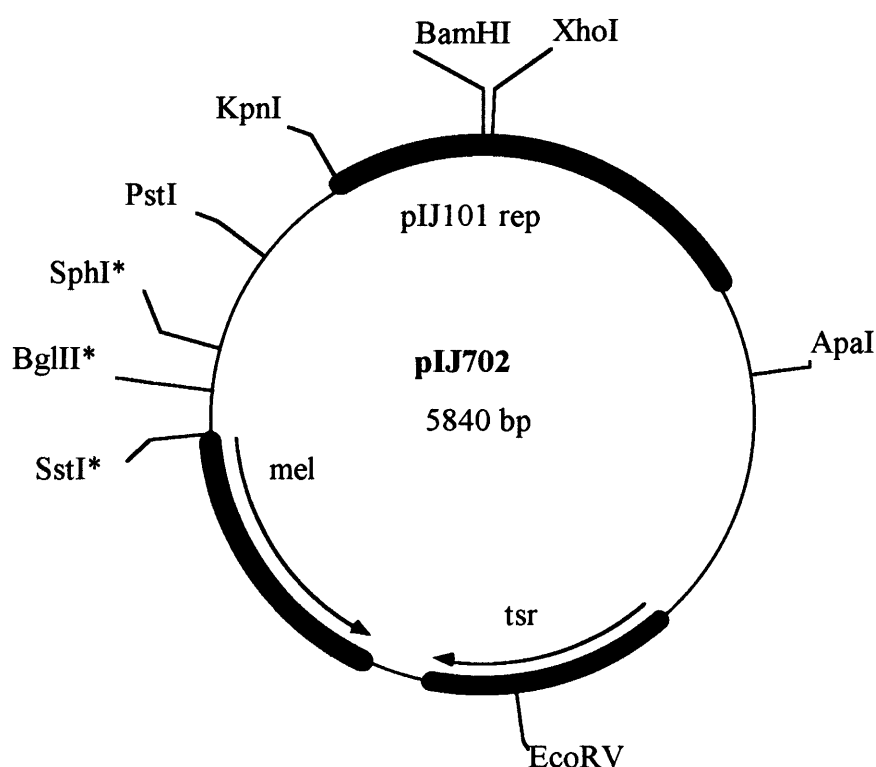
* This was the annealing temperature. It was adjusted according to the melting temperature of the oligonucleotides used. If the melting temperature was too high then the oligonucleotide would not anneal. If it was too low then the oligonucleotide could anneal in multiple places.

Once PCR amplification was complete the DNA was analysed by gel electrophoresis as described in section 2.11.

2.17 Plasmids

Presented here are all the vectors used for cloning purposes during this project.

2.17.1 pIJ702 (Kieser et al 1982)

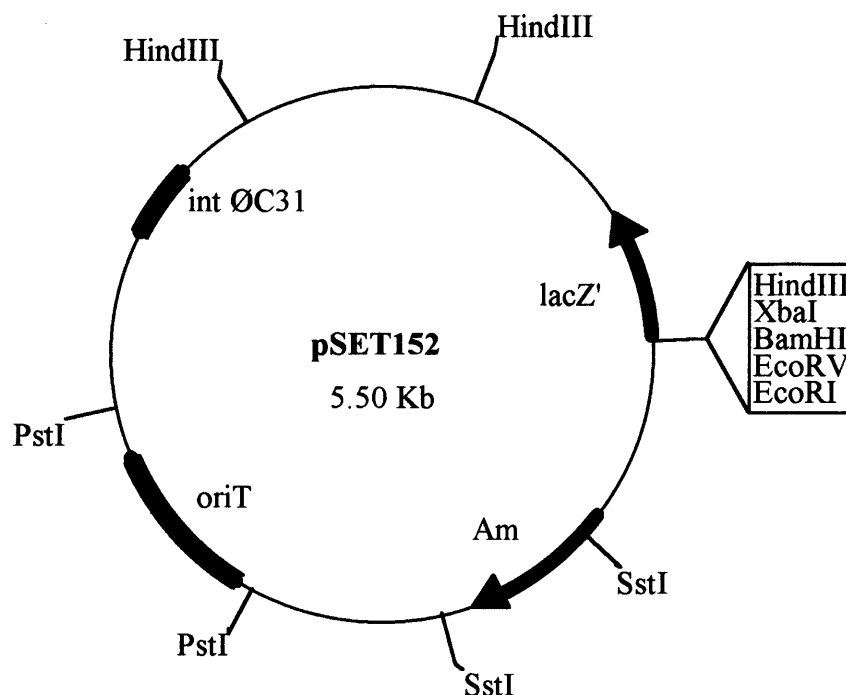


pIJ702 is a non transmissible plasmid with a broad host range. It is derived from pIJ101, acquiring the origin of replication. pIJ702 has an unusually high copy number of between 40-300 per chromosome and is therefore very stable.

The gene, *mel*, encodes tyrosinase, necessary for the biosynthesis of a brown pigment known as melanin (Katz et al 1983). The *SphI*, *BglII*, and *SstI* sites are present in the regulatory region of this gene. The cloning of DNA at one of these restriction sites causes insertional inactivation of the *mel* gene. This results in the absence of pigmentation, and colonies are white instead of brown.

The gene, *tsr*, encodes a methylase that confers resistance to thiostrepton (further details can be found in Chapter 1 section 1.4.1)

Figure 2.17.2 pSET152 (Biermann et al. 1992)



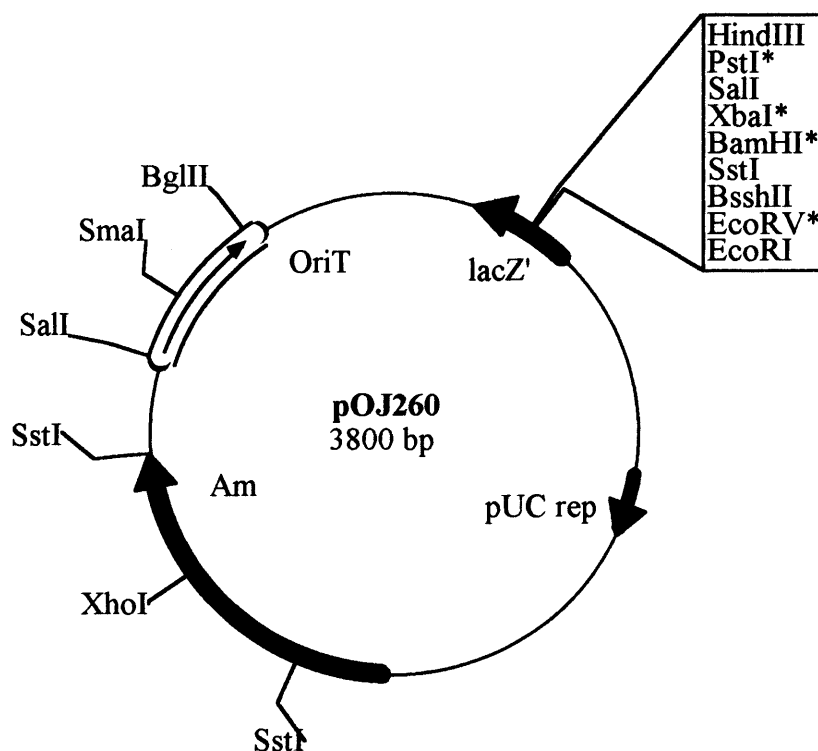
pSET152 is an integrative plasmid which can integrate site-specifically using the bacteriophage ØC31 integration functions (*int* ØC31). pSET152 can be used to insert homologous or heterologous DNA into the *Streptomyces* chromosome, at the unique ØC31 *att* site.

The plasmid carries an origin of transfer, *oriT*, which enables it to be introduced into *Streptomyces* from *E. coli* by conjugal transfer. The transfer functions for mobilisation of the plasmid are provided in trans by the appropriate host organism.

The plasmid possesses the apramycin resistance marker, represented by *Am* which can be selected for in both *E. coli* and *Streptomyces*.

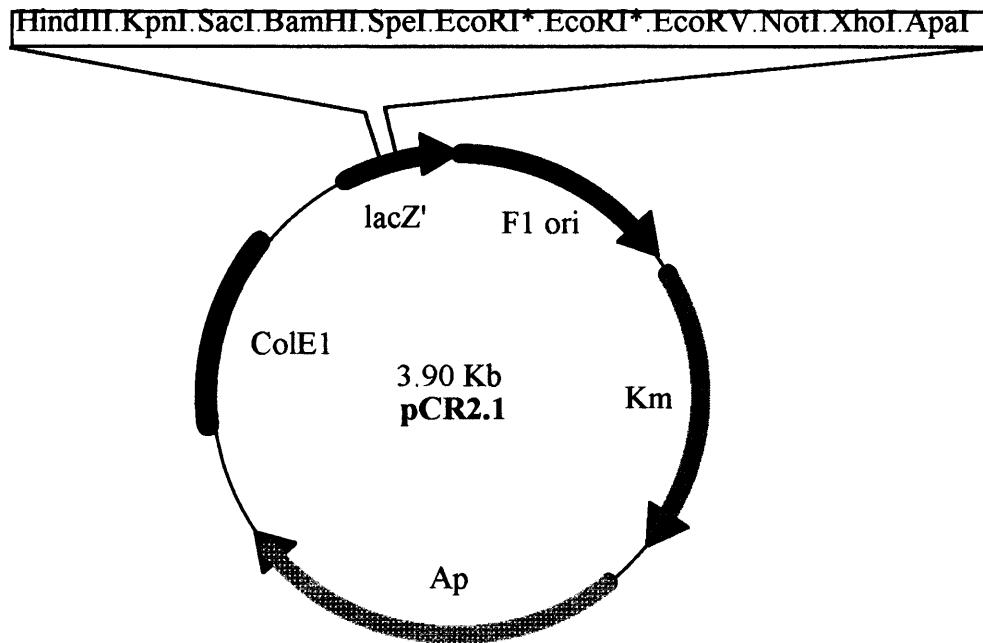
The plasmid contains a pUC18 polylinker containing unique sites *Xba*I, *Bam*HI, *Eco*RV and *Eco*RI for cloning DNA. Ligation of DNA in this polylinker can be detected by blue/white selection, due to insertional inactivation of *lacZ* (section 2.14.2).

Figure 2.16.3 pOJ260 (Bierman et al. 1992)



pOJ260 does not replicate in *Streptomyces* spp and has a pUC origin of replication for *E. coli*. It is useful for the disruption and replacement of genes in *Streptomyces*. pOJ260 contains the α -terminal end of the *lacZ* gene (*lacZ'*). The polylinker contains unique sites, *HindIII*, *PstI*, *XbaI*, *BamHI*, *EcoRV* and *EcoRI*. Ligation of DNA at any of these sites can be detected by blue/white selection (section 2.14.2) The plasmid also carries a selection marker (*Am*) which confers resistance to apramycin, and an origin of transfer (*oriT*) which enables the plasmid to be introduced into *Streptomyces* by conjugal transfer. Functions required for mobilisation are provided in *trans* by the host organisms.

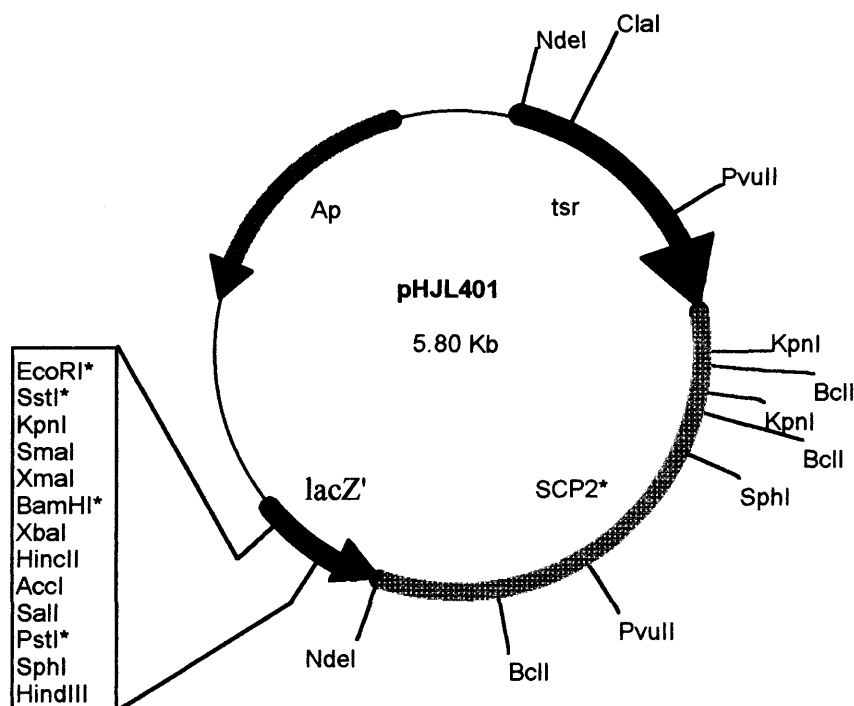
Figure 2.17.4 pCR2.1 (Mead et al 1991)



Taq polymerase adds single deoxyadenosine residues to the 3' ends of PCR-amplified products. The linearised vector pCR2.1 has single deoxythymidine residues which allow PCR-amplified inserts to ligate efficiently with the vector. The thymidine residues are flanked by *EcoRI* sites (*) for excision of the insert. The plasmid also possesses ampicillin (*Ap*) and kanamycin (*Km*) resistance genes for selection in *E. coli*.

pCR2.1 possesses a pUC origin of replication (*ColEI*) and *lacZ'* for blue/white selection (section 2.14.2).

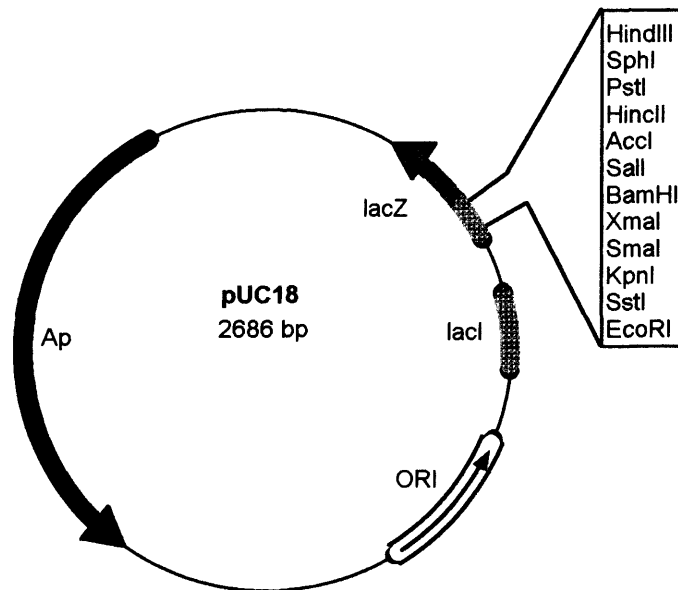
Figure 2.17.5 pHJL401 (Larson and Hershberger 1986)



pHJL401 is a shuttle vector for *E. coli* and *Streptomyces*. The plasmid possesses a pUC18 origin of replication which gives it a high copy number in *E. coli*. pHJL401 also possesses the origin of replication from SCP2* which gives it a moderate copy number in *Streptomyces*.

tsr confers resistance to thiostrepton in *Streptomyces* and *Ap* represents the β -lactamase gene that confers resistance to ampicillin in *E. coli*. The polylinker contains unique sites, *HindIII*, *PstI*, *XbaI*, *BamHI*, *XmaI*, *SmaI*, *SstI*, *EcoRI*. Ligation of DNA in this polylinker can be detected by blue/white selection, due to the insertional inactivation of *lacZ'* (section 2.14.2)

Figure 2.17.6 pUC18 (Yanisch-Perron et al. 1985)



pUC18 is a very high copy number plasmid containing portions of PBR322 and M13mp19. It has a *colEI* origin of replication, a *lacZ* promoter and *lacZ α* for blue/white selection. All the sites in the polylinker are unique. The plasmid also possess are β -lactamase gene, represented here as Ap, which is selected for in the presence of ampicillin.

Chapter 3

Introduction of *grmA* into *M. purpurea*

Chapter 3

Introduction of *grmA* into *M. purpurea*

3.0 Aim

Preliminary investigations prior to this work determined the site of action of GrmA in *S. lividans*. This ribosomal methyltransferase methylates a specific guanine residue at position 1389 (*E.coli* 1405) of the 16S rRNA. However, these investigations also suggested that this site is not methylated in *M. purpurea*. Is *grmA* therefore silent in its native host?

The aim of this part of the work was to introduce further copies of *grmA* into *M. purpurea*. Extra copies may titrate out the effects of a putative repressor of *grmA*, thereby permitting its expression. If so, changes occurring in the level of *grmA* transcript or the methylation of the 16S rRNA might subsequently be observed.

3.1 Introduction

3.1.1 The expression and regulation of ribosomal methyltransferases from aminoglycoside producers

Comparisons with ribosomal methyltransferases found in other actinomycetes may give some insight into the expression and regulation of *grmA* in *M. purpurea*. There are four models, now established concerning the regulation of resistance genes from antibiotic-producing actinomycetes, which are discussed in Chapter 1 (section 1.11). Particular attention is paid here to the mechanism that controls the regulation of resistance determinants in the aminoglycoside producers, *Micromonospora zionensis* (*sgm*) and *Streptomyces tenebrarius* (*kgmB*).

3.1.2 *sgm* and *kgmB*

M. zionensis, producer of G-25 (6-*N*-methyl-sisomicin) possesses an aminoglycoside resistance determinant, *sgm* (Kojic et al. 1992), conferring resistance to sisomicin, gentamicin, kanamycin, tobramycin and hygromycin B but not neomycin or apramycin.

The control mechanism for *sgm* is translational autoregulation (Kojic et al. 1996). Interestingly, *sgm* has tandem promoters also identified in other *Micromonospora* spp (described further in Chapter 1, section 1.10.3) possibly allowing differential expression of the gene during different phases of antibiotic production. In contrast, *grmA* has a single promoter, similar to those found in *E. coli* (Kelemen et al. 1991).

The coding regions for *sgm* and *grmA* are very similar. Comparisons of the deduced amino acid sequences reveals 90% identity between Sgm and GrmA (Kojic et al. 1992). The 5' untranslated regions (UTR) differ in length and sequence, being 72 nucleotides for *sgm* and only 11 for *grmA*. The transcriptional start site for *grmA* was mapped by SI nuclease protection in *S. lividans* and found to be 11 nucleotides upstream of the translational start site. However the transcriptional start site for *grmA* has yet to be identified in *M. purpurea* (Kelemen et al. 1991).

Kojic et al. (1996) compared the levels of protein synthesised from *grmA* and *sgm* driven by the *lacZ* promoter in an *E. coli* minicell system. Despite the fact that both genes conferred gentamicin resistance, only GrmA was detectable on SDS polyacrylamide gels. Variations in the 5' UTR could be one factor involved in the different level of expression from these two genes.

It was established that Sgm represses its own translation by binding to the mRNA 14 bp upstream of the ribosome binding site. This site encompasses the hexanucleotide CCGCCC. This hexanucleotide is also present in the C1400 region of 16S rRNA where the Sgm protein is expected to methylate. Although no similarities have been found between the secondary structure of the 16S rRNA and the 5' UTR, the hexanucleotide is involved in the formation of a hairpin loop recognised by the methylase (Kojic et al. 1996).

Only a few copies of the methylase are required to methylate ribosomes conferring resistance over the whole cell. Once all the ribosomes have been methylated, excess copies of the enzyme bind to the mRNA upstream of the RBS preventing unnecessary translation of the protein.

It has been suggested that the mechanism regulating *sgm* is different to that for *grmA*. This is because they do not share the same features in the 5' untranslated region and the GrmA protein is detectable in *E. coli* whereas Sgm is not (Kojic et al. 1996).

S. tenebrarius is the producer of the nebramycin complex which is a combination of modified kanamycin B derivatives mainly tobramycin and apramycin. The methyltransferase encoded by *kgmB* (kanamycin-gentamicin resistance methylation) modifies residue G1389 in the 16S rRNA. This gene may be regulated by a similar mechanism to that described for *sgm* (Holmes and Cundliffe 1991) but has a different type of promoter. The 5' end of the transcript has been mapped to reveal a single promoter that does not fall into the category of *Streptomyces* promoters that are similar to *E. coli* ones (Holmes and Cundliffe 1991). Within the 5' UTR of *kgmB* lies a sequence CGUCA 21 bp upstream of the initiation codon. This pentameric sequence is also located around the methylation site for *kgmB* (G1405 in *E. coli* sequence) in the 16S rRNA (Holmes and Cundliffe 1991). The pentameric sequence is directly repeated in the 16S rRNA of *S. tenebrarius*. It would be interesting to observe if this also serves as an additional methylation site for *kgmB* in *S. tenebrarius*.

3.2 Introduction of DNA into *M. purpurea* by conjugation

Due to the lack of success with attempting to introduce DNA into *M. purpurea* by transformation, another approach was used which has been developed for *Streptomyces* spp. This involved the transfer of DNA from *Escherichia coli* to *M. purpurea* by conjugation.

3.2.1 Conjugation

Conjugation is a natural process by which plasmid DNA is transferred from a donor cell to a recipient cell. It was first thought to occur only between Gram negative microorganisms, however it was soon discovered that DNA could be transferred between Gram positive organisms and members of both groups.

The mechanism of conjugation is not fully understood. What is known, however, is that an extracellular filament is produced by the plasmid-bearing donor cell and makes contact with one or more recipient cells. This leads to the formation of a mating pair or an aggregate. DNA is transferred as a single strand in the 5'-3' direction initiating at the nick site of the origin of transfer (*oriT*), following which, circularisation and replication occurs in the recipient cell. This process is sometimes referred to as rolling circle replication (reviewed by Frost et al. 1994, Lanka and Wilkins 1995).

The single stranded nature of the DNA during transfer has become a very useful tool for the introduction of DNA into certain actinomycetes, which would otherwise be very difficult to manipulate. Some (eg *S. fradiae*) cannot be transformed easily due to host restriction enzymes. The efficiency with which DNA can be introduced into *Micromonospora purpurea* is very low. Attempts were made to transform *M. purpurea* with pIJ702, following protocols used for *Streptomyces* spp and other *Micromonospora* spp. (Kelemen et al. 1988, Kojic et al. 1991, Matsushima and Baltz 1988), however, without success.

There is a well established method for conjugal transfer of DNA into *Streptomyces* spp. from *E. coli* (Bierman et al. 1992). The donor organism is *E. coli* S17-1 which carries the transfer (*tra*) genes from the broad host range IncP type plasmid, RP4, integrated in its genome (Simon et al. 1983). The conjugative plasmids designed by Bierman et al. (1992) all possess the origin of transfer (*oriT*) obtained from another IncP plasmid, RK2 (Guiney and Yakobson 1983).

There is no account of the same procedure being used in *Micromonospora* spp. so little is known about the plasmids that can be introduced and maintained. As a result the above method had to be adapted and optimised for *M. purpurea*.

3.2.2 Method of conjugation

AS-1 Agar (Baltz 1980)

The following were added together:

Difco yeast extract	1 g
L-alanine	200 mg
L-arginine	200 mg
L-asparagine	500 mg
Soluble starch	5 g
Sodium chloride	2.5 g
Sodium sulphate	10 g

The ingredients were dissolved in 1 litre of SQ water and 20 g of agar was then added. This was adjusted to pH 7.5 using 1 M KOH.

R2YE soft agar (Baltz 1980)

The following ingredients were added together:

Sucrose	51.5 g
(K ₂ SO ₄)	0.125 g
(MgCl ₂ .6H ₂ O)	5.06 g
Glucose	5 g
Difco casamino acids	0.05 g

The ingredients were dissolved in 400 ml of SQ water and the solution was divided between ten 50 ml bottles before 0.7% agar was added to each. Before use, the medium was remelted and a mixture of the following ingredients was added to each bottle of 50 ml.

0.5% (KH ₂ PO ₄)	0.5ml
3.68% (CaCl ₂ .2H ₂ O)	4 ml
20% L-Proline	0.75 ml
5.73% TES	
(adjusted to pH7.2 using 2MNaOH)	5 ml
Trace element solution	0.1 ml
1 M NaOH	0.25 ml

Procedure

10 ml of YEME/PEG was inoculated with 100 µl of *M. purpurea* hyphal fragments from a stock stored at -70°C and incubated for 48 hours at 30°C. After this time the 10 ml culture was homogenised and allowed to grow for a further 16 hours. At the same time as homogenisation, a 50 ml culture of LB broth was set up, inoculated with 120 µl of *E. coli* S17-1 glycerol stock containing the conjugative plasmid, and incubated at 37°C. Antibiotic was added for the selection and maintenance of cells which contained the plasmid.

After 16 hours of incubation, *M. purpurea* was diluted by a factor of 5 in 10 ml of YEME/PEG and grown for a further 3 hours. The *E. coli* was also left incubating for this period.

10 ml of the S17-1 culture and the diluted culture of *M. purpurea* were spun down at 3000 rpm for 5 minutes, washed in the media they had been grown in and resuspended in 2 ml of the same medium. At this stage physiological changes in the mycelia (at a magnification of x100) were identified indicating that they were ready for conjugation (Figure 3.6).

To set up the conjugation, 100 µl of S17-1 (10⁸ donors) was pipetted onto an AS-1 agar plate and 50 µl of *M. purpurea* (10⁷ recipients) was added. The inoculum was spread evenly over the plate and left to dry.

The plates were incubated at 30°C for 24 hours and overlaid with 4 ml of R2YE soft agar containing nalidixic acid (60 µg/ml) and the antibiotic required for selection of the conjugative plasmid. Since *E.coli* is sensitive to nalidixic acid this ensured that only *M. purpurea* cells containing the plasmid grew through the overlay.

After a few more days of growth, depending on the vector conjugated, colonies were picked and plated onto AS-1 agar containing nalidixic acid (60 µg/ml) and the antibiotic to select for the vector, in order to check for transconjugants.

3.3 Conjugal transfer of pSET152 into *M. purpurea*

pSET152 is an integrative plasmid (Bierman et al. 1992) (Figure 2.17.2). It does not possess an origin of replication in *Streptomyces spp.* The plasmid integrates into the chromosome at the attachment/integration site for phage int ϕ C31. pSET152 was conjugated into *M. purpurea* as described in section 3.2.2. Transconjugants containing the plasmid were selected for with apramycin (25 µg/ml) and nalidixic acid (60 µg/ml). Approximately 400 exconjugants grew through the overlay (Figure 3.0), all of which continued to grow when replica plated onto AS-1 containing nalidixic acid (60 µg/ml) and apramycin (25 µg/ml).

3.3.1 Southern analysis to check the presence of pSET152 in *M. purpurea*

Genomic DNA was prepared from wild type *M. purpurea* and from a transconjugant of *M. purpurea* which was believed to contain pSET152. The procedure is described in section 2.3.6. Cells were grown in YEME/peg containing apramycin (25 µg/ml). Southern analysis was performed (Materials and Methods, section 2.8) to confirm the integration of pSET152 in the *M. purpurea* genome (Fig 3.1.1 to 3.1.2). The probe used was a 900 bp *Sst*I fragment from pSET152 which contained most of the

apramycin resistance gene. The probe was labelled by the incorporation of [α - ^{32}P] dCTP using random hexamer priming and Klenow enzyme.

Figure 3.0 *M. purpurea* transconjugants containing pSET152



Figure 3.0

M. purpurea containing pSET152 grown on AS-1 agar for 5 days, following overlay with nalidixic acid (60µg/ml) and apramycin (25µg/ml).

3.4 Construction of pLST0013; high copy number conjugative vector.

Figures 3.1.1 and 3.1.2

Integration of pSET152 into the *M. purpurea* genome.

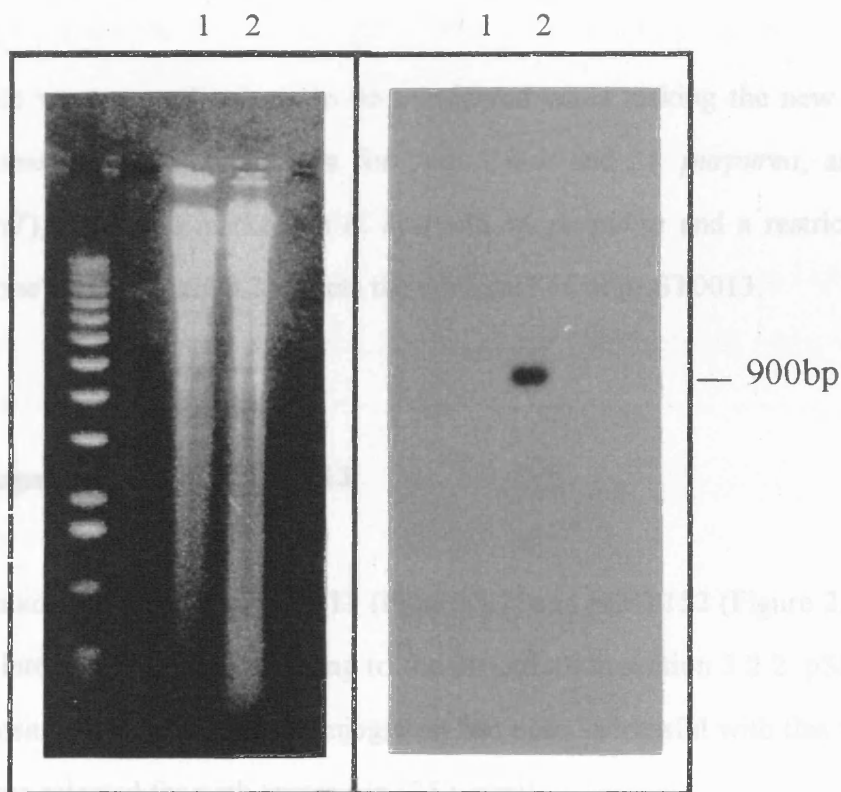


Figure 3.1.1

Figure 3.1.2

3.4 Construction of pLST0013; high copy number conjugative vector.

Using the vector pSET152 (Figure 2.17.2), conjugation was shown to be a potentially useful method for introducing DNA into *M. purpurea*. The crucial experiment was to introduce *grmA* into *M. purpurea* at a high copy number in order to over-express it. None of the plasmids available possessed all the necessary characteristics for this experiment.

There were several criteria to be considered when making the new plasmid. It had to possess origins of replication for both *E.coli* and *M. purpurea*, an origin of transfer (*oriT*), selectable markers in *E. coli* and *M. purpurea* and a restriction site in which to clone *grmA*. Figure 3.2 depicts the construction of pLST0013.

3.4.1 Conjugal transfer of pLST0013

Plasmids pLST0012, pLST0013 (Figure 3.2) and pSET152 (Figure 2.17.2) were conjugated into *M. purpurea* according to the procedure in section 3.2.2. pSET152 was used as a positive control because conjugation had been successful with this vector. The plasmids were selected for with apramycin (25 µg/ml).

It was important at this stage to observe the morphology of *M. purpurea* growth and the efficiency of conjugation. As there was no published information on such an experiment in this microorganism, data of this kind were particularly valuable.

Putative transconjugants containing pLST0012 and pLST0013 took 7-10 days to grow through the overlay and were approximately 1 mm in diameter and orange. Those containing pSET152 only took 4 days to grow and were 3-4 mm in diameter and purple. These characteristics are typical of mature colonies of *M. purpurea*.

The average number of colonies on each plate for plasmids pSET152, pLST0012 and pLST0013 was 300, 30 and 3 respectively. Twenty colonies from each type of transconjugant were picked and replica plated onto AS-1 agar containing apramycin (25 µg/ml) and nalidixic acid (60 µg/ml). Those possessing pSET152 grew within four days

whereas those thought to contain pLST0012 and pLST0013 appeared not to be growing at all. However, the original inoculum was picked off the plate and used to inoculate 10 ml of YEME/PEG containing apramycin (25 µg/ml) and nalidixic acid (60 µg/ml). These cultures were incubated at 30°C until there was sufficient biomass from which to prepare plasmid. Transconjugants containing pLST0012 grew in about 10 days and plasmid was isolated from 10 of these. Those containing pLST0013 did not grow in agar or broth.

The method used to isolate plasmid from *Streptomyces* (Hopwood et al. 1985) was applied to *M. purpurea* with the following modifications. The first problem encountered was that the mycelium would not grow in YEME/sucrose broth. The solution was to change the broth to YEME/PEG.

A second problem was that cells had been growing for some time before plasmid was prepared. As a consequence of this, they probably had tough cell walls, which were resistant to lysozyme.

Another possible explanation for why *M. purpurea* did not lyse easily lay in the chemical composition of the cell walls. It has been observed that some *Micromonospora* spp. do not possess β-N acetylmuramic acid recognised by lysozyme (β N-acetylmuramidase) in their peptidoglycan, but instead possess β-N-glycolylmuramic acid (Kawamoto et al. 1981). This problem was solved by the addition of twice the quantity of 10% SDS used in the lysis step (see Materials and Methods, section 2.3.1).

Despite the problems encountered the method was successful and pLST0012 was isolated from all 10 transconjugants. The plasmid DNA extracted was barely visible when separated by gel electrophoresis. It was used to transform *E.coli* NM522. Further plasmid was made from the transformants of NM522. It was digested with *Pst*I and separated by gel electrophoresis (Figure 3.3)

This conjugation experiment was repeated on three separate occasions with similar results each time. Plasmids pSET152 and pLST0012 were successfully conjugated into *M. purpurea* and pLST0013 was not.

3.4.2 Transformation of *S. lividans* with pLST0012 and pLST0013

S. lividans (OS456) was transformed with plasmids pLST0012 and pLST0013 according to the method described in section 2.3.2. Transformation plates were overlayed with apramycin (25 µg/ml). Regenerated protoplasts grew and sporulated within 4 days. Colonies were picked and plasmid prepared. Transformation of *S. lividans* with the two plasmids was successful.

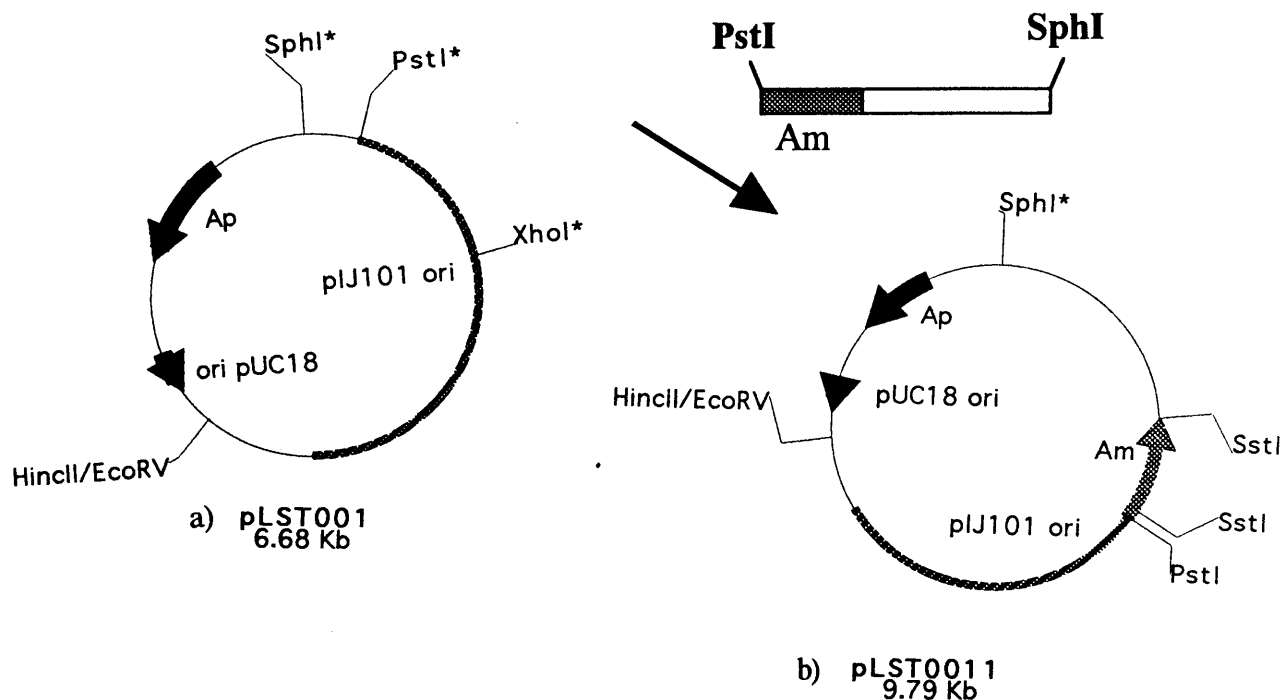


Figure 3.2
Construction of a high copy
conjugative plasmid pLST0013

Key

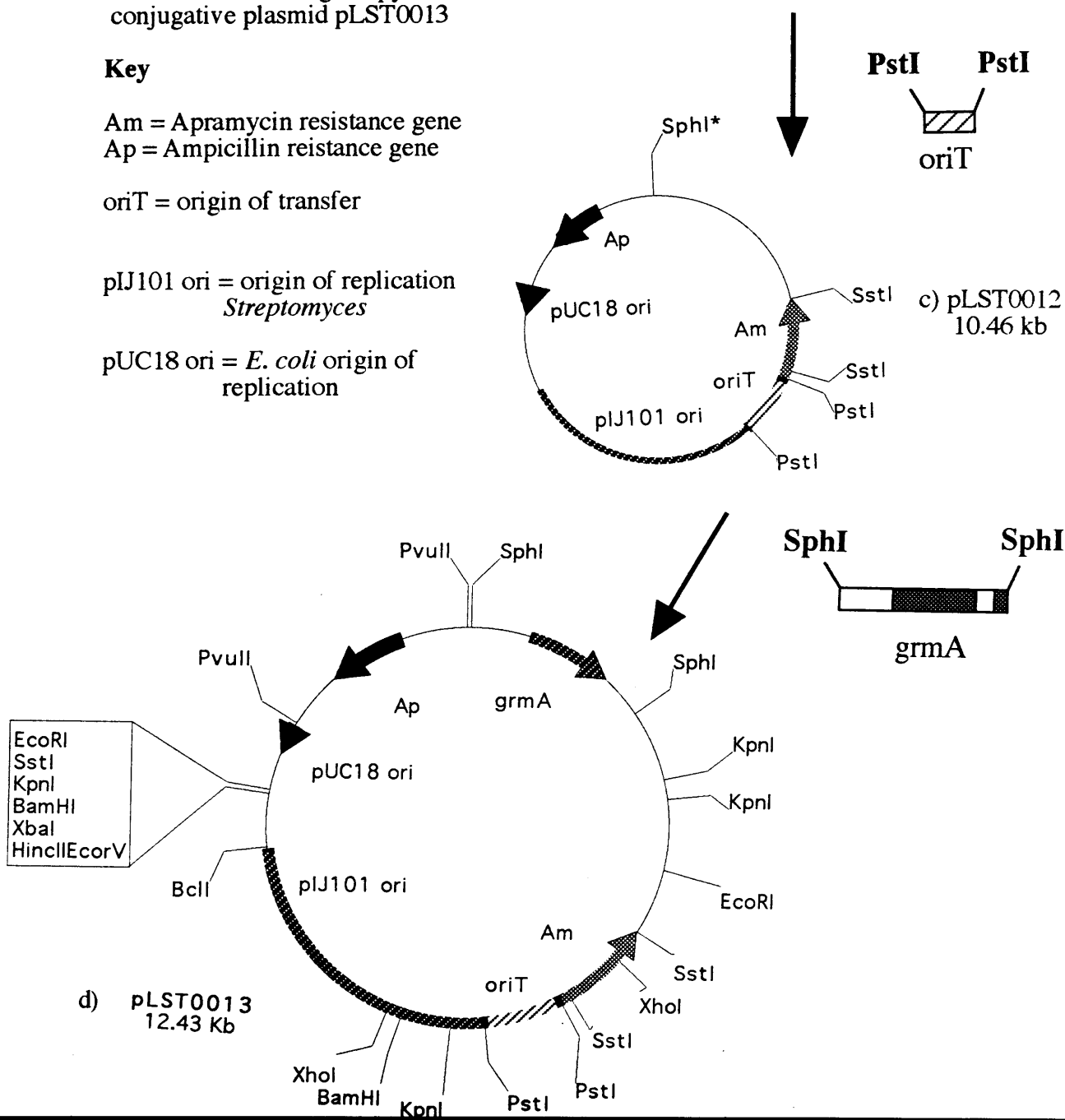
Am = Apramycin resistance gene

Ap = Ampicillin resistance gene

oriT = origin of transfer

pIJ101 ori = origin of replication
Streptomyces

pUC18 ori = *E. coli* origin of
replication



3.5 Expression of *grnA* using a constitutive promoter

Figure 3.3

Restriction analysis of plasmid DNA extracted from *E.coli* NM522 containing pLST0012.

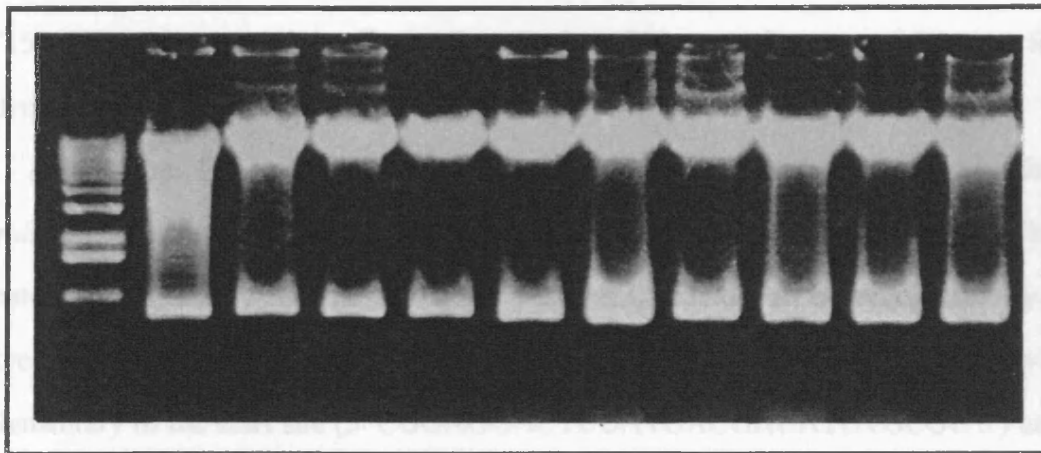


Figure 3.3

Plasmid DNA was extracted from ten putative transconjugants of *M. purpurea* containing pLST0012 (Materials and Methods section 2.3.1). However to confirm that the plasmid was pLST0012, the DNA was introduced into *E. coli* NM522 by transformation (Materials and Methods, section 2.3.4).

The gel presented here shows plasmid isolated from ten *E.coli* transformants, and restricted with *PstI*. This released the *oriT* fragment giving two bands shown here, of sizes 760 bp and 9.7 kb.

3.5 Expression of *grmA* using a constitutive promoter

An alternative approach to the introduction of *grmA* on a high copy number plasmid, was used to overexpress *grmA* in *M. purpurea*. The expression of *grmA* was driven by a powerful constitutive promoter different to the *grmA* promoter. For the purpose of this experiment a promoter isolated from the actinomycete *Saccharopolyspora erythraea* was used (*ermEp**) (Bibb et al. 1985). Plasmid pLST9828 was constructed (personal communication Dr A. Butler) by cloning *ermEp** into pSET152 (Figure 2.17.2) at the *EcoRI/BamHI* sites. This served as a useful vector for the introduction of *grmA*.

A PCR-amplified (Materials and Methods, section 2.16) product of *grmA* lacking the promoter region and flanking DNA was introduced downstream of the *ermEp**. This eliminated the possibility that the promoter for *grmA* could still be recognised by a putative repressor in *M. purpurea*. The primers were two 27 mers. One was complementary to the start site (5' CGGAGGACTCGATGACGACATCTGCGC 3') and the other was complementary to a sequence a few nucleotides downstream of the end of the ORF (5' TCTAGAGTACACGCACGACGCCATCC 3') The template used was a 1.9 kb *BamHI* restriction fragment of pLST1381a (Chapter 5, Figure 5.1) carrying *grmA*. This was obtained by shotgun cloning as described in Chapter 5, section 5.1. The fragment was excised from pLST1381a by digestion with *BamHI*. Approximately 50 ng of this DNA was used as a template for PCR with an annealing temperature of 60°C.

The amplified product was identified by gel electrophoresis, purified using Jetsorb (AMS biotechnology) and cloned into vector pCR2.1 (Figure 2.17.4). For the purposes of cloning in pLST9828, an *XbaI* site was engineered at the 3' end of the *grmA* ORF during amplification. The *grmA* was then excised from pCR2.1 with *XbaI* and cloned (Materials and Methods section 2.14) in the *XbaI* site downstream of *ermEp** to give plasmid pLST1382a. The orientation of the insertion in pLST1382a (Figure 3.4) was confirmed by *BamHI/EcoRV* digestion. Figure 3.4 shows a restriction map of pLST1382a.

3.5.1 Conjugal transfer of plasmids into *M. purpurea*

Conjugation experiments were set up as before with plasmids pSET152, pLST1382a, and pLST9828. Colonies containing these plasmids were large and purple 4 days after being overlayed with apramycin (25 µg/ml) and nalidixic acid (60 µg/ml). There were approximately three hundred colonies on each plate. Forty colonies were picked from each plate and replica plated onto apramycin (25 µg/ml) and nalidixic acid (60 µg/ml). They all grew.

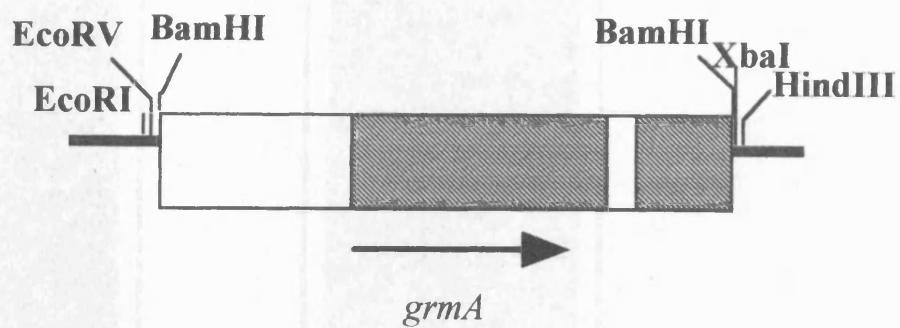
Other conjugations were set up at the same time as those already described, using plasmids pLST1381c and pLST1385 (Figure 3.4), derived from pSET152 and pLST9828 respectively. Conjugal transfer of pLST1385, pLST1381c and pLST0013. was unsuccessful. There was an obvious difference between those vectors that could be introduced and those that could not. pLST1385, pLST1381c and pLST0013 all carried the *grmA* promoter and DNA flanking the gene. pLST1382a however possessed only the *grmA* ORF. The significance of these findings is discussed later in section 3.7. Subtle differences in the restriction maps of pLST1385, pLST1381c and pLST1382a can be identified in Figure 3.4.

Southern analysis was performed, to confirm the integration of a second copy of *grmA* in the *M. purpurea* genome (Figure 3.5).

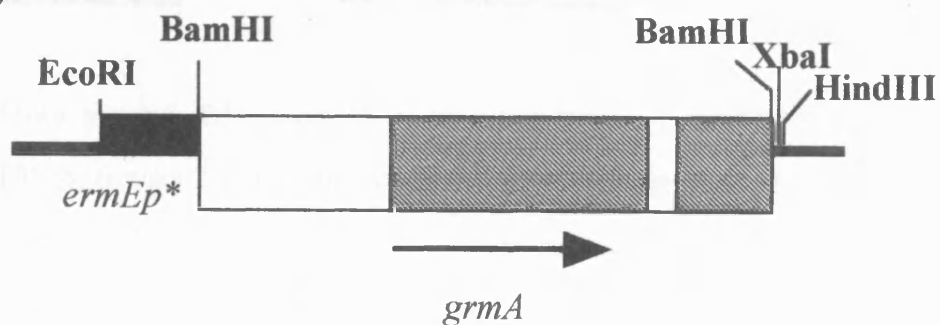
Figure 3.4

Restriction maps of pLST1381c, pLST1382a and pLST1385

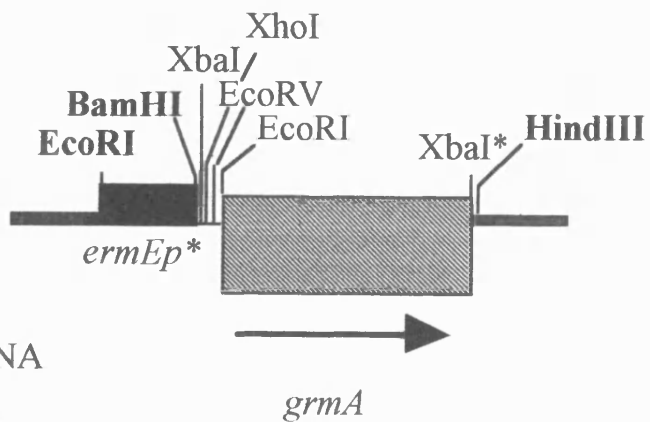
pLST1381c



pLST1385



pLST1382a



200 bp



M. purpurea DNA

pSET152 DNA

Figure 3.5

The integration of a second copy of *grmA* in the *M. purpurea* genome was confirmed by Southern analysis

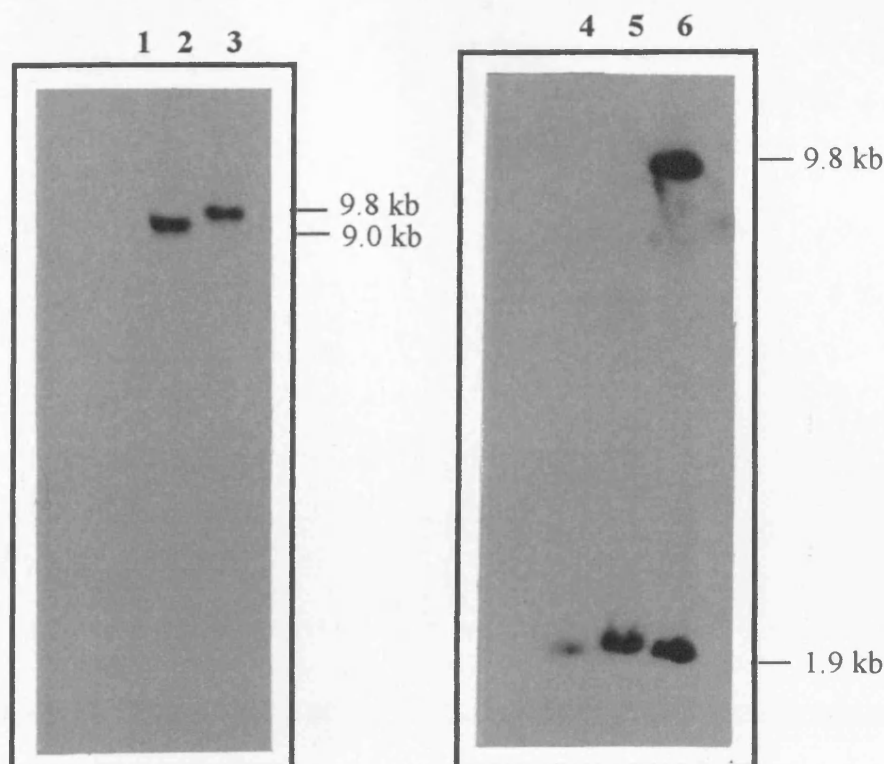


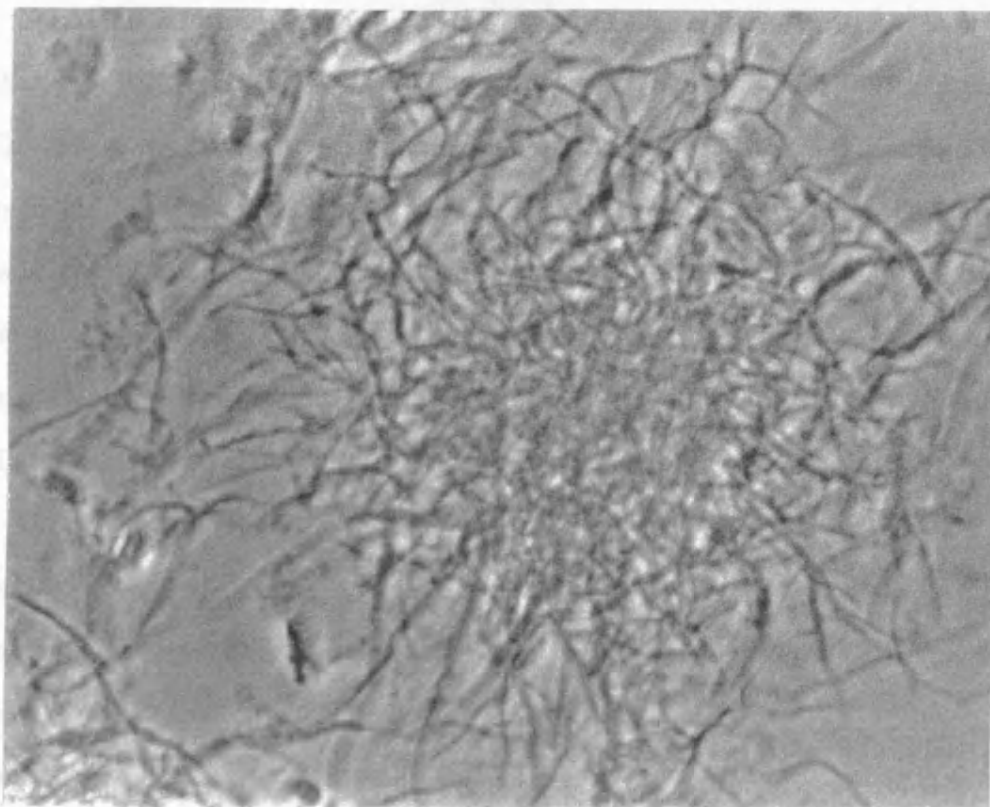
Figure 3.5

10 μ g of total genomic DNA from *M. purpurea* wt (tracks 1 and 4), *M. purpurea* containing pLST9828, (tracks 2 and 5) and *M. purpurea* containing pLST1382a (tracks 3 and 6) was digested with *Bam*HI for 12 hours and then subjected to Southern blotting. The membranes were probed with a 200bp *Bgl*II fragment internal to *grmA* (tracks 4-6) and the 900 bp *Sst*I fragment from pSET152 containing most of the apramycin resistance gene (tracks 1-3.). The membrane was washed at high stringency (Materials and Methods, section 2.8).

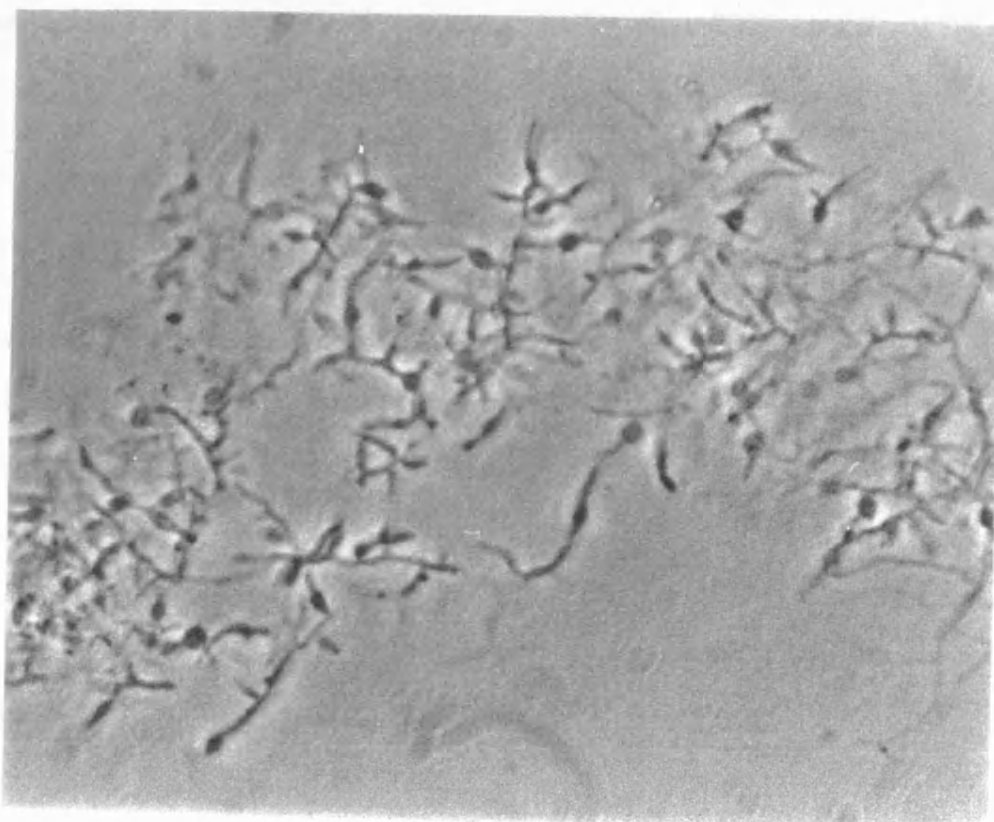
The *grmA* probe hybridised with two copies of *grmA* in the strain that contained pLST1382a (9.8 kb and 1.9 kb fragments), but with only one copy of *grmA* in the wt and the strain that contained pLST9828 (1.9 kb). The *Sst*I probe hybridized with pLST9828 in the strains that contained pLST9828 and pLST1382a but not with DNA from the wt.

Figure 3.6 *expressed in a 5T14Gag-3.0 vector*

(A)



(B)



3.5.2 Is *grmA* expressed in pLST1382a in *S. lividans* ?

pLST1382a was introduced into *S. lividans* TK21 by transformation to establish whether the PCR-amplified *grmA* conferred gentamicin resistance when driven by *ermEp**. *S. lividans* was also transformed with pLST1381c, pLST9828 and pLST1385, at this time. There was confluent growth from the transformed strains, four days after the plates had been overlayed with 1 ml of SSQ containing gentamicin (1 mg/ml). Minimum inhibitory concentration (MIC) values of gentamicin were determined for each strain. It was evident that the PCR-amplified product of *grmA* functioned efficiently in *S. lividans*. See Table 3.0 for the MIC results.

Table 3.0

Minimum inhibitory concentrations for strains of *M. purpurea* and *S. lividans* on gentamicin (gentamicin sulphate supplied by Sigma).

Organism	Plasmid contained	MIC gentamicin (µg/ml)
<i>M. purpurea</i>	wild type	>2000
	pLST9828	>2000
	pSET152	>2000
	pLST1382a	>2000
<i>S. lividans</i> (TK21 & OS456)	wild type	1
	pLST9828	200
	pSET152	200
	pLST1381c	>2000
	pLST1382a	>2000
	pLST1381	>2000
	pLST1385	>2000

3.6 Is *grmA* transcribed in *M. purpurea*?

Following the preparatory work described previously, the expression of *grmA* could be investigated. The aim was to compare the levels of *grmA* transcript in *M. purpurea* wt and *M. purpurea* containing pLST1382a. At this stage it had not been established whether *grmA* transcription was initiated in *M. purpurea*. The levels of *grmA* transcript from *S. lividans* (TK21) transformed with pLST1381c, pLST1382a and pLST9828 were also observed and served as interesting comparisons with *M. purpurea*.

To present clearly the subtle differences between vectors used in these experiments, details of their restriction maps can be found in Figure 3.4.

3.6.1 Preparation and analysis of total RNA

Total RNA was prepared (section 2.5) from the following strains: *M. purpurea* wt, *M. purpurea* containing pLST1382a, *S. lividans* wt (TK21), *S. lividans* (TK21) containing pLST1382a, pLST1381c and pLST9828. The maintenance of integrated plasmids was selected for with apramycin (25 µg/ml). The RNA was quantified and analysed by gel electrophoresis (Figure 3.7).

3.6.2 Dot blot analysis of the *grmA* transcript

Dot blot analysis (Materials and Methods section 2.6) was performed on the RNA prepared in section 3.6.1, to compare the levels of *grmA* transcript in each strain. For the initial dot blot, the RNA was probed with a DNA fragment derived from the 5' end of *grmA*, because at this stage it was unclear whether any *grmA* transcript was being produced in *M. purpurea*. (Figure 3.8).

The RNA was then probed with a DNA fragment derived from the 3' end. This would suggest whether transcript extended to the end of the gene (Figure 3.9). This dot

blot was a more controlled experiment than the initial one, as a single-stranded, end-labelled probe was used. This would specifically hybridise to the *grmA* transcript rather than mRNA being transcribed from the opposite strand of DNA. RNA from *S. lividans* containing pLST9828 was used as a negative control so that non-specific hybridisation with the vector could also be ruled out.

The levels of hybridisation could vary between samples due to an unequal loading of the RNA. To rule out this possibility, a control blot was set up and probed with a fragment of 16S ribosomal DNA (rDNA) originating from *M. purpurea* (Figure 3.10). The rDNA had been generated by amplification (Materials and Methods section 2.16) from the *M. purpurea* genome using PCR (Chapter 4, section 4.2.3).

The RNA was always loaded vertically in the blotter in the following order:

- *M. purpurea* wt
- *M. purpurea* + *grmA* (pLST1382a)
- *S. lividans* + pLST9828*
- *S. lividans* + *grmA* (pLST1385)
- *S. lividans* + *grmA* (pLST1382a)

*Except for Figure 3.8, where RNA from *S. lividans* wt was used.

Details of the amount of RNA loaded and the probe used in each case varies. These parameters are described with each figure. For information about the probes used, see Chapter 1, Figure 1.1, for a restriction map of *grmA*.

Figure 3.7

Electrophoretic analysis of total RNA from *M. purpurea* and *S. lividans* strains.

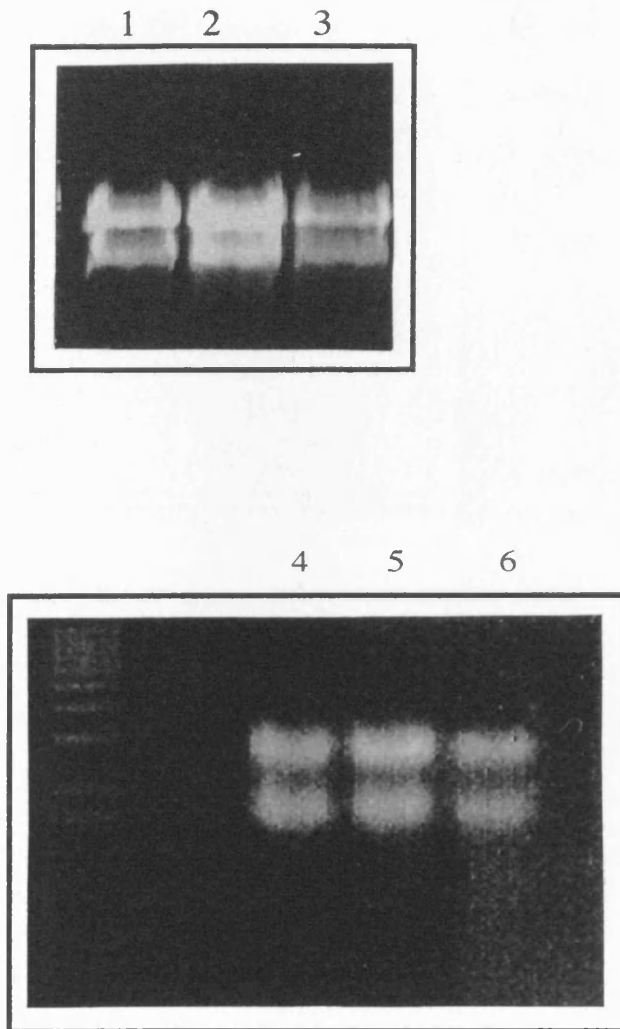
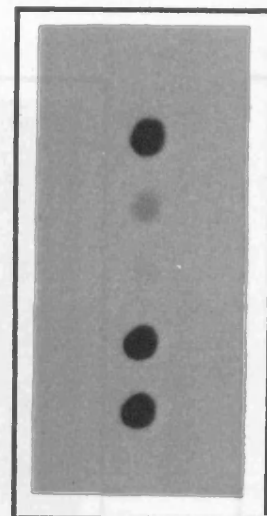


Figure 3.7

Total denatured RNA (10 μ g) from the following strains was separated by gel electrophoresis using 1% agarose containing 2.2 M formaldehyde: *M. purpurea* wt (track 1); *M. purpurea* containing pLST1382a (track 2); *S. lividans* wt (track 3); *S. lividans* containing pLST1382a (track 4); pLST1385 (track 5) and pLST9828 (track 6). The bands for 23S and 16S ribosomal RNA are clearly visible and appear to be undegraded.

Figure 3.8

Total RNA from various strains was probed with a fragment of DNA derived from the 5' end of *grmA*.



M. purpurea + pLST1382a

M. purpurea wt

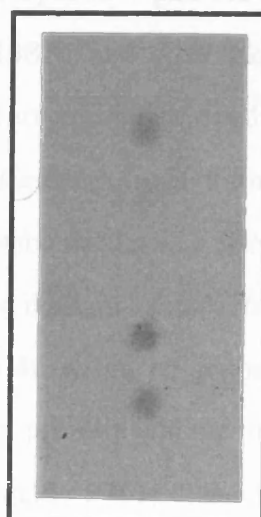
S. lividans TK21 wt

S. lividans TK21 + pLST1381c

S. lividans TK21 + pLST1382a

Figure 3.9

Total RNA from various strains probed with a fragment of DNA derived from the 3' end of *grmA*.



M. purpurea + pLST1382a

M. purpurea wt

S. lividans TK21 + pLST9828

S. lividans TK21 + pLST1381c

S. lividans TK21 + pLST1382a

Figure 3.10 of RNA hybridisation analysis

Total RNA from various strains was probed with a fragment of 16S rDNA from *M. purpurea* wild type.

In Figures 3.8 and 3.9 show a similar pattern of hybridisation. The negative control (RNA from *S. lividans* + pLST9828) showed no signs of hybridisation and gave a clean background to compare with the other strains.

A similar level of hybridisation was observed for RNA from all strains when

tested for *M. purpurea* + pLST1382a. This result confirmed that similar quantities

of RNA were used in each strain. The results of the hybridisation analysis are shown in Figures 3.8 and 3.9.

There was no hybridisation observed for *S. lividans* + pLST9828 (Figure 3.8) and *S. lividans* + pLST1381c (Figure 3.9).

Hybridisation was observed for *M. purpurea* + pLST1382a, *M. purpurea* wt, *S. lividans* + pLST1382a and *S. lividans* + pLST1381c.

The intensity of the hybridisation signal was similar for all strains tested. This could be a useful

method for studying the genetics of *Mitrospira* spp. when combined with the method of conjugation. The level of transcript in *M. purpurea* wt was low compared with the

other strains containing two copies of genes (Figure 3.8 and 3.9) suggesting that

there is a difference in the expression of the genes in *M. purpurea* wt compared with the other strains.

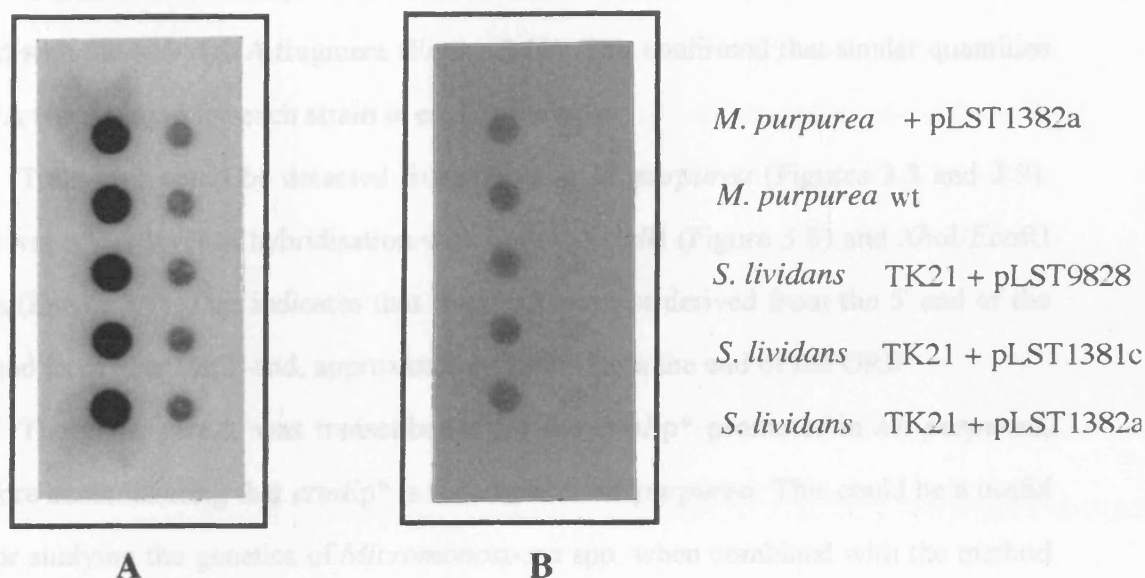
For study of the genetics of *Mitrospira* spp. when combined with the method of conjugation. The level of transcript in *M. purpurea* wt was low compared with the

other strains containing two copies of genes (Figure 3.8 and 3.9) suggesting that

there is a difference in the expression of the genes in *M. purpurea* wt compared with the other strains.

Figure 3.10

The wells were loaded with 0.1 µg (left hand lanes) or 0.1 ng (right hand lanes) of RNA from *M. purpurea* + pLST1382a, *M. purpurea* wt, *S. lividans* + pLST9828, *S. lividans* + pLST1381c and *S. lividans* + pLST1382a. Blotting, hybridisation and washing were performed as described in section 2.6. The X-ray film was allowed to develop for two different periods of time. In **A** the developing time was 1 hour and in **B** 15 minutes. The probe used was a rDNA fragment from *M. purpurea* that encodes 100 bp of the 3' minor domain of the 16S rRNA. This fragment was obtained by PCR-amplification from *M. purpurea* genomic DNA (Chapter 4, section 4.2.3) and labelled by random hexamer priming and the incorporation of [α - 32 P]dCTP by Klenow enzyme (Materials and Methods 2.9.1). The different intensities of the dots are analysed in section 3.7.



3.7 Results of RNA hybridisation analysis

The dot blots in Figures 3.8 and 3.9 show a similar pattern of hybridisation. The negative control (RNA from *S. lividans* + pLST9828) showed no signs of hybridisation and gave a clean background for comparisons with the other strains.

A similar level of hybridisation was observed for RNA from all strains when probed with the 16S rDNA fragment (Figure 3.10). This confirmed that similar quantities of RNA were loaded for each strain in each experiment.

Transcript could be detected from *grmA* in *M. purpurea* (Figures 3.8 and 3.9). There was a low level of hybridisation with both the *Bgl*III (Figure 3.8) and *Xho*I/*Eco*RI probes (Figure 3.9). This indicates that there is transcript derived from the 5' end of the gene and from near the 3' end, approximately 250bp from the end of the ORF.

The gene, *grmA*, was transcribed from the *ermEp** promoter in *M. purpurea*, therefore demonstrating that *ermEp** is functional in *M. purpurea*. This could be a useful tool for studying the genetics of *Micromonospora* spp. when combined with the method of conjugation. The level of transcript in *M. purpurea* wt was low compared with the level in the strain containing two copies of *grmA* (Figures 3.8 and 3.9) suggesting that *ermEp** is a stronger promoter than the native *grmA* promoter and more efficient at driving the expression of *grmA* in *M. purpurea*.

The scenario appears to be different in *S. lividans*. If the levels of transcript for *grmA* driven by *ermEp** (pLST1382a) are compared with *grmA* driven by its own promoter (pLST1381c), the same level of hybridisation can be observed (Figures 3.8 and 3.9). This suggests that like *ermEp**, the *grmA* promoter is constitutive and very effective at driving the expression of *grmA* in *S. lividans*.

3.8 Discussion

3.8.1 Conjugal transfer of DNA into *M. purpurea*

Four plasmids were successfully introduced into *M. purpurea* via conjugation and maintained using the adapted method. These were pSET152, pLST9828, pLST1382a and pLST0012. The number of transconjugants containing pSET152 was a factor of 10 higher than those carrying pLST0012. This was no surprise as integrated plasmids seem to be more stable than replicating ones. Different morphological characteristics could be identified in *M. purpurea* depending on which plasmid the organism was carrying.

A problem arose when pLST0013 was introduced into *M. purpurea* via conjugal transfer. Transconjugants thought to contain pLST0013 would not grow when replica plated onto apramycin. This is a curious finding because it suggests that the plasmid had become unstable and was lost, although why DNA containing *grmA* should elicit this response is not clear.

There could be several explanations for this phenomenon. An increase in the size of the plasmid may have affected its stable inheritance in *M. purpurea*. This seems an unlikely explanation for the following reasons. Firstly, pIJ101 derivatives have a copy number of 40 to 300 per chromosome (Kieser et al. 1982). Such densities reduce the probability of genetic instability during subsequent rounds of bacterial growth and sporulation. Secondly, during construction of pLST0013 the sites for the insertion of DNA did not interrupt those functions believed essential for the stability and replication of the plasmid. Finally, successful transformation of *S. lividans* with both pLST0012 and pLST0013 showed that *grmA* did not render plasmids unstable in *S. lividans*.

Plasmid replication depends on one or more plasmid-encoded products as well as a range of host-encoded replication proteins (reviewed by Thomas 1988). Subtle differences in the action of these proteins between *M. purpurea* and *S. lividans* may have resulted in variations in the stability of pLST0013. However, this also seems unlikely as both pIJ702 (Kelemen et al. 1988) and pLST0012 can be maintained and recovered from *M. purpurea*.

A more likely explanation than the afore-mentioned, is that the introduction of extra copies of *grmA* were toxic to *M. purpurea*. This view is supported by the fact that other plasmids that were successfully introduced by conjugation (pSET152 and pLST9828), were unsuccessfully introduced when *grmA* was cloned into them (pLST1381c and pLST1385 in section 3.5.1). The average number of colonies on each conjugation plate for plasmids pLST0012 and pLST0013 was 30 and 3 respectively. The fact that the cell count was lower with the introduction of pLST0013, suggests that the presence of *grmA* may have been lethal to *M. purpurea*.

Further experiments would be needed to determine whether the toxicity was caused by expression from the *grmA* promoter, or the mere presence of the *grmA* DNA. These experiments would involve the conjugation of pLST0012 and pSET152, with the insertion of *M. purpurea* genomic DNA carrying a disrupted copy of *grmA*. Successful conjugation would confirm that it was expression from the *grmA* promoter that was in some way lethal to *M. purpurea*. It should be noted that a disrupted copy of *grmA* was successfully introduced during attempts to knock *grmA* out in *M. purpurea* using the suicide vector pOJ260. (Chapter 5).

Data obtained prior to this work indicated that *grmA* and an adjacent gene downstream were cotranscribed (Kelemen et al. 1991). A putative regulatory protein which controls the transcription of *grmA* by binding to the promoter, would also control the transcription of the downstream gene and any other genes that were transcriptionally linked to *grmA*.

On the basis of these assumptions the behaviour of *grmA* in *M. purpurea* may be explained by the following hypothesis: Genomic copies of *grmA* would normally be saturated by the bound regulatory protein. It follows that *grmA* and transcriptionally linked genes would either be repressed or transcribed at a very low level. However, this equilibrium would be shifted if further copies of *grmA* together with the native promoter were introduced. A certain percentage of the genomic copies of the *grmA* promoter would remain unbound as the protein binds to the copies introduced by conjugation. This in turn would allow expression of the wild type copy of *grmA* and downstream genes. It is possible that the product of a downstream gene is toxic to *M. purpurea*

The outcome of the introduction of *grmA* driven by *ermEp** also fits this hypothesis. The regulatory protein would not recognise a foreign promoter such as *ermEp** which would allow efficient transcription of plasmid encoded *grmA*. Subsequently genomic copies of the *grmA* promoter would remain bound by the putative regulatory protein so that genomic copies of *grmA* and (perhaps more importantly) downstream genes would remain unexpressed or transcribed at a low level.

3.8.2 Transcript analysis

The results clearly show a low level of transcript specific to *grmA* in *M. purpurea*. Assumptions cannot be made at this stage as to whether the transcript is full length, although the data strongly suggest it. Further work involving mapping the 3' end of the transcript needs to be done in order to establish the size of the transcript. The possibility of transcriptional attenuation, as a mechanism of regulation for *grmA*, would also need to be ruled out, although the present data (Figure 3.9) indicate that any putative truncated transcript produced in the uninduced state would be marginally shorter than the full gene length.

Taken together, the data suggest that the *grmA* promoter is as effective as *ermEp** at driving the expression of *grmA* in *S. lividans* when comparing the levels of transcript for *grmA* from the two promoters (Figure 3.9).

The results in this chapter also indicate that *grmA* is transcriptionally regulated in *M. purpurea* but not in *S. lividans*. RNA dot blot analysis revealed a higher level of transcript specific to *grmA* in *S. lividans* containing an extra copy of the gene (driven by the *grmA* promoter in pLST1381c) than in *M. purpurea* wt (Figure 3.9). Furthermore, regulation of *grmA* in *M. purpurea* appeared to be alleviated by the introduction of *grmA* driven by *ermEp**.

Chapter 4

Methylation of 16S Ribosomal RNA by GrmA

Chapter 4

Methylation of 16S Ribosomal RNA by GrmA

4.0 Aims

The aims behind these experiments were to establish whether GrmA methylates 16S rRNA at guanine 1389 (1405 in *E. coli*) in *M. purpurea*, and find out if an extra copy, introduced on plasmid pLST1382a, would overcome or titrate out the effects of a putative repression or regulation mechanism.

The objectives were to isolate ribosomal RNA from *M. purpurea*, *M. purpurea* containing an extra copy of *grmA*, *S. lividans* and *S. lividans* containing *grmA* and carry out primer extension using the 16S rRNA as a template for reverse transcriptase to identify pauses due to methylation by GrmA.

4.1 Introduction

4.1.1 The structure and function of 16S rRNA

Ribosomal RNAs are very highly conserved between species. This is probably due to the fact that they have a central role in both the structure and function of ribosomes (Noller 1991, Noller et al. 1995). This introduction will concentrate mainly on the structure and relevance of 16S rRNA. However, the importance of 5S rRNA and 23S rRNA should be remembered.

At one time rRNA was considered to be a structure for the positioning of ribosomal proteins. It was assumed that ribosomes were a multi-enzyme complex driving translation and rRNA was merely the structural component. However, with the discovery of self splicing introns and catalytic RNA (Cech et al. 1981, Guerrier-Takada et

al. 1983) it became widely accepted that rRNA has a potentially important role in the biochemical processes of translation.

To elicit the structure of 16S rRNA, studies were carried out using a set of chemical probes, specific for each nucleotide (Stern et al. 1989). The susceptibility of the RNA to modification by these probes was assessed by primer extension, using the modified RNA as a template and synthetic oligimers as primers. Reverse transcriptase paused at the sites of modification causing premature termination. The cDNA was separated by polyacrylamide gel electrophoresis and the pause sites were identified. Intramolecular and intermolecular interactions protected bases from the chemical probes and these were revealed as an absence of bands or bands of reduced intensity. Various other methods have been employed since to confirm these findings. One of the most recent methods used is hydroxyl radical footprinting (Heilek et al. 1995, Noller et al. 1995).

Proteins play an important role in the stability and tertiary structure of rRNA. Mutations in proteins can alter the conformation of 16S rRNA (Allen and Noller 1989, Gutell et al. 1985, Noller 1991). Proteins involved in the late stages of ribosomal assembly may be responsible for the fine tuning of rRNA functional sites and induction of conformational changes, which can in turn activate sites required for ribosomal function. Mutations in assembly proteins S4 and S12 can influence the efficiency of translational proof reading, despite the fact that these mutations are clustered, on the opposite side of the 30S subunit from the site of codon-anticodon interaction. S4 and S12 may indirectly influence events in the decoding region through changes in the conformation of the 16S rRNA.

The omission of individual proteins sometimes fails to show all or nothing effects on translation and often result in only modest decreases in ribosome function. However, some proteins have a direct role in the process of translation. An example of such a protein is S21, which is essential for initiation. Located around the cleft in the small subunit, S21 is required for the correct base pairing of the Shine-Dalgarno sequence of the mRNA to the 3' end of the 16S rRNA (Backendorf et al. 1981). Some proteins

appear to be positioned at major bends in the RNA. Their role may be to stabilise these bends.

In tertiary folding, base-protein, protein-protein and base-base interactions are important for ribosome function. Different domains in the 16S rRNA can be spaced quite far apart but are folded in such a way that they are in close proximity to each other in pseudoknot-like structures (Gutell and Woese 1990).

Cunningham et al. (1993) demonstrated that mutations in base pairs G1401:C1501, C1404:G1497 and G1405:C1496 (by changing a C to a G or *vice versa* in one or other of the residues) inhibited tRNA binding to the ribosomal A and P sites. This provided further evidence for the role of rRNA in translation. Double mutations in these base pairs largely restored tRNA binding. However, synthesis of fMet-Val was perturbed, indicating that the primary sequence of the 16S rRNA is also an important factor in ribosome function.

4.1.2 16S rRNA interacts with transfer RNA and messenger RNA

Translation functions efficiently due to close interactions between tRNA, rRNA, and mRNA. These interactions are important in the three fundamental aspects of translation; codon and anticodon interaction at the first stage of initiation, peptide bond formation during elongation, and finally translocation, which is the movement of tRNA and mRNA with respect to the ribosome. A classic example is the specific base pairing between the purine-rich sequence 5' to the initiation codon in the mRNA, known as the Shine-Dalgarno site (Shine and Dalgarno 1974) and a pyrimidine-rich sequence near the 3' end of the 16S rRNA. Base pairing may also occur at other sites along the rRNA and mRNA, the purpose of which may be to retain the correct reading frame during translation (Trifonov 1987).

Another important function of the 16S rRNA is the alignment of tRNA with mRNA in the decoding region (the A and P sites) in the early stages of initiation. Moazed and Noller (1986) used chemical probing to look at the interactions between

tRNA and 16S rRNA. Ribosomes were subjected to chemical probing in the presence and absence of bound tRNA. Bases protected by tRNA were in areas where the secondary structure was very highly conserved around the A and P sites (Moazed and Noller, 1986).

Residues involved in P site interactions in the 16S rRNA seem to be more widely distributed than those around the A site (Ahsen and Noller 1995, Moazed and Noller 1990). Here, tRNA only protects two clusters, the 1400-1500 region and the 530 loop, which is located at the opposite side of the 30S subunit from the decoding region. Tertiary folding brings these regions close together. The 530 loop may have a role in the binding of elongation factor EF-Tu to the ribosome (Moazed et al. 1995). The 1400-1500 loop is an area of interest because many aminoglycoside antibiotics bind here.

4.1.3 Interactions of antibiotics with ribosomal RNA.

Many Antibiotics are ribosomal ligands that have been fundamentally important in the study of ribosome structure and function. They are found to interact with specific regions of the rRNA. Streptomycin is shown to protect residues 911 to 915 in the 16S rRNA. In the middle of this region is residue 912 which, when mutated, confers resistance to streptomycin (Maozed and Noller 1987). It is also in this region of the 16S rRNA that mutations of the assembly protein S12 confer streptomycin resistance or dependence. Neomycins and related antibiotics induce translational errors and inhibit translocation by binding closely to the site of codon recognition in the small subunit. They interfere with tRNA binding and protect residues A190, G791, A909, A1394, A1394, A1413, and G1487 (in *E. coli*) from chemical probes during the procedures described previously in sections 4.1.1 and 4.1.2.

Post transcriptional modifications of rRNA are clustered in conserved regions around residues 1400-1500. These modifications can either be attributed to methylation that confers resistance to antibiotics (Beauclerk and Cundliffe 1987) or that which is not associated with any known phenotype (Hall and Dunn 1968).

The close association between the interaction of antibiotics, the decoding region of the 16S rRNA (Moazed and Noller 1987, Purohit and Stern 1994), and clusters of modified residues indicates that antibiotics may have had an important function in the evolution of the translation apparatus (Davies 1990). This is discussed further in Chapter 1 (section 1.1).

4.1.4 Resistance to antibiotics

Post transcriptional modification of 16S rRNA is not the only mechanism shown to result in antibiotic resistance. Point mutations at the 3' end of the 16S rRNA have been shown to confer resistance to aminoglycosides. Two examples of this are a C to G substitution in the 16S rRNA gene of yeast mitochondria (equivalent to C-1409 in *E. coli* 16S rRNA) which results in resistance to paromomycin (Li et al. 1982) and a U to C transition in the 16S rRNA of *Tetrahymena* (equivalent to U-1495 in *E. coli*) giving rise to hygromycin resistance (Spangler and Blackburn 1985). These two organisms are useful for the analysis of these mutations as they possess only single copies of the rRNA genes. Mutations in *E. coli* are difficult to isolate because there are seven rRNA operons. A point mutation in one of these genes might escape detection because only a small fraction of the ribosomes would be altered.

The exact position of post transcriptional modification can be determined by primer extension. Reverse transcriptase assays have been used extensively to locate the sites of action of ribosomal methylases in 16S and 23S rRNA (Chapter 1, Table 1). The modified rRNA is used as a template for reverse transcriptase in the presence of an oligonucleotide primer and [α - 35 S]dATP. The enzyme transcribes the RNA into cDNA but modification of the template causes the enzyme to pause at the preceding nucleotide. cDNA is separated by polyacrylamide gel electrophoresis.

Methylation of guanine at N7 of the purine ring has no effect on cDNA extension by reverse transcriptase. If the RNA template is treated first with sodium borohydride followed by aniline (Peattie 1979), scission of the phosphodiester backbone occurs 3' to

7-methylguanosine and this terminates transcription (Inoue and Cech 1985). In order to confirm that GrmA methylates at G1389 (1405 in *E.coli*), rRNA had to be cleaved with sodium borohydride and aniline before methylation of guanine at N7 could be identified.

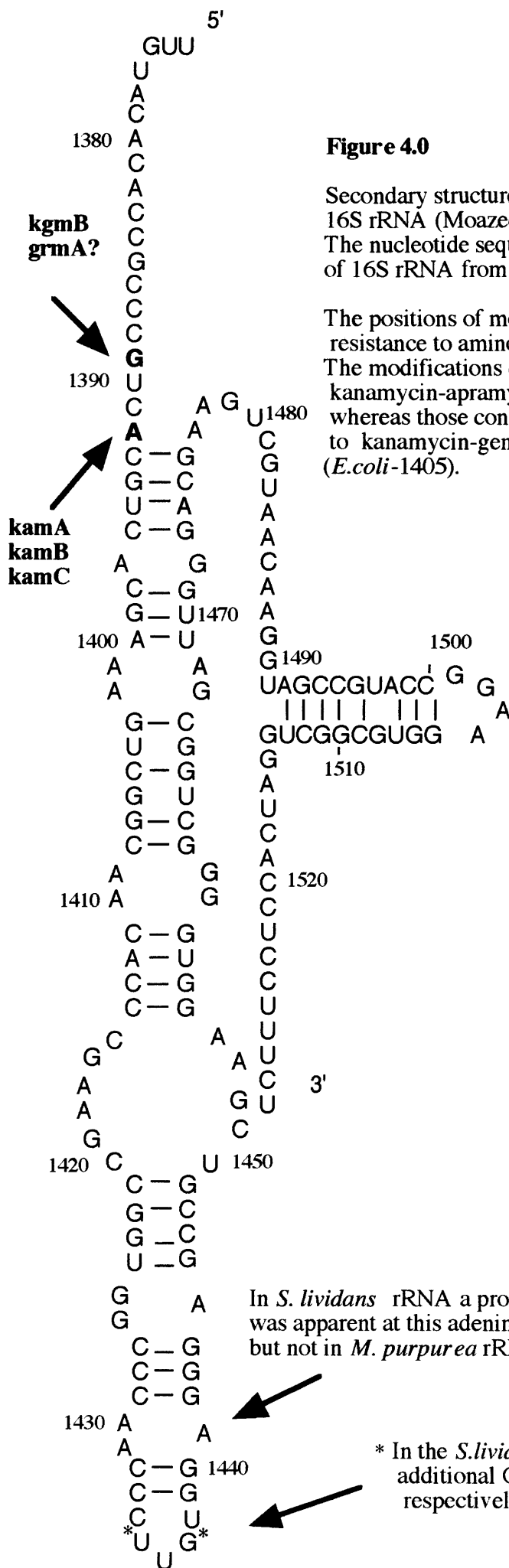


Figure 4.0

Secondary structure model for eubacterial
16S rRNA (Moazed et al 1986).
The nucleotide sequence is that
of 16S rRNA from *M. purpurea*

The positions of modification in 16S rRNA giving
resistance to aminoglycosides are shown here.
The modifications conferring resistance to
kanamycin-apramycin are at position 1392
whereas those conferring resistance
to kanamycin-gentamicin are at position 1389
(*E.coli*-1405).

In *S. lividans* rRNA a prominent pause
was apparent at this adenine residue,
but not in *M. purpurea* rRNA. See figure 4.1

* In the *S.lividans* 16S rRNA there is an
additional C and G at 1434 and 1440
respectively .

4.2 Methods

4.2.1 Strand scission of rRNA using sodium borohydride and aniline

The method was an adaptation of an earlier protocol (Peattie 1979). Ribosomal RNA (10 µg, either as a dry pellet or dissolved in 1-2 µl of super Q) was added to 10 µl of Tris HCl (pH 8.2) in a large eppendorf tube. The RNA was left to dissolve in the Tris-HCl for 5 minutes on ice. To this, 10 µl of 0.2 M sodium borohydride was added. This was mixed gently with the end of a plastic pipette tip and left in the dark, on ice, for 60 minutes. After this time 0.6 M sodium acetate and 600 µl of ice cold ethanol were added to the reactions and they were precipitated overnight at -20°C. Once precipitated, the RNA was spun down for 10 minutes in a microcentrifuge and vacuum-dried for 10 minutes. The RNA was not dried to completion as this caused problems when redissolving the RNA in the next step.

The RNA was resuspended gently in 20 µl of 1 M aniline acetate (pH 4.5) and left in the dark for approximately 5 minutes to dissolve. After this time the RNA was frozen in a bath of dry ice and IMS. The eppendorf tubes were opened and covered in Nescofilm. Holes were then punched in the Nescofilm with a hyperdermic needle and the RNA was lyophilized for approximately two hours until the pellet was dry. The RNA pellet was then dissolved in 100 µl of SSQ water and lyophilised again, to remove the residual aniline. Finally, it was dissolved in 8 µl of SSQ water. 80% recovery was assumed giving a concentration of 1 µg/µl.

4.2.2 Primer extension of 16S rRNA

The protocol used was an adaptation of a method used earlier in this laboratory (Beauclerk and Cundliffe 1987).

Reagents

Annealing buffer

10mM Hepes KOH (pH 7.0)

50mM KCl

Extension buffer

50mM Tris-HCl (pH 8.3)

10mM MgCl₂

50mM KCl

20mM dithiothreitol.

Superscript II storage buffer

20mM Tris-HCl (pH7.5)

100mM NaCl

0.1mM Na₂EDTA

50% glycerol.

4.2.2.1 Preparation of the annealing mixture

Ribosomal RNA (1 µg) was added to 2 pmol of oligonucleotide, PE1(3), in 3 µl of annealing buffer contained in a small eppendorf tube (0.5 ml).

4.2.2.2 The annealing reaction

The annealing mixture was heated in a Hybaid oven at 75°C for 5 minutes. The temperature setting was altered to 45°C. The annealing reactions were left to cool to this temperature. The cooling process took between 10 and 15 minutes. The samples were

removed from the oven and the magnesium concentration was raised to 10 mM by the addition of 1.5 μ l of annealing buffer containing 30 mM magnesium chloride. Incubation was continued for a further 20 minutes on the bench at 25°C.

4.2.2.3 The extension reaction

To the annealing mixture was added 4.5 μ l of extension buffer containing 125 μ Mol of each dNTP (except for dATP), 3.2 μ Ci [α -³⁵S] dATP (100Ci mmol⁻¹ Amersham) and 1 unit of reverse transcriptase (Superscript II RNase H supplied by Gibco BRL catalogue number 18064-022). The Superscript II had previously been diluted in storage buffer. The extension mix was incubated in the Hybaid oven at 45°C for 40 minutes after which time the samples were removed and 2 μ l of chase solution was added. This consisted of 500 mM of each dNTP in extension buffer. The samples were then returned to the oven and incubated for a further 20 minutes at 45°C. The reaction was then stopped by quick freezing using a dry ice/IMS bath.

4.2.3 Generation of a sequence ladder of 16S rRNA from *M. purpurea*

4.2.3.1 The source of the template

A sequence ladder was generated from DNA, which is complementary to the region of 16S rRNA around G1389. The ladder would serve as a set of markers to locate pause sites in the primer extension reactions. To obtain the relevant stretch of DNA for sequencing, primers were designed around this region and the ribosomal DNA (rDNA) was amplified by PCR (Materials and Methods, section 2.16) from genomic DNA isolated from *M. purpurea*. The PCR-amplified region extended over approximately 100 bp of DNA.

4.2.3.2 Amplification of the 16S rDNA

The two primers used to amplify the ribosomal DNA were derived from nucleotides 1376 to 1393 and from nucleotides 1476 to 1493 inclusive. Amplification was carried out using *Taq* polymerase, (Gibco BRL) at an annealing temperature of 50°C.

4.2.3.3 Ligation and transformation of the amplified product

Once amplified, the rDNA fragment was gel isolated using jetsorb (AMS Biotechnology) and ligated with PCR vector PCR2.1 (Invitrogen) (Figure 2.17.4), following the protocol for ligation and transformation set out by Invitrogen's TA cloning kit (catalogue number K2030-01). This kit was ideal for this purpose because *Taq* polymerase adds a single deoxyadenosine to the 3' ends of the amplified product. The linearised PCR 2.1 vector, supplied with the kit, has single 3' deoxythymidine residues allowing for efficient sticky-ended ligation. Transformation was also pursued using competent *E. coli* INFαK' cells supplied with the TA cloning kit. Plasmid was prepared from the transformants and restricted with *Eco*RI to confirm the presence of the insert.

4.2.3.4 Sequencing

Manual sequencing of the rDNA fragment was performed using the T7 sequencing kit from Pharmacia. This provided all of the solutions, reagents and detailed protocols required for all stages of the DNA sequencing. The sequence was first determined using universal primer. Plasmid pCR 2.1 possesses a modified pUC18 multicloning site, complementary to universal and reverse primer. For convenience, the rDNA was sequenced in this vector.

Sequence generated using the universal primer was compared with that of *M. purpurea* 16S rRNA, found in the Nucleotide Query database (at <http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/quer>). The sequences were found to be the same apart from a nucleotide change. The residue at 1393 was different. In the sequence generated here it was a C and in their sequence it was a G.

To generate the ladder needed for analysis of the primer extension data, sequencing was executed using primer PE1(3) (section 4.2.3.5).

4.2.3.5 Preparation of Oligonucleotide PE1(3) used to prime reverse transcriptase

The primer used was complementary to residues 1476 to 1493 of the *M. purpurea* 16S rRNA. The sequence of the primer was 5' CTACCTTGTTACGACTTC 3'. It was prepared and purified by Genosys. The method of purification used was separation by polyacrylamide gel electrophoresis.

4.3 Results

The results are presented in Figures 4.1, 4.2, and 4.3. Three experiments were performed to confirm the results. The original preparation of rRNA was used on each occasion. However, this RNA had been aliquoted. A different aliquot was used for each experiment. More importantly, the cleaved template was taken from different cleavage reactions for each primer extension experiment.

16S rRNA was isolated from the four strains: *M. purpurea* wt, *M. purpurea* containing *grmA* (pLST1382a), *S. lividans* wt (OS456) and *S. lividans* (OS456) containing *grmA* (pLST1381). All the strains were grown in YEME/PEG as described in the protocol. The *S. lividans* strain containing pLST1381 was grown in gentamicin (20 µg/ml) and the *M. purpurea* strain containing pLST1382a in apramycin (25 µg/ml).

The rRNA was used as a template for reverse transcriptase after it was primed with PE1(3). cDNA was also generated from a region of 16S rDNA (section 4.2.3) using a commercial sequencing kit (Pharmacia). The cDNA transcripts were separated by gel electrophoresis. cDNA derived from the 100 bp fragment of rDNA (section 4.2.3) served as a ladder for the identification of methylation dependent pauses associated with the action of *grmA*.

To expose the methylation of guanine using reverse transcriptase, the RNA was first cleaved with sodium borohydride and aniline. Primer extension was performed on untreated RNA to confirm that the aniline treatment had progressed to completion. A strong pause present in the cleaved samples, which was absent in the uncleaved samples, could then be attributed to the N7-methylation of guanine.

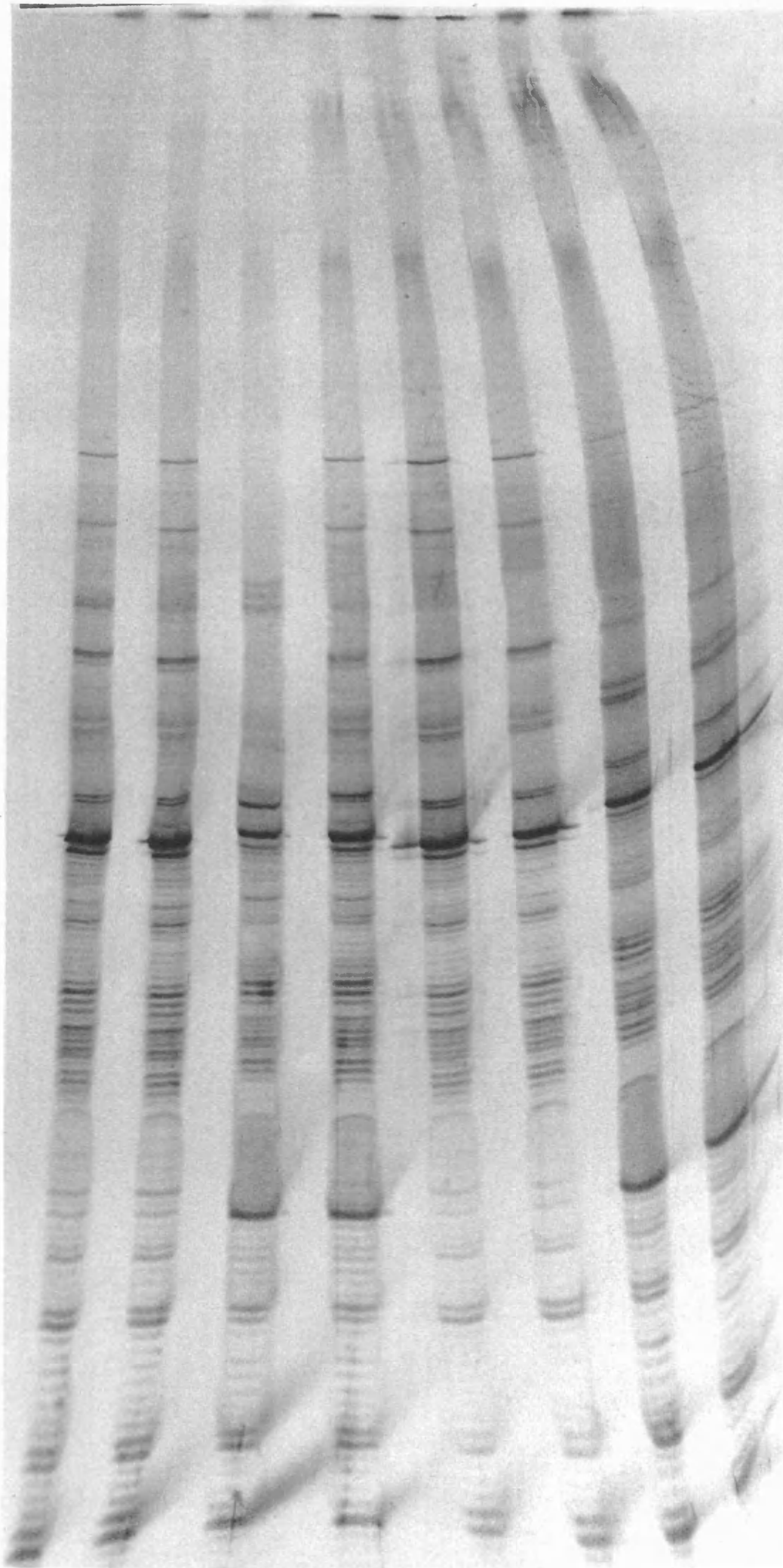
The results of the experiments with un-cleaved RNA clearly showed that reverse transcriptase extended the primer through the region of interest. The pattern of pauses was consistent between species. This was unsurprising as the sequence and secondary structure in this region of the 16S rRNA is very highly conserved. Pausing of this sort can be attributed to the modification of residues not associated with an antibiotic resistance phenotype (Hall and Dunn 1968, Fellner and Sanger 1968).

In the experiments where the RNA had been chemically treated with sodium borohydride and aniline, one prominent methylation-dependent pause site was seen in the *S. lividans* sample that contained *grmA*. This was unequivocally attributed to the action of *grmA*. The conversion of residue G1389 to 7-methylguanosine, followed by strand scission by sodium borohydride and aniline caused the termination of reverse transcription. This interpretation is reinforced by the fact that the strong pause identified at G1389, was not present in the *S. lividans* wt.

The results in this chapter confirm that GrmA does not methylate residue 1389 in *M. purpurea* 16S rRNA under the conditions described. A strong pause could not be observed at residue 1389 in *M. purpurea* or in *M. purpurea* containing an integrated copy of *grmA*.

Figure 4.1

1 2 3 4 5 6 7 8



U1374

C1387

U1395

A1440

Figure 4.2

Primer extension was performed to determine the site of methylation by GrmA

1 2 3 4 A C G T 5 6 7 8

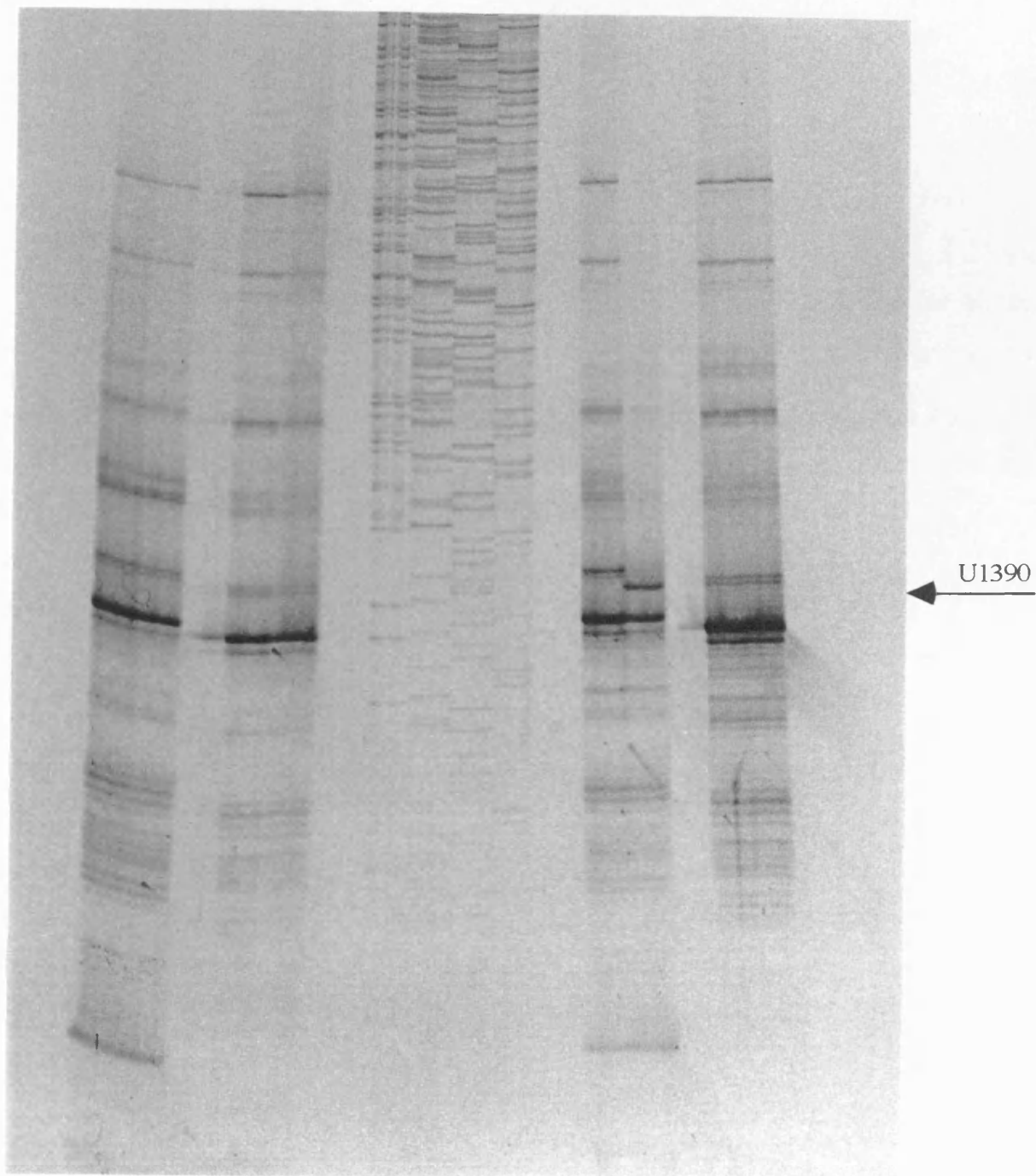
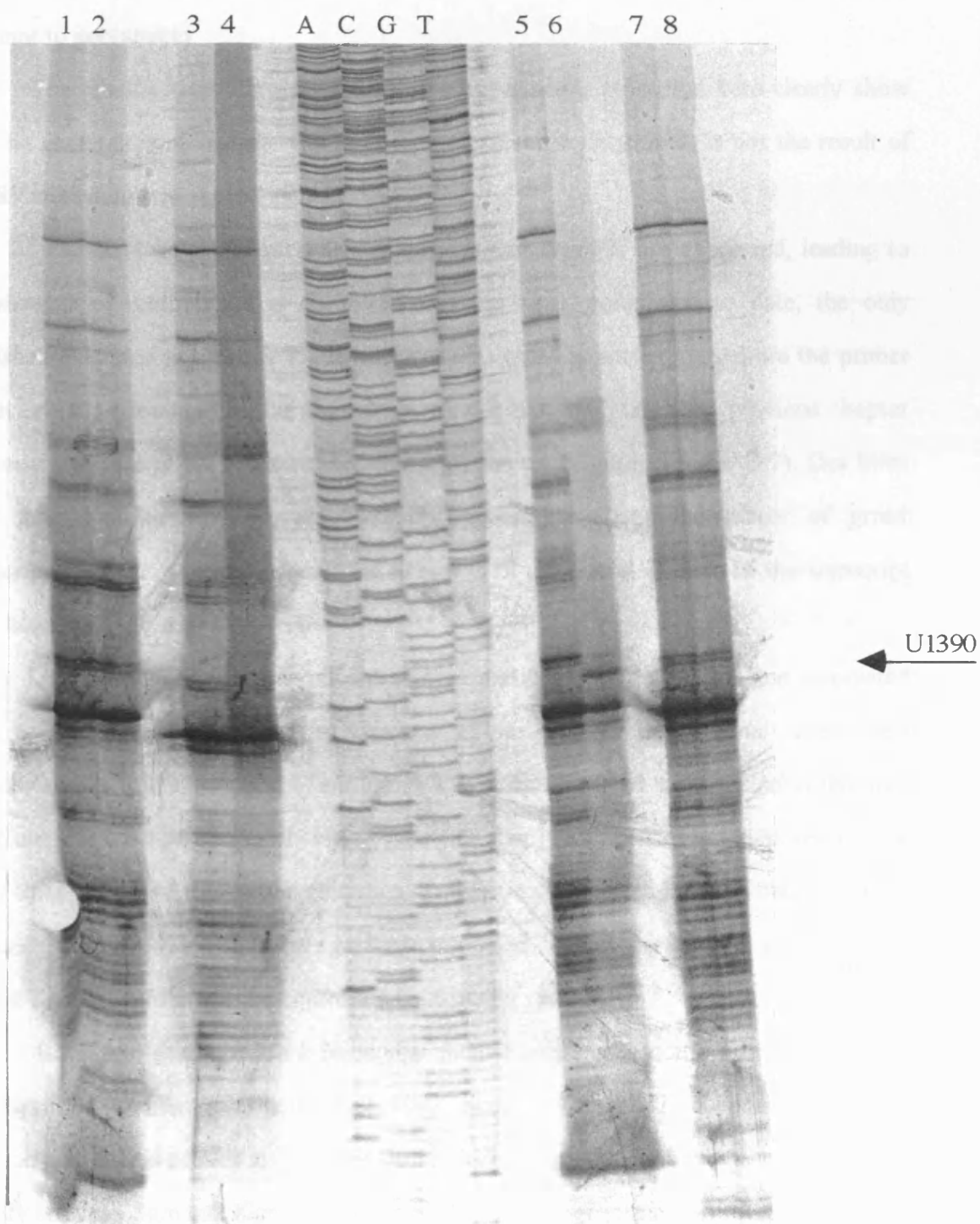


Figure 4.3

Primer extension analysis to confirm the site of methylation by GrmA



4.4 Discussion

Ribosomes were extracted from *M. purpurea* in the gentamicin-producing and non-producing phases (Piendl and Böck 1982). These ribosomes were assayed for protein synthesis in the presence of gentamicin (up to 500µg/ml) using polyuridylic-acid-dependent polyphenylalanine synthesis. They found the ribosomes to be constitutively resistant to gentamicin.

The results from the primer extension experiments presented here clearly show that the gentamicin resistance, exhibited by *M. purpurea* ribosomes, is not the result of G1389 methylation in the 16S rRNA.

The obvious explanation at this stage is that *grmA* is not expressed, leading to the absence of methylation at G1389. From the work completed to date, the only published evidence supporting the hypothesis that *grmA* is not expressed are the primer extension data presented in this chapter. RNA dot blot analysis in the previous chapter demonstrated that *grmA* is transcribed in *M. purpurea*. (Figures 3.8 and 3.9). Dot blots have their limitations, however. Definite conclusions about the extent of *grmA* transcription in *M. purpurea* cannot be drawn until the 5' and 3' ends of the transcript have been mapped in this organism.

A long 5' UTR that contains extensive secondary structure is often associated with transcriptional attenuation (Kelemen et al. 1994), translational attenuation (Weisblum 1984, Mayford and Weisblum 1989) or translational autoregulation (Holmes and Cundliffe 1991, Kojic et al. 1996) (see Chapter 1, section 1.11, for more details). *grmA* does not share these characteristics. Translational autoregulation is believed to be the mechanism by which *sgm* and *kgmB* are regulated. This is discussed in more detail in section 3.1.2. Could *grmA* be controlled by a similar mechanism?

Both *sgm* and *kgmB* are ribosomal methyltransferases conferring resistance to kanamycin and gentamicin (Kojic et al. 1992, Skeggs et al. 1987). Comparisons of the deduced amino acid sequences revealed 90 % identity between Sgm and GrmA, and 54% identity between Sgm and KgmB (Kojic et al. 1992). Closer inspection reveals, however,

that the promoter regions of these three elements differ considerably (Kojic et al. 1996, Kelemen et al. 1991, Holmes et al. 1991).

The transcriptional and translational elements, which are involved in the regulation of *sgm*, *grmA* and *kgmB*, appear to have evolved independently of the coding regions for these genes. It follows that *grmA* could be regulated by a mechanism that has also evolved independently, and may therefore be different to existing models.

A putative mechanism of regulation for *grmA* was discussed in Chapter 3, sections 3.1.2 and 3.8.2. The conclusions drawn from the data in Chapter 3 were that *grmA* is subject to transcriptional control and that the *grmA* promoter is an essential component in this regulation. However, due to the lack of analysis of the *grmA* transcript in *M. purpurea*, no definite conclusions could be drawn.

If *grmA* is silent in *M. purpurea* gentamicin resistance must be due to another ribosomal resistance gene. Ohta and Hasegawa (1993) claimed to have cloned a novel gentamicin resistance gene from *M. purpurea* that gave a similar resistance phenotype to *grmA*. The gene was found by probing *M. purpurea* genomic DNA with a fortimicin resistance gene (*fmrO*) cloned from the astromycin producer, *M. olivasterospora*. The amino acid sequence of *fmrO* shows a sequence identity of 30.8% to *grmA* and confers resistance to gentamicin, kanamycin and astromycin. Constitutive resistance to gentamicin in *M. purpurea* could possibly be due to an *fmrO*-like gene.

Two further questions need to be answered: Is the *grmA* transcript translated in *M. purpurea*? Does it methylate 16S rRNA at an alternative site to G1389? In the decoding region there is a close association between the locations of aminoglycoside binding sites in the decoding region, and methylated bases giving resistance to those antibiotics. Therefore, it seems highly unlikely that *grmA* would methylate rRNA outside this region. However, the pentameric sequence (GCUCA), embracing G1389, is directly repeated (Figure 4.0). The GrmA protein may recognise a guanine residue at position 1394 within the repeated sequence.

Differences in the secondary or tertiary structure of the 16S rRNA between *S. lividans* and *M. purpurea* may account for an alternative methylation site for GrmA in *M. purpurea*. Assuming that there are structural differences, one interpretation of the

data is that residues G1394 and G1389 are recognised by GrmA in *S. lividans* but in *M. purpurea* GrmA has access to only one binding site at G1394. The data in this chapter show a strong pause in the region of G1394. However, there is a strong pause present in this region in every sample, including *S. lividans* wt.

Although there are differences between *M. purpurea* (Koch et al. 1996) and *S. lividans* (Suzuki and Yamada 1988), in the nucleotide sequence of the 1400-1500 loop, the secondary structure appears to be conserved for the two species. Sequence alignments presented at internet site <http://www-rrna.uia.ac.be> were analysed to establish if there are differences in the secondary structure of the 16S rRNA between *M. purpurea* and *S. lividans*. Internal loops or bulges appear to interrupt the normal helical structure around nucleotides G55, C80 and C81, U181 and U182, and G1120 in *M. purpurea* 16S rRNA. However, these regions of rRNA have not been shown to interact with the 1400-1500 bp loop in the decoding region. These are the only apparent differences according to the information presented at www-rrna.uia.ac.be.

Alterations in proteins can affect the tertiary structure of the rRNA (Allen and Noller, Gutell 1985, Noller 1991). Although *M. purpurea* and *M. melanosporea* are closely related organisms, which exhibit a high degree of ribosomal protein homology, there are differences in four proteins in the 30S subunit and three proteins in the 50S subunit (Piendl and Böck 1982). This may contribute to the different levels of gentamicin resistance exhibited by the 50S subunits isolated from these two organisms. The large subunit in *M. purpurea* may be altered in such a way that gentamicin cannot interact with it.

It would be interesting to study the level of homology between ribosomal proteins in *S. lividans* and *M. purpurea*. Differences in their proteins may affect the tertiary structure of the rRNA and subsequently change the site of methylation of GrmA.

If GrmA was acting elsewhere in the 1400-1500 region, primer extension analysis, described in this chapter, would reveal a strong pause in the 16S rRNA isolated from *M. purpurea*. This would be absent in 16S rRNA isolated from *S. lividans* wt. However, this was not observed under the conditions used in these experiments. The

possibility of an alternative methylation site for GrmA should not be disregarded before the 16S rRNA has been scrutinised further, by primer extension analysis.

In the past, the sites of methylation of other aminoglycoside resistance genes were determined once they had been cloned in *E. coli* or *S. lividans*. (Beauclerk and Cundliffe 1987, Holmes et al. 1991, Skeggs et al. 1985). Have primer extension experiments been performed on 16S rRNA from aminoglycoside producers? Such experiments using rRNA isolated from *S. tenebrarius* and *S. tenjimariensis*, would confirm the site of methylation of their resistance determinants. It would be very interesting to establish whether an absence of methylation at G1389 was observed for KgmB, for example.

Earlier in this discussion, it was stated that an alternative methylation site for GrmA would be predicted to lie in the decoding region. The same could be said for an *fmrO*-like gene. However, the data obtained here present a flaw in this argument. There are no methylation-dependent pauses specific to *M. purpurea* in this region.

Chapter 5

**Is *gmrA* the only gentamicin resistance gene in
M. purpurea?**

Chapter 5

Is *grmA* the only gentamicin resistance gene in *M. purpurea* ?

5.0 Aim

There were two possible explanations for the data obtained in Chapter 4. The GrmA protein could be methylating 16S rRNA at a site other than G1389. Alternatively, *grmA* could be silent in *M. purpurea*, in which case there must be another resistance determinant responsible for the constitutively resistant phenotype. The aim of the work in this chapter was to address the question: Is *grmA* the only gentamicin resistance gene in *M. purpurea*?

The objective was to shotgun clone genomic DNA isolated from *M. purpurea* into *S. lividans* and select for gentamicin resistant colonies. Restriction analysis would reveal if the DNA, conferring gentamicin resistance, contained *grmA* or an alternative gene.

5.1 Shotgun cloning

5.1.1 The use of pIJ702 and *Streptomyces lividans* for gene cloning

Shotgun cloning of gentamicin resistance genes from *M. purpurea* involved the use of a host strain, in this case *S. lividans*, and a cloning vector, pIJ702. The two have been used together to clone a number of resistance determinants from many actinomycetes. pIJ702 has a wide host range that extends beyond the *Streptomyces* spp. It was derived from pIJ101, which is an 8.9 kb plasmid present in *S. lividans* ISP5434. Some of the important features of pIJ702 are discussed in Figure 2.17.1. The high copy

number of pIJ702 reduces the genetic instability of the plasmid and enables the overproduction of extracellular proteins (reviewed by Gusek and Kinsella 1992). It is a non-conjugative vector; which is a useful characteristic for shotgun cloning, because conjugative vectors can cause superinfection during protoplast regeneration.

S. lividans is ideal as a host for cloning DNA: It possesses the ability to secrete foreign proteins, recognise a wide range of foreign promoters and seems to lack an endonuclease restriction system. *Streptomyces* spp are ubiquitous in soil and compost. They naturally excrete enzymes capable of degrading leaf litter such as proteases, amylases, xylanases, and chitinases. *Streptomyces* spp. make good hosts for the excretion of foreign proteins. *S. lividans* is particularly suitable because of the fact it excretes very low levels of proteases (Gusek and Kinsella 1992)

5.1.2 Preparation of *M. purpurea* genomic DNA for shotgun cloning

The likelihood of there being another gentamicin resistance determinant in *M. purpurea* was discussed in Chapter 1, section 1.9. The *M. purpurea* genome was probed with a fortimicin A resistance gene, *fmrO* (Ohta et al. 1993), from the fortimicin producer, *M. olivasterospora* (see section 1.3). The gene hybridised with a 6.1kb *Bam*HI fragment rather than a 1.9kb *Bam*HI fragment (Figure 1.1) as would have been expected for *grmA* (Kelemen et al. 1991).

The fragments of *M. purpurea* genomic DNA used in shotgun cloning, needed to be between 1kb and 6kb in size, as this would include *grmA* and the putative gene found (Ohta et al. 1993). Initially, a *Bam*HI total digestion was used.

An ostensibly total digestion of *M. purpurea* genomic DNA was performed. 80 µl (80 µg) of genomic DNA was added to 5 units of *Bam*HI, 10 µl of 10x reaction buffer 3, supplied with the enzyme (Gibco BRL), and 5 µl of SSQ water. The DNA was digested at 37°C for 4 hours. The reaction was stopped by the addition of 0.2 M EDTA (disodium salt). The DNA was purified by phenol extraction and then precipitated by the addition of 1/10 volume of 0.3 M sodium acetate and 3 volumes of 100% ethanol. It was

spun down at 12, 500 rpm for 10 minutes and left to dry on the bench. Once dry, the DNA was resuspended in 40 µl of SSQ water. Approximately 60% of the DNA was recovered.

5.1.3 Transformation of *S. lividans* with totally digested genomic DNA from *M. purpurea*

Shotgun cloning was initially performed using the procedure described by Hopwood et al. (1985). 5µg of *Bam*HI digested *M. purpurea* genomic DNA was mixed with 1 µg of pIJ702 (Katz et al. 1983), which had been linearized with *Bgl* II (Gibco BRL). Ligation of the DNA was performed in a total volume of 20 µl, in the presence of 4 µl of 5 x ligase buffer and 1 unit of T4 DNA ligase. The ligation mixture was incubated for 24 hours at 16°C.

Protoplasts were prepared from *S. lividans* OS456 (Materials and Methods, section 2.3.2.1). 4.2×10^9 (50 µl) of these were transformed (Materials and Methods, section 2.3.2.2) with 5 µl of the ligation mixture. These protoplasts were allowed to regenerate on 6 R2YE agar plates (Thompson et al. 1982) at 30°C. After 20 hours the plates were overlayed with SSQ water containing thiostrepton (20 µg/ml) and incubated at 30°C for a further 4 days, to select for primary transformants containing pIJ702. These were then replica plated onto NEF agar containing thiostrepton (20 µg/ml) and gentamicin (20 µg/ml). Incubation was continued at 30°C. After 6 days, 10 colonies had grown and sporulated. All were picked for analysis.

5.2 Analysis of the gentamicin resistant transformants

Spores were isolated from the 10 colonies and streaked across an NEF plate containing thiostrepton (20 µg/ml) and gentamicin (20 µg/ml) to obtain more biomass for making stocks. Once the strains had sporulated, they were collected, resuspended in 20% glycerol, and frozen at -20°C.

The next step was to isolate plasmid from them (Materials and Methods, section 2.3.1). Cells were grown in YEME/sucrose containing gentamicin (10 µg/ml) and thiostrepton (20 µg/ml).

9 out of the 10 strains did not grow in gentamicin (10 µg/ml) and were discarded. Plasmid (pLST138) was isolated from the remaining strain, digested with *Bam*HI and separated by electrophoresis. pLST138 contained 4 *Bam*HI fragments, of sizes 5.5 kb, 3.0 kb, 2.5 kb and 1.9 kb. They were excised from the agarose and purified using Jetsorb (AMS Biotechnology) according to manufacturer's guidelines.

Each *Bam*HI fragment was ligated with pIJ702, (Materials and Methods, section 2.14) which had been linearised with *Bgl*II. This was used to transform *S. lividans* OS456. Transformants were initially selected on thiostrepton (20 µg/ml) and then replica plated onto thiostrepton (20 µg/ml) and gentamicin (20 µg/ml). Colonies grew containing the 1.9 kb *Bam*HI fragment of *M. purpurea* DNA (pLST1381). Plasmid was extracted and used to retransform *S. lividans* (Materials and Methods, section 2.3.2), for confirmation of the presence of a gentamicin resistance gene. The 5.5 kb, 3.0 kb and 2.5 kb *Bam*HI fragments did not confer gentamicin resistance. Minimum inhibitory concentrations of gentamicin were established for *S. lividans* carrying pLST138 and pLST1381. A restriction map of pLST138 can be found in Figure 5.0.

The 1.9 kb *Bam*HI fragment in pLST1381 was also cloned into pUC18 (Materials and Methods section 2.14) which had previously been linearised with *Bam*HI. This plasmid was denoted pLST1381a (Figure 5.1). Restriction analysis confirmed that the 1.9 kb *Bam*HI fragment contained *grmA* (Figure 5.2). It was noted at this stage, that *grmA* was expressed in NM522. The minimum inhibitory concentration of gentamicin for NM522 containing pLST1381a was > 20 µg/ml.

Further subcloning of pLST138 was performed using pHJL401 (Figure 2.17.5) to confirm that *grmA* was the only gentamicin resistance gene cloned on this plasmid (Figure 5.0).

Southern analysis confirmed that the gene cloned was *grmA*, originating from *M. purpurea* (Figure 5.6).

5.3 Problems with subsequent shotgun cloning experiments.

After the success of finding *grmA* in the initial experiment, a further 5 experiments were performed in an attempt to isolate a gentamicin resistance gene other than *grmA*. These experiments were unsuccessful, because they failed to clone *grmA*.

The number of transformants that grew during the primary selection on thiostrepton was very low. Between 10^5 and 10^6 colonies were expected to grow (Hopwood et al. 1985). This number includes cells transformed with religated pIJ702 and those transformed with pIJ702 containing a fragment of *M. purpurea* DNA. However, approximately 1000 colonies grew.

Cells containing *M. purpurea* genomic DNA were distinctive, as they were white (Figure 2.17.1). The lack of pigment was caused by insertional inactivation of the *mel* gene in pIJ702. There was a higher ratio of white to brown colonies (3 : 1). The reduction in the number of colonies seemed to be due to the number of cells that were transformed, and not the percentage of pIJ702 molecules that had ligated with *M. purpurea* DNA. The protocol according to Hopwood et al. (1985) was optimised. Further steps were taken to increase the number of transformed cells.

The ligation reaction was set up with an increase in the quantity of vector and *Bam*HI digested genomic DNA from *M. purpurea*. 2 µg of linearised pIJ702 was ligated with 10 µg of genomic DNA, in a total volume of 20 µl, containing 4 µl of 5 x ligase buffer and 1 unit of T4 DNA ligase.

1.7×10^{10} (200 µl) protoplasts were transformed (Materials and Methods, section 2.3.2.) with all 20 µl of the ligation mixture. The quantity of transformation buffer used

was increased proportionally, to 800 µl. The protoplasts were spread equally between 20 regeneration plates. Transformants were selected for as previously described. The modifications to the protocol resulted in an increase in the number of primary transformants: the previous total of 1000 colonies was increased to a lawn of colonies on each of the 20 plates. Despite this, *grmA* was still not cloned. Two new approaches were used to rectify this.

Firstly, it was realised that the totally digested genomic DNA could include sizes that were too small to contain the gene of interest. Small fragments of DNA may have been preferentially ligated to pIJ702 over larger ones. This may explain why *grmA* was not cloned. The DNA was partially digested instead and purified differently to that described previously. Partial digestions were set up as follows: The digestion mixture consisted of 80 µg (in 80 µl SSQ) of genomic DNA, 1 unit of *Bam*HI, 9 µl of SSQ and 10 µl of 10x reaction buffer 3, supplied with the enzyme. Several different incubation times were tested. The DNA was digested for 5, 10 15, and 20 minutes at 37°C. The reaction was stopped by the addition of 20 mM EDTA.

5 µl of blue gel dye was added to each of the following; 10 µg of DNA from each partial digestion, 10 µg of DNA that had been totally digested with *Bam*HI, and 10 µg of uncut genomic DNA. These samples were separated on 1 % LMP agarose for 16 hours at 15 volts (Materials and Methods, section 1.11.2). The time point used to obtain the sizes of DNA, required for shotgun cloning was 10 minutes.

To purify the DNA, 80 µg of restricted DNA was separated by electrophoresis on 1 % LMP agarose for 17 hours at 13 volts. DNA fragments between the sizes 3 kb and 10 kb, were excised from the agarose and purified by CTAB/butanol extraction (Materials and Methods, section 2.12). From 80 µg of DNA originally digested with *Bam*HI, approximately 10 µg of DNA was recovered.

The second new approach was to overcome the large number of mutants growing during the secondary selection on thiostrepton (20 µg/ml) and gentamicin (20 µg/ml). From 4 shotgun cloning experiments 50 colonies grew. MICs were established for these strains on gentamicin. Out of 50 colonies only 8 grew on 20 µg/ml. Plasmid

was prepared from these 8. They were found only to contain pIJ702. The conclusion was that they were mutants.

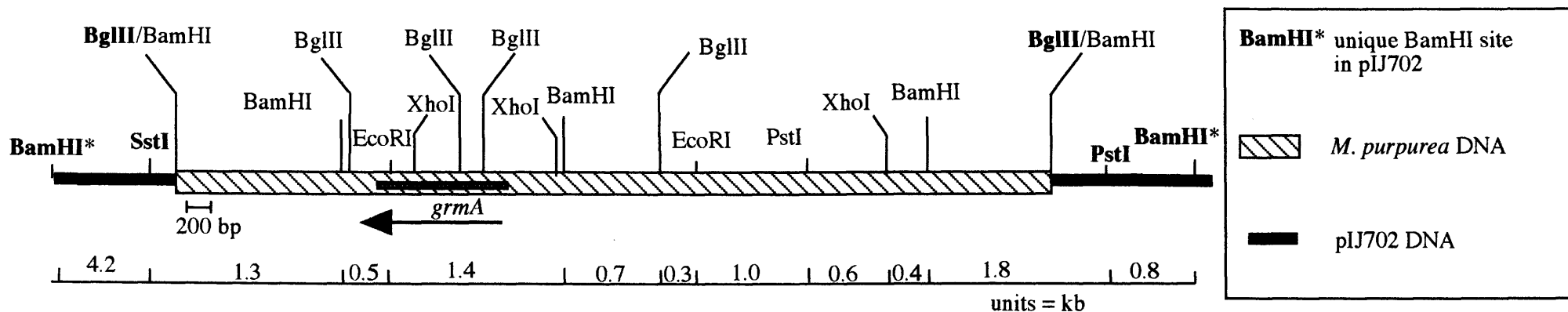
To minimise the problem two courses of action were taken. Firstly, a decision was made to use strain *S. lividans* TK21 instead of OS456 in future experiments. OS456 is a derivative of TK21. The two strains are identical apart from the fact that OS456 has inactivated *lrm* and *mgt* genes. The gene pair was disrupted in TK21 by the replacement of 1.4 kb (which contained part of both genes) (Pernodet et al. 1996) with a Ω HYG cassette (Blondelet-Rouault 1997). This cassette confers resistance to hygromycin B in *Streptomyces* and *E.coli*. It encodes a hygromycin phosphotransferase. The tandem gene pair, *lrm-mgt* confers inducible resistance to macrolide-lincosamide-streptogramin B antibiotics. Their presence has hindered previous attempts to analyse cloned MLS genes. However, in OS456, the hygromycin cassette may have been presenting a problem in these cloning experiments: by conferring a low level of gentamicin resistance, even though a hygromycin phosphotransferase would not be expected to modify gentamicin. Secondly, a higher concentration of gentamicin (100 μ g/ml) was also used to select against mutant strains.

Using a combination of all the changes described, 3 gentamicin resistant clones were obtained from 3 consecutive experiments. These were analysed as described for pLST138 in section 5.2. Restriction maps of the plasmids isolated from these clones can be found in Figures 5.3 to 5.5. Subcloning revealed that these too, did not contain any gentamicin resistant determinants apart from *grmA* (Figures 5.3 to 5.5).

The gene, *grmA*, was cloned on four separate occasions. No other gentamicin resistance determinants were found. The question posed at the beginning of this chapter has still not been answered: Is *grmA* the only gentamicin resistance gene in *M. purpurea*? Using the shotgun cloning, the question could only be answered if there was a positive outcome and an alternative gentamicin resistance gene had been found. Another strategy was required to say for definite whether such a gene exists.

pLST138

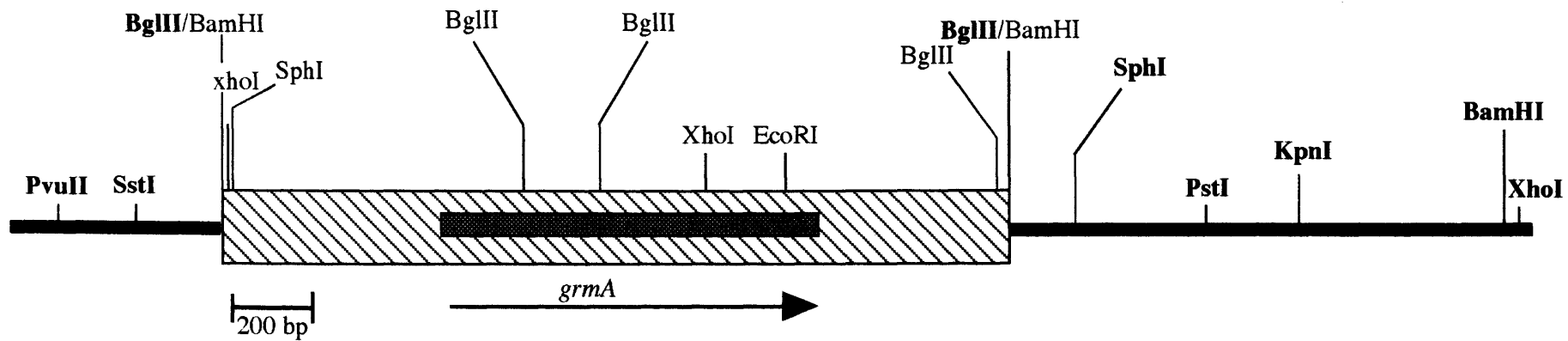
Figure 5.0 Restriction map and subcloning of pLST138



	plasmid name	Vector used for subcloning	Gentamicin MIC
	pLST1381	pIJ702	> 2000 µg/ml
	pLST1381a	pUC18	> 20 µg/ml
	pHJL401	pHJL401	1 µg/ml
	pHJL401	pHJL401	1 µg/ml

Figure 5.1 Restriction maps of pLST1381 and pLST1381a

a) pLST1381



b) pLST1381a

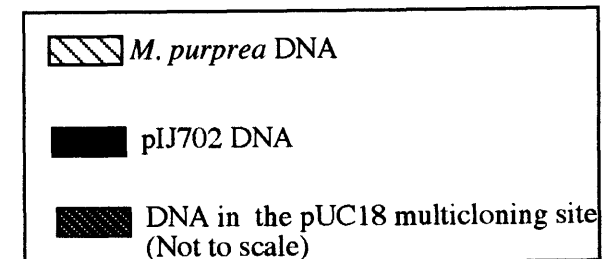
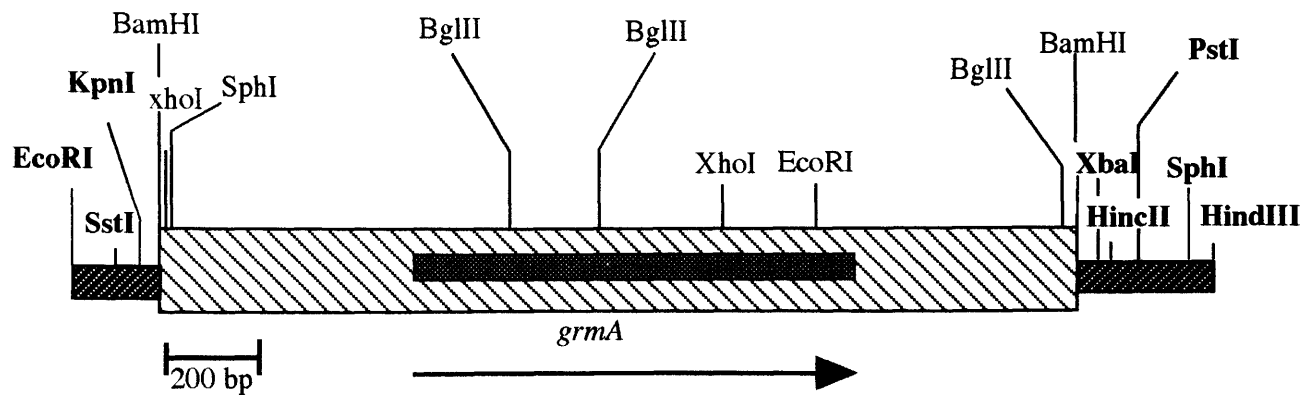


Figure 5.2 a

Restriction analysis of pLST1381a

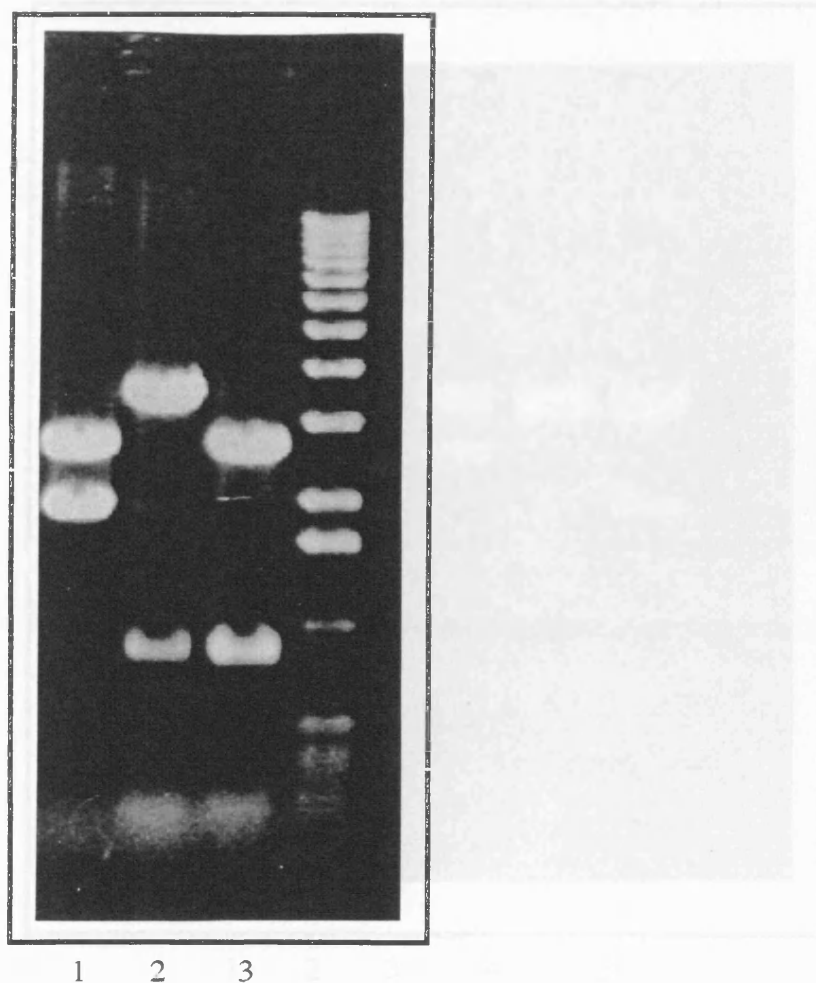


Figure 5.2a

The gel depicts 10 μ g of pLST1381a, digested with *Bam*HI (track 1), *Bgl*II (track 2), and *Bgl*II/*Bam*HI (track 3) (Materials and Methods, section 2.11). The DNA was separated by electrophoresis on 0.7% agarose for 120 minutes at 90 volts.

Figure 5.2b

Further restriction analysis of pLST1381a

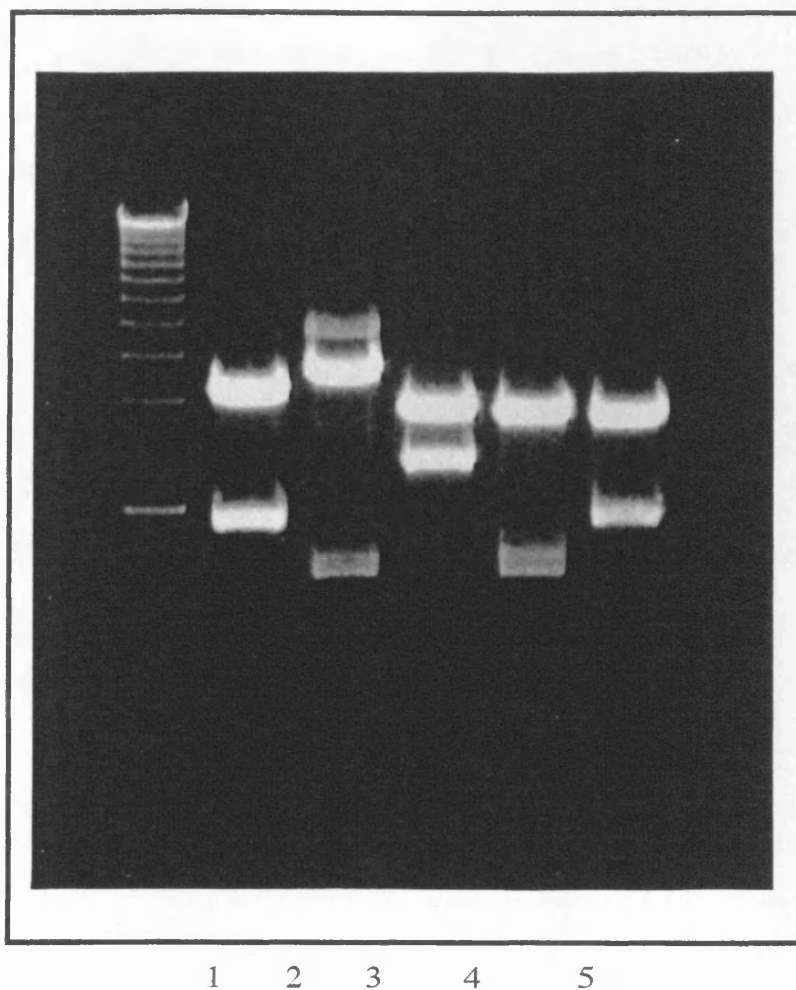
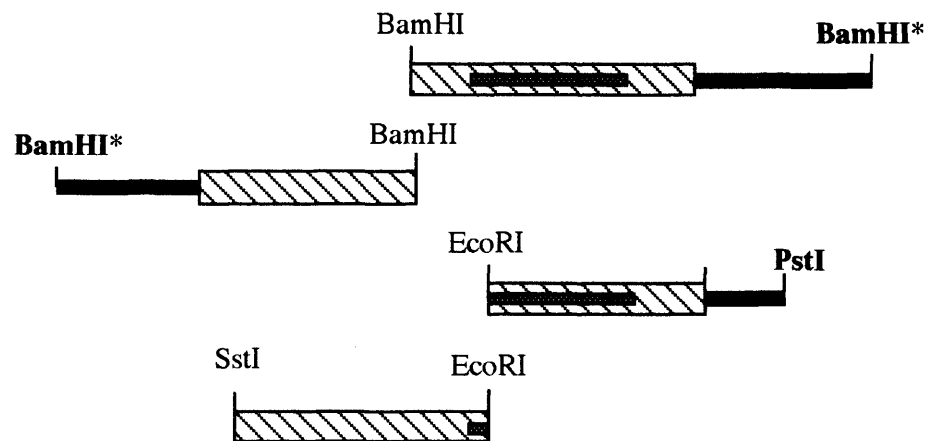
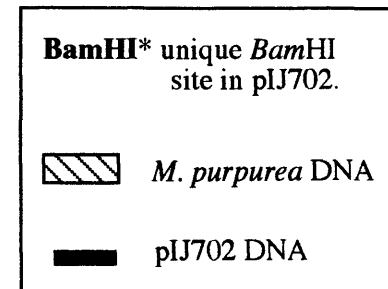
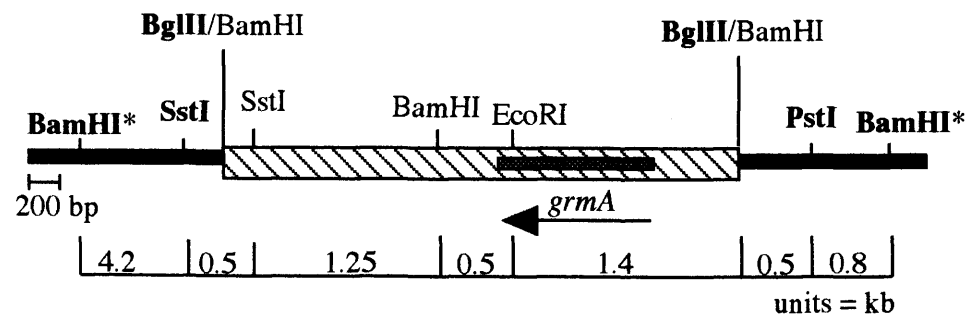


Figure 5.2b

The gel shows 10 µg of plasmid pLST1381a digested with *Eco*RI (track 1), *Bgl*II (track 2), *Bam*HI (track3), *Bam*HI/*Bgl*II (track 4), and *Xho*I (track 5) (Materials and Methods, section 2.11). The DNA was separated by electrophoresis on 0.7% agarose for 3 hours at 90 volts.

Figure 5.3 Restriction map and subcloning of pLST139



Vector used for subcloning	Gentamicin MIC
pHJL401/pIJ702	> 2000 µg/ml
pHJL401/pIJ702	1 µg/ml
pHJL401	1 µg/ml
pHJL401	1 µg/ml

Figure 5.4 Restriction map and subcloning of pLST139a

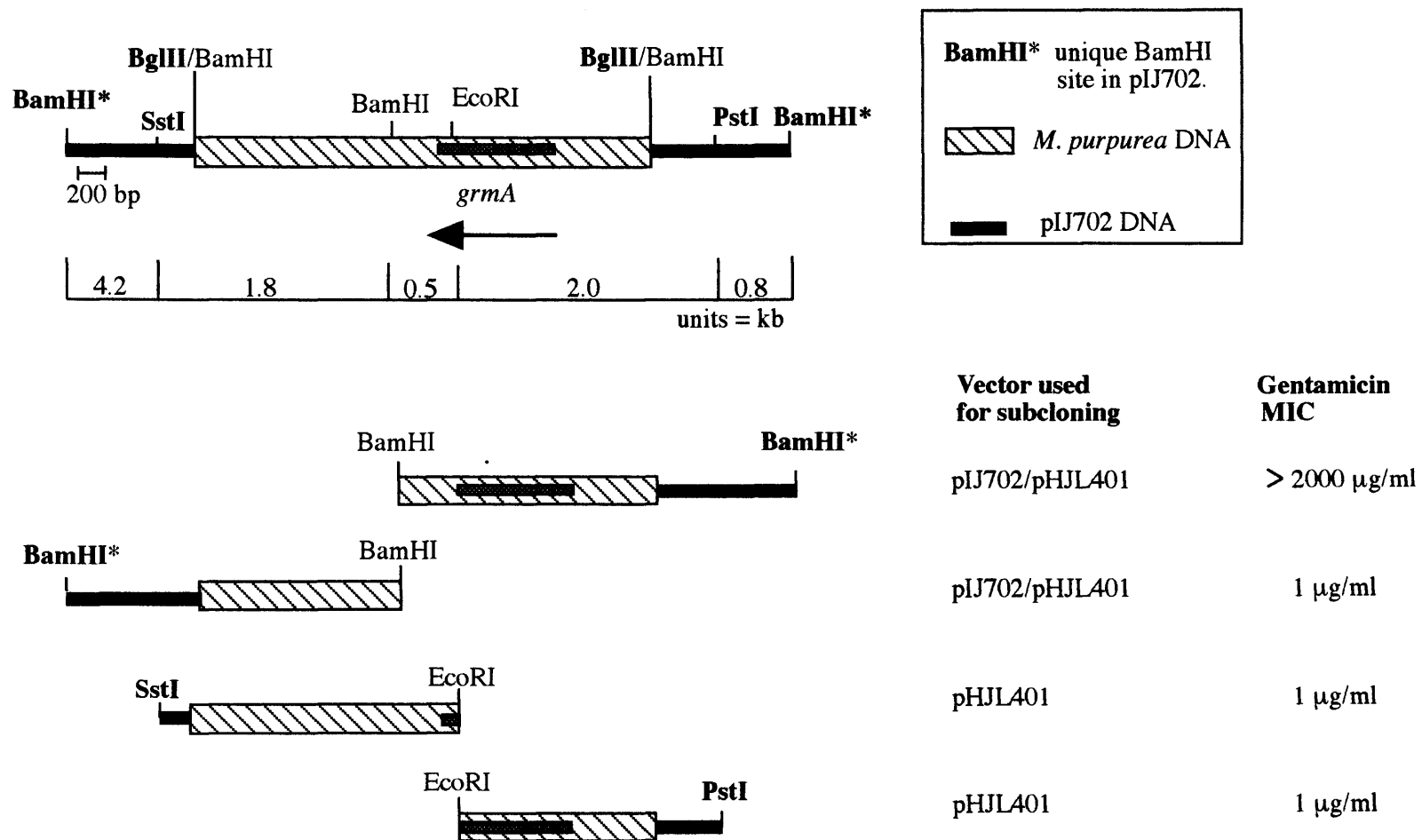


Figure 5.5 The restriction map and succloning of pLST139b

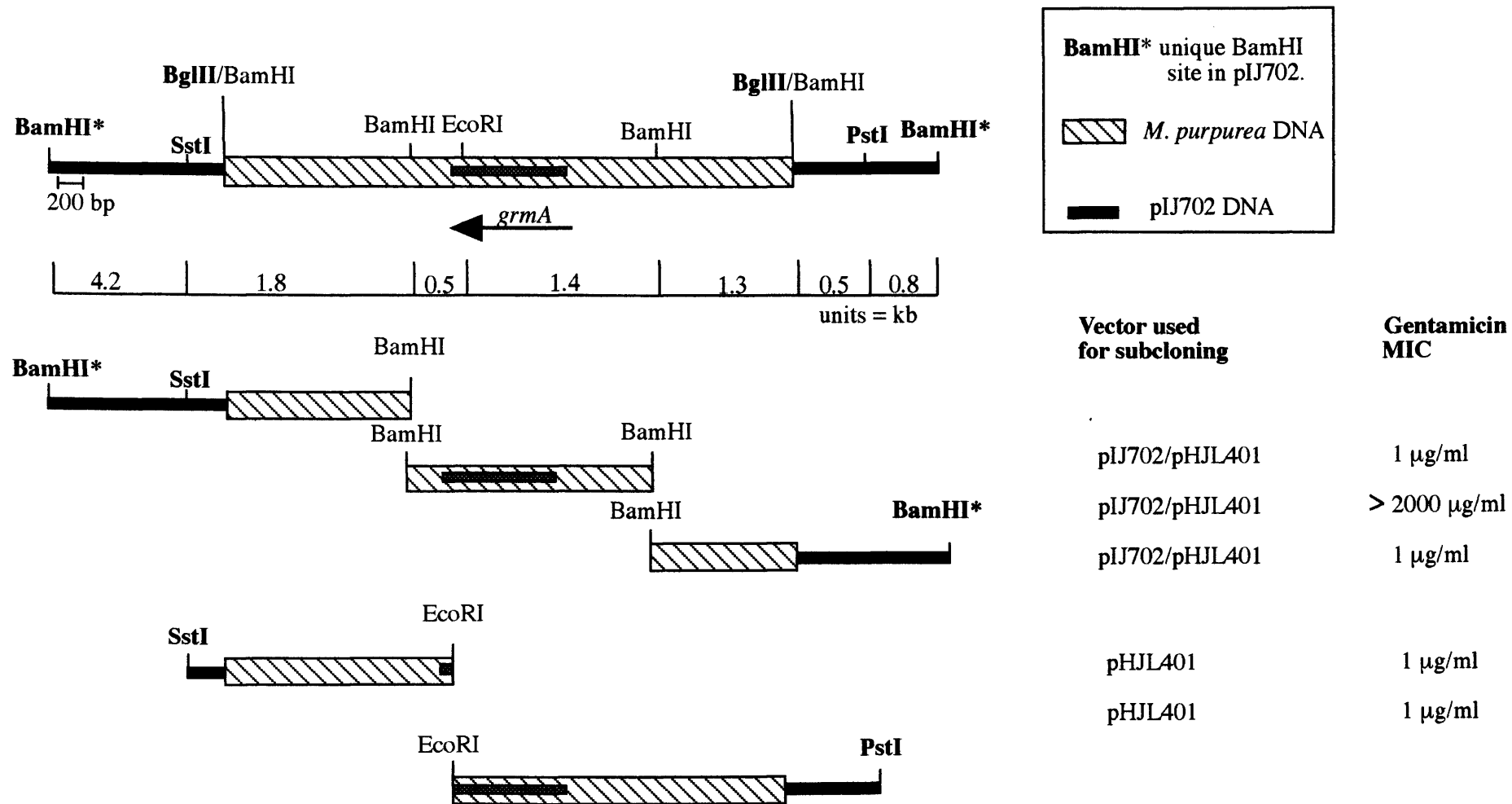


Figure 5.6 The cloning of *grmA* from *M. purpurea* was confirmed by Southern analysis

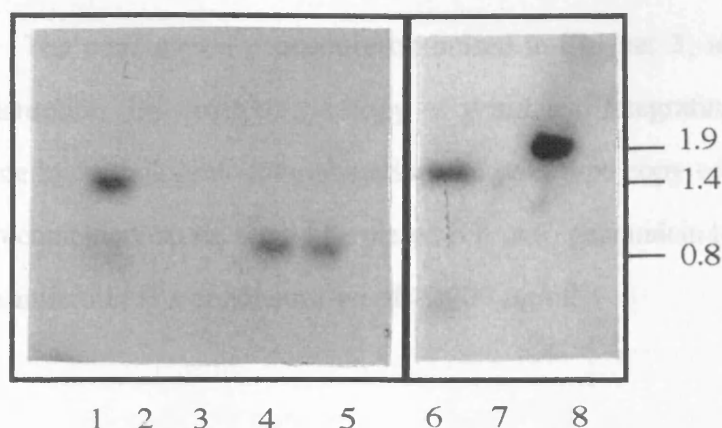


Figure 5.6

10 μ g of total genomic DNA from *M. purpurea* was digested with different enzymes and probed with either a 200 bp *Bgl*II fragment internal to *grmA* (tracks 6-8) or an 800 bp *Xho*I/*Bgl*II fragment that started 600 bp upstream of the transcriptional start site of *grmA* and extended approximately 200 bp into the gene (tracks 1-5). A restriction map of *grmA* can be found in Chapter 1, Figure 1.1. The *M. purpurea* DNA was digested with *Xho*I/*Bam*HI (track 1), *Xho*I/*Bgl*II (track 4), *Bam*HI/*Bgl*II (track 5) *Eco*RI/*Bam*HI (track 6) and *Bam*HI (track 8). 10 μ g genomic DNA from *S. lividans* OS456 (track 2), and 1 μ g of pUC18 (tracks 3 and 7) were digested with *Bam*HI (negative controls). The membranes were washed at high stringency (Materials and Methods, section 2.8).

The *Xho*I/*Bgl*II probe hybridised with an *Xho*I fragment of size 1.3 kb (track 1) an *Xho*I/*Bgl*II fragment 0.8 kb in size (track 4), and a *Bam*HI/*Bgl*II fragment of 0.8 kb (track 5). The *Bgl*II probe hybridised with a 1.4 kb *Eco*RI/*Bam*HI fragment, and a 2 kb *Bam*HI fragment. Neither of the probes hybridised with the negative controls (tracks 2, 3, and 6). These results confirmed that *grmA* had originated from *M. purpurea*.

5.4 Disruption of *grmA*

The conjugation procedure optimised in Chapter 3, section 3.2.2, was used for the disruption. By disrupting a copy of *grmA* and integrating it into the *M. purpurea* genome by homologous recombination the wild type copy of *grmA* would be replaced. This recombinant strain would be plated out onto gentamicin to see if it was still resistant to the antibiotic at a concentration of >2000 µg/ml.

5.4.1 Gene replacement

Gene replacement has been fundamental in the study of genetics and regulation of antibiotic biosynthesis in actinomycetes (reviewed by Baltz 1998). It has aided the construction of recombinant strains that can produce higher yields of antibiotic and novel compounds. The technique exploits the natural process of homologous recombination. Various methods have been employed. Two such methods are the introduction of DNA by either a temperature sensitive or non replicative plasmid. The only way that DNA inserted in these vectors can be maintained by the cell, is by integration into the genome.

The function of a specific gene can be disrupted by replacing it with a defective copy. This can be achieved by introducing a copy with the transcriptional start and stop sites excised. However this approach does not allow selection for the integration of foreign DNA. An alternative approach which avoids this problem, is the insertion of a resistance marker in the coding region of the gene.

5.4.2 Homologous recombination

Homologous recombination involves homology matching and strand exchange mediated by RecA. As the name indicates, the formation of a recombinant chromosome occurs between two DNA molecules that share a reasonably large region of sequence similarity. This is also referred to as generalized recombination as it is likely to occur in any area, where there is high similarity to the incoming DNA. This can have important implications when using homologous recombination to replace a specific gene, because other, unpredictable recombination events may take place in the genome.

During homologous recombination one strand of one molecule of DNA becomes nicked. The ends of this broken strand are paired with the unbroken complementary duplex. This part of the process is mediated by RecA which binds single stranded DNA forming a protein-DNA complex that can bind double stranded DNA. This process is ATP dependent. Breakage of the complementary strand mediated by RecBC occurs and the two are joined together. Isomerization of these two linked molecules results in the unbroken strand of each forming a cross over bridge. Cleavage and joining of the bridging strands then separates the two molecules. A region of heteroduplex DNA has been produced near the cross over point. If this process is repeated further along the same piece of DNA, a double cross over event will occur resulting in the replacement of one piece of DNA for another.

5.5 Construction of pLST1383

The plasmid used for the disruption of *grmA* was constructed from the suicide vector pOJ260 (Bierman et al. 1992). A restriction map of pOJ260 can be found in Chapter 2 Figure 2.17.3. *grmA* was restricted using *Bam*HI and inserted into pOJ260 at the *Bam*HI site. A unique *Aat*II site was found in *grmA* that was not present in pOJ260. The DNA used to disrupt *grmA* was the thiostrepton gene, *tsr*, isolated from *S. azureus*. This was cloned into the *Aat*II site, generating "arms" of *M. purpurea* DNA (700bp and 1.2 kb) on either side of *tsr* (Materials and Methods, section 2.14). The position of *Aat*II within the *grmA* coding region can be identified in Figure 5.7.

tsr was obtained by PCR-amplification (Materials and Methods section 2.16) from pIJ702. Primers were designed that generated *Aat*II sites at the 3' and 5' ends. An annealing temperature of 60°C was used. The PCR-amplified product was cloned into pCR2.1 (Materials and Methods, section 2.14). A restriction map of pCR2.1 can be found in Chapter 2, Figure 2.17.4. *tsr* was excised from pCR2.1 using *Aat*II and cloned into the *Aat*II site of *grmA* in pOJ260 to give pLST1383 (Figure 5.7). There are two *Aat*II sites in *tsr* so it was excised from pCR2.1 using partial digestion (Materials and Methods, section 2.13).

5.5.1 The conjugal transfer of pLST1383 into *M. purpurea*

pLST1383 was introduced into *M. purpurea* using the protocol previously described in section 3.2.2. Transconjugants were overlaid with thiostrepton (20 µg/ml) and nalidixic acid (60 µg/ml) to select for *M. purpurea* cells carrying *tsr*. A mass of colonies grew on one side of the plate within 5 days. These were scraped from the plate and resuspended in 1 ml of YEME/PEG containing 5% DMSO to make a mycelial stock, which was stored at -70°C.

Different quantities (10 µl or 150 µl) of this mycelial stock were streaked onto AS-1 agar containing thiostrepton (20 µg/ml) to obtain single colonies for analysis. The

transconjugants were analysed to determine if homologous recombination had occurred on both sides *tsr*. Those that grew on thiostrepton were replica plated onto AS-I agar containing thiostrepton (20 µg/ml) and apramycin (25 µg/ml). A total of 2000 colonies were screened from 5 conjugation experiments and all of them were resistant to thiostrepton and apramycin, suggesting that homologous recombination may have occurred on one side of *tsr* resulting in the integration of the entire vector. A diagrammatic representation of the predicted single cross over events can be found in Figure 5.8

Southern analysis was performed to confirm that pLST1383 had integrated into the genome of *M. purpurea* (Figure 5.10). Genomic DNA was prepared from two recombinant strains (SRE1 and SRE2) and the wild type. The data suggested that pLST1383 integrated into the *M. purpurea* genome in SRE2 by a single cross over event. The data for SRE1 was difficult to interpret. Mycelial stocks were also prepared from SRE1 and SRE2.

The data in Figure 5.10 did not indicate where in the genome the single cross over event may have taken place. It would be considered a grossly inaccurate interpretation of the data to assume that homologous recombination occurred between the *grmA* sequence in the genome and the disrupted copy. Further Southern analysis was performed to determine whether pLST1383 had integrated into the region of DNA around *grmA* or elsewhere in the genome. Genomic DNA from *M. purpurea* wild type and 9 recombinant strains (including SRE2) was digested with *SphI*. The genomic DNA was probed with a 200 bp *BglII* fragment internal to *grmA* (Figure 5.11).

The diagram in Figure 5.8 was used to determine which fragment sizes the probe was expected to hybridise with. This was dependent upon which side of *tsr* the recombination event had occurred. If a single cross over had occurred upstream of *tsr*, the probe would hybridise with two copies of *grmA* on *SphI* fragments of 11 kb and 6.8 kb, whereas a recombination event downstream would generate *SphI* fragments of 12 kb and 5.7 kb containing *grmA*. Only 1 strain (SRE2) showed the correct pattern of hybridisation for one of these scenarios. Recombination had occurred upstream of *tsr*. Genomic DNA from SRE2 was analysed further to confirm that recombination had occurred within the 1.9 kb *BamHI* fragment (Figure 5.12).

It was difficult to assess how the other 8 recombinant strains had been generated. A curious feature which was consistent with all 8 strains was the subtle differences in the sizes of the bands between strains and the fact there were always two bands. Another feature common to all 8, was the absence of an 11 kb band which corresponds to the size of the *SphI* fragment containing *grmA* in the genome. If an integration event had occurred outside the 11 kb *SphI* fragment, it would remain undisrupted and the *grmA* probe would hybridize with a fragment of this size. One interpretation of the results is that regions of DNA from pLST1383 had randomly integrated into the 11 kb *SphI* fragment thereby increasing its size. The data indicate that pLST1383 may also have integrated randomly at other sites in the genome.

5.5.2 Obtaining double cross overs from single cross overs

Recombinant strain, SRE2, was grown in media that contained no selection for pLST1383. Firstly, 100 µl of the SRE2 mycelial stock was inoculated into 10 ml of YEME/PEG in a plastic universal. This was incubated at 30°C for 2 days whilst shaking horizontally at 150 rpm. 1 ml of this was then removed, inoculated into 10 ml of YEME/PEG and incubated for 2 days in the same manner. This process was repeated again. At each 2 day interval 10 µl and 150 µl of cells were removed. These were streaked onto NEF agar containing thiostrepton (20 µg/ml) to obtain single colonies that contained *tsr*. These colonies were tested for apramycin resistance, to observe if the vector had been lost. A total of 500 colonies were screened. All of them were apramycin resistant.

5.6 Modification of pLST1383

Due to the lack of success in replacing the wild type *grmA* with a disrupted copy, an alternative construct was designed. From experience, researchers have found that a bias towards a double crossover could be favoured by equal arms of DNA on either side of the disrupting insert.

pLST1383 was modified to give approximately 600 bp of DNA on either side of the thiostrepton gene. This was achieved by PCR-amplification (Materials and Methods 2.16) of *grmA* (disrupted with *tsr*), using pLST1383 as the template. The primers were designed so that the PCR-amplified product was generated with *Bam*HI sites at the 3' and 5' ends. The amplified product was cloned into PCR2.1 (Materials and Methods, section 2.14), excised with *Bam*HI and then cloned into the *Bam*HI site of pOJ260 to give pLST1383a (Materials and Methods, section 2.14). A restriction map of pLST1383a can be found in Figure 5.7

5.6.1 Conjugation with pLST1383a

Conjugation was performed using vectors pLST1383 and pLST1383a as described above. Only 1 thiostrepton resistant transconjugant (SRE3) resulted from 5 conjugation experiments using pLST1383a. Further analysis revealed that this recombinant strain was thiostrepton and apramycin resistant, indicating that a single cross event had occurred, resulting in the integration of pLST1383a. This was confirmed by Southern analysis (Figure 5.9).

Genomic DNA from *M. purpurea* wt, SRE2 and SRE3 was digested with *Eco*RI/*Bam*HI and probed with a 200 bp *Bgl*II fragment internal to *grmA*. In *M. purpurea* wt, the probe hybridised with a 1.4 kb *Bam*HI/*Eco*RI fragment containing the wt copy of *grmA*. The sizes of DNA fragments predicted to hybridise with the probe can be found in Figures 5.8 and 5.9 for SRE2 and SRE3 respectively. In SRE2, the probe hybridised with the genomic copy of *grmA* and with a 2.5 kb *Bam*HI/*Eco*RI fragment

This confirmed that the single cross over had occurred within the 1.9 kb *Bam*HI fragment containing *grmA*. In SRE3 the probe hybridised with two *Eco*RI/*Bam*HI fragments of sizes 1.4 kb and 2.0 kb. This confirmed that pLST1383a had integrated into the 1.9 kb *Bam*HI fragment containing *grmA*, downstream of the *Aat*II site (ie. downstream of *tsr* in pLST1383).

Figure 5.7 Restriction maps of pLST1383 and pLST1383a

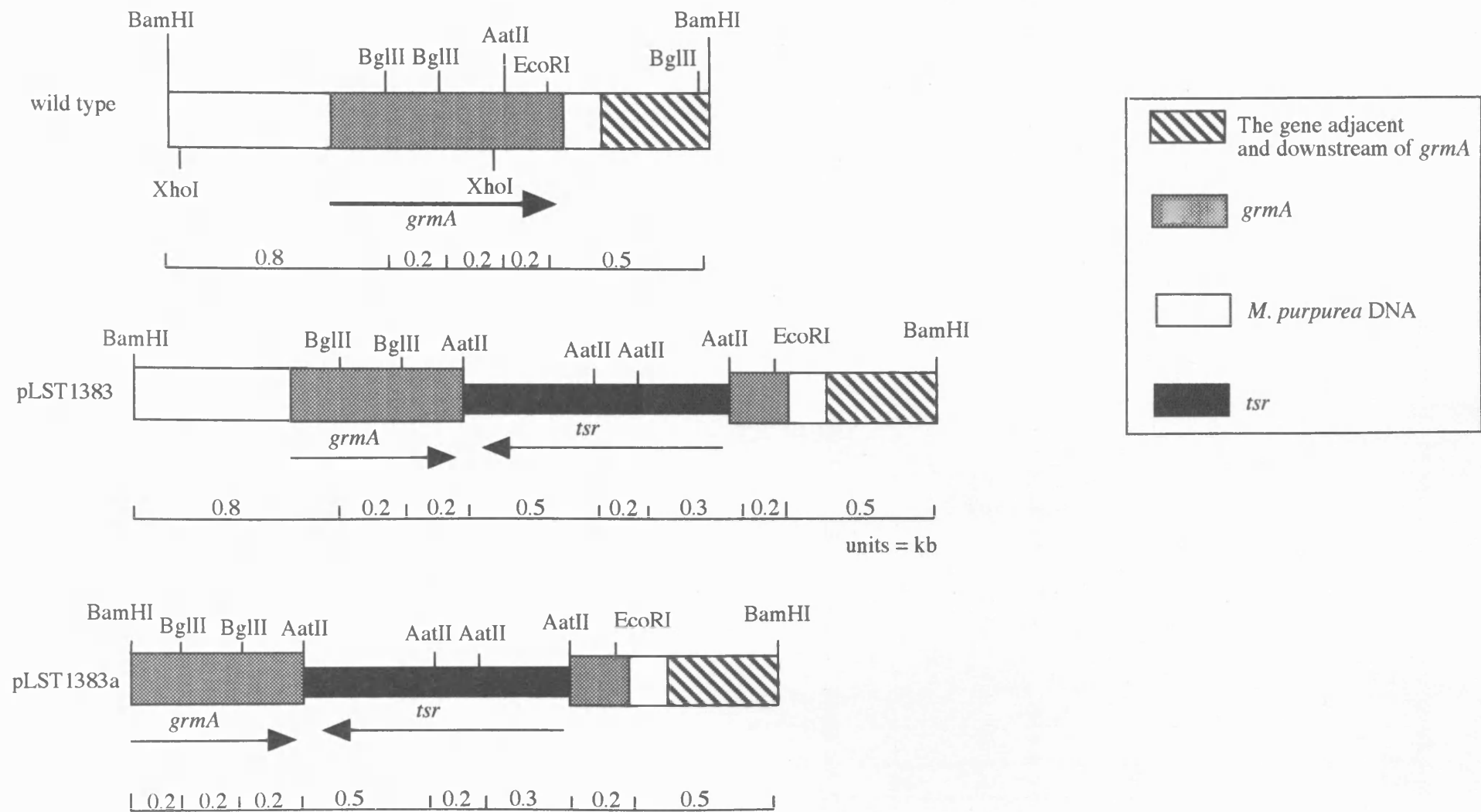


Figure 5.8

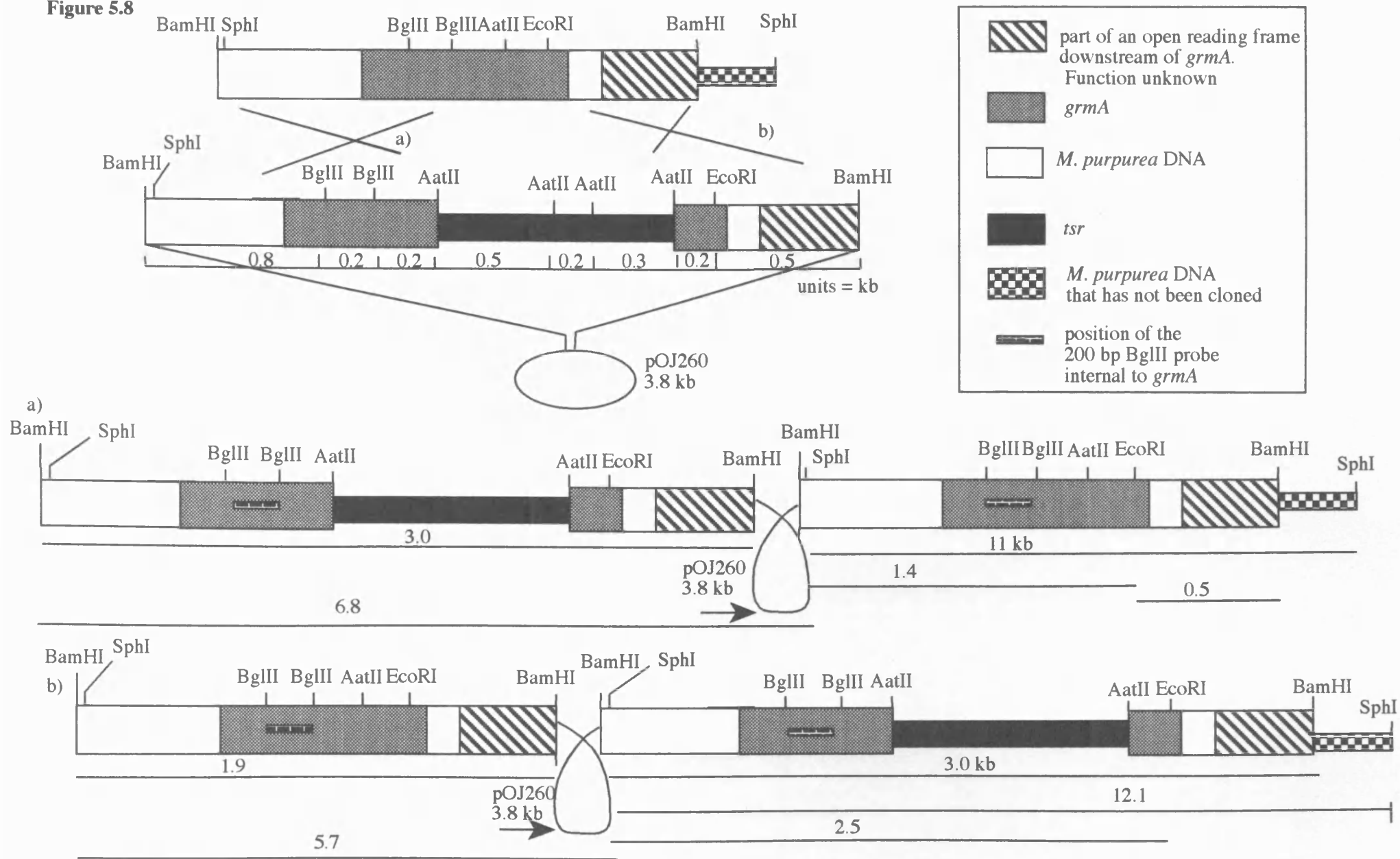




Figure 5.9

Predicted homologous recombination events between pLST1383a and genomic DNA in and around *grmA*

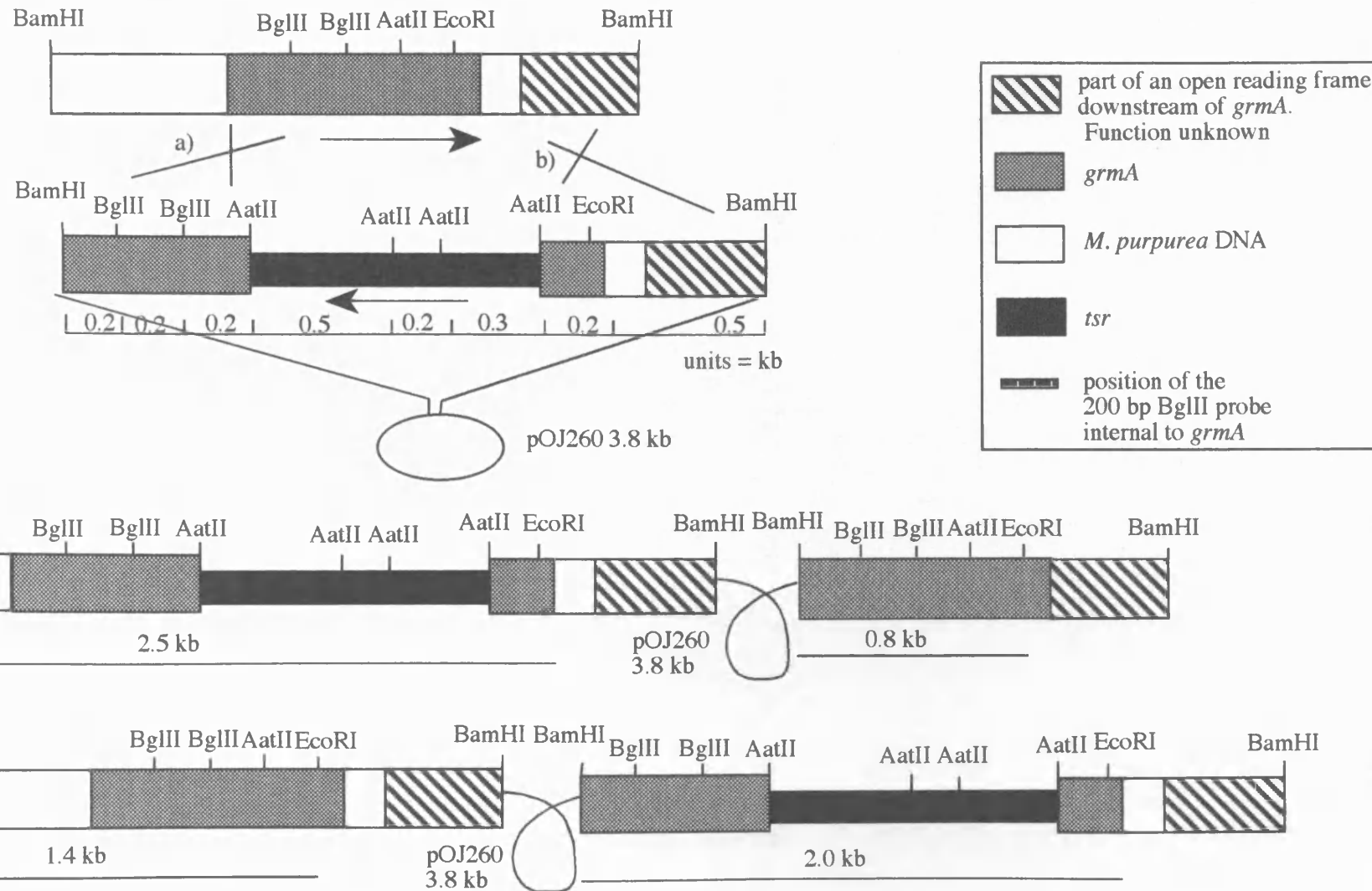




Figure 5.10

Integration of pLST1383 into the *M. purpurea* genome, investigated by Southern analysis

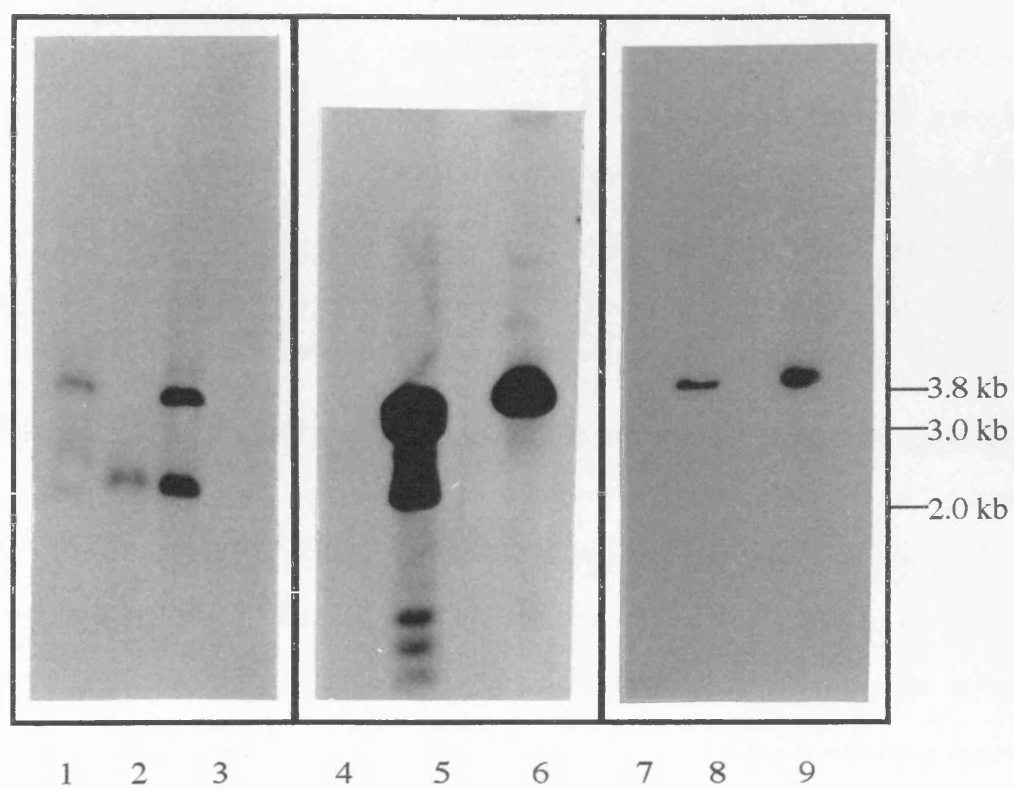
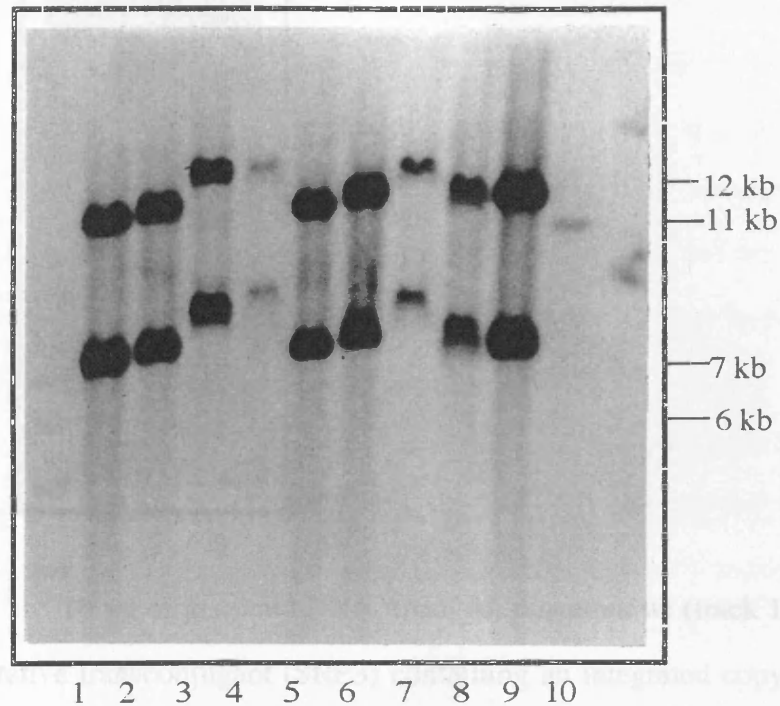




Figure 5.11

Determination of the site of integration of pLST1383 by Southern analysis



... (track 1), SRB3 (track 1) and the ...
 ... integrated copy of pLST1383a (track 3)
 was digested with *Bam*HI/*Eco*RI for 12 hours. The DNA was then subjected to
 Southern blotting (Materials and Methods, section 2.8). The membranes were probed
 with a 200 bp *Hpa*II fragment internal to *gms4* (Materials and Methods, section 2.8).
 The membrane was washed at high stringency (Materials and Methods, section 2.8).
 The probe hybridized with the genomic copy of *gms4* present on a 1.4 kb *Bam*HI/*Eco*RI
 fragment (a 36 basepair overlap) (track 1).

In genomic DNA from SRB2, the probe hybridized with the genomic copy of
gms4 and a 2.5 kb *Bam*HI/*Eco*RI fragment (track 2). Together with the results from
 Southern analysis presented in Figure 5.10, these results indicate that the integrated
 copy of pLST1383a is located on a 2.5 kb fragment.

... (track 1) and the ...

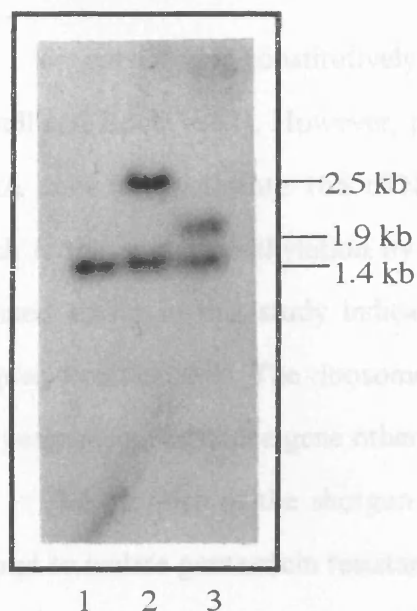
... (track 1) and the ...

... (track 1) and the ...

... (track 1) and the ...

Figure 5.12

Determination of the site of integration of pLST1383 and pLST1383a by Southern analysis



10 µg of genomic DNA, from *M. purpurea* wt (track 1), SRE2 (track 2) and the putative transconjugant (SRE3) containing an integrated copy of pLST1383a (track 3) was digested with *Bam*HI/*Eco*RI for 12 hours. The DNA was then subjected to Southern blotting (Materials and Methods, section 2.8). The membranes were probed with a 200 bp *Bgl*II fragment internal to *grmA* (Materials and Methods, section 2.8). The membrane was washed at high stringency (Materials and Methods, section 2.8). The probe hybridised with the genomic copy of *grmA* present on a 1.4 kb *Bam*HI/*Eco*RI fragment in *M. purpurea* wt (track 1).

In genomic DNA from SRE2, the probe hybridised with the genomic copy of *grmA* and a 2.5 kb *Bam*HI/*Eco*RI fragment. Taken together with the results from Southern analysis presented in Figures 2.10 and 2.11, it seems that pLST1383 integrated by homologous recombination into the 1.9 kb *Bam*HI containing *grmA*

In SRE3, the probe hybridised with the genomic copy of *grmA* and a 2.0 kb *Bam*HI/*Eco*RI fragment suggesting that pLST1383a had integrated into the 1.9 kb *Bam*HI fragment containing *grmA* in the *M. purpurea* genome, by homologous recombination downstream of *tsr* as depicted in the predicted single cross over event in Figure 5.9

5.7 Discussion

5.7.1 Shotgun cloning gentamicin resistance genes from *M. purpurea*.

M. purpurea is constitutively resistant to gentamicin at the level of the ribosome (Piendl and Böck 1982). However, primer extension analysis in Chapter 4, revealed that GrmA does not methylate 16S rRNA in *M. purpurea* at guanine 1389 (*E.coli* 1405), which is the site of methylation by GrmA when it is cloned in *S. lividans*. The data obtained so far in this study indicate that *grmA* may not be expressed (discussed in Chapter 4 section 4.4). The ribosomes in *M. purpurea* may be resistant due to the action of a gentamicin resistance gene other than *grmA*.

The purpose of the shotgun cloning experiments described in this chapter, was attempt to isolate gentamicin resistance genes other than *grmA*, from *M. purpurea*. Four *S. lividans* strains containing *grmA* were obtained from 4 separate experiments. The *M. purpurea* DNA from these four strains was subcloned (Materials and Methods, section 2.14), to establish if gentamicin resistance genes other than *grmA* were present (Figures 5.0 and 5.3 to 5.5).

There could be several explanations as to why another gentamicin resistance gene was not cloned in these experiments. Firstly, the gene may have been cloned without its promoter. The putative gene could be co-transcribed with other genes from a promoter a long way upstream. Secondly, the gene could be positively activated by the product of a regulatory gene in *M. purpurea*. Unless the two genes were cloned together, the gentamicin resistance gene may not be switched on. A good example that demonstrates both these phenomena is the streptomycin resistance gene, *aphD*, in *S. griseus*. *aphD* is transcribed by readthrough from the promoter of a regulatory gene *strR*, which lies upstream of the streptomycin biosynthetic cluster. *strR* is activated by a cascade of events that occurs in response to the production of the hormone, A factor (described in more detail in Chapter 1, section 1.10.1). Both problems may have been overcome by using an alternative cloning strategy. If a cosmid library of the *M. purpurea* genome had

been created instead, the fragments of *M. purpurea* DNA obtained, would be large enough to carry clusters of genes.

The third, most obvious explanation is that *grmA* is the only gentamicin resistance gene in *M. purpurea*. As discussed in Chapter 4, section 4.4, the gene product may confer gentamicin resistance in *M. purpurea* by methylating the 16S rRNA at a site other than G1389.

An alternative approach, gene replacement, was attempted, to determine if *grmA* is the only gentamicin resistance gene in *M. purpurea*.

5.7.2 Disruption of *grmA*

A copy of *grmA* was disrupted by *tsr* in plasmid pLST1383. This was introduced into *M. purpurea* by conjugal transfer, with the hope that the disrupted copy of *grmA* would replace the genomic copy. The replacement of *grmA* was unsuccessful. The reason for this is not understood as there could be several explanations for this phenomenon.

The first, manifest explanation is that *grmA* cannot be disrupted in the *M. purpurea* genome. Replacement of *grmA* may be a lethal event. However, this seems unlikely because the function of *grmA* could be considered non-essential for survival of the cell.

A further experiment would be needed, to establish whether the disruption of *grmA* in *M. purpurea* is a lethal event. During the experiments performed in this chapter, a recombinant strain (SRE2) was generated by the introduction of pLST1383, using conjugal transfer. Integration of pLST1383 into the 1.9 kb *Bam*HI fragment containing *grmA* in the *M. purpurea* genome, was confirmed by Southern analysis (Figures 5.10, 5.11 and 5.12). However, these data did not indicate whether the *grmA* ORF or the flanking DNA was disrupted. A single cross over event anywhere in the 1.9 kb *Bam*HI fragment, would result in the reconstitution of a functional copy of the gene, due to the fact the whole of the *grmA* ORF was introduced on pLST1383. Even if the *grmA* ORF

was disrupted in the genome, the strain would remain gentamicin resistant because of the reconstituted copy. Disruption of the gene's function would not necessarily require a double cross over event. It could be achieved by a single cross over event, as long as a second copy of *grmA* was not reconstituted. The 3' and 5' ends of the gene could be removed to prevent expression of the gene.

A double cross over was needed for *grmA* to be replaced in the *M. purpurea* genome. A second explanation for the lack of success in replacing *grmA*, is that the most efficient experimental approach was not employed. The use of a suicide vector may not have been the best approach, because it may not have allowed enough time for a double cross over event to take place. The vector, pOJ260, cannot replicate in *Streptomyces spp.*. For a disrupted copy of *grmA* to be maintained in *M. purpurea*, the DNA has to integrate into the genome, before the vector becomes unstable and is lost. This approach seems to have allowed sufficient time for a single homologous recombination event to occur but not a double one.

To allow more time for a double cross over to occur, the DNA could be introduced on a temperature sensitive plasmid. Once introduced, the plasmid would be able to replicate at the permissive temperature, giving plenty of time for the DNA to recombine. The temperature could then be raised to a non-permissive level, and the plasmid would be lost.

The approach of subculturing the recombinant strain, SRE2, in non selective media to encourage a second recombination event, was unsuccessful. The integration of pLST1383 was very stable.

The genomic DNA from 9 strains thought to contain pLST1383 was studied by Southern analysis. Only 1 strain (SRE2) definitely contained pLST1383 integrated into the region of interest. Curiously, the others appeared to have been generated by random integration events which may have involved deletions or tandem integration of regions of the vector (Wohlleben and Muth 1993). This phenomenon has been observed by other researchers who have used pOJ260 to replace antibiotic biosynthetic genes in *S. fradiae* (personal communication Dr S. A. Fish).

The frequency of thiostrepton resistant strains generated by the introduction of pLST1383a was much lower than that for pLST1383. One recombinant strain (SRE3) was generated by the introduction of pLST1383a. This was shown to contain the vector integrated within the 1.9 kb *Bam*HI fragment containing *grmA*. One possible explanation for the difference in the frequency of recombinant strains, generated from the two different vectors, could be that the extra DNA in pLST1383 has a nucleotide sequence which is very similar to other sequences in the *M. purpurea* genome. Recombination can occur between partially homologous sequences as well as homologous ones (Hosted and Baltz 1996). Removal of the extra DNA from pLST1383, may have reduced the frequency of partially homologous recombination events between the vector and the *M. purpurea* genome.

Chapter 6

General Discussion

Chapter 6

General Discussion

6.0 Primer extension analysis of *M. purpurea* 16S rRNA

Investigations prior to this study suggested that GrmA methylates 16S rRNA at residue G1389 when the gene is cloned in *S. lividans* (personal communication Dr G. H. Kelemen). However, these investigations also indicated that G1389 of the 16S rRNA is not methylated in *M. purpurea*, even though ribosomes extracted from this organism are constitutively resistant to gentamicin (Piendl and Böck 1982).

The data obtained during this work (Chapter 4, Figures 4.1, 4.2, and 4.3) confirm that GrmA methylates *S. lividans* 16S rRNA at G1389 (*E. coli* 1405), but does not methylate at this site in *M. purpurea*.

The aims of the work were to introduce a second copy of *grmA* (driven by *ermEp**) into *M. purpurea* by conjugal transfer and establish if the effects of a putative repressor or regulation mechanism were titrated out. However, primer extension analysis revealed that G1389 is not methylated in *M. purpurea* containing an extra copy of *grmA*. This seems surprising, as this strain possesses a higher level of *grmA* transcript than the wild type (Chapter 3, Figures 3.8 and 3.9). Regulation of *grmA* in *M. purpurea* appeared to have been alleviated by the introduction of *grmA* driven by *ermEp**. Either the extra copy of *grmA* is also repressed at the transcriptional level, or there could be a second mechanism, which controls synthesis or action of the methylase. There is no evidence to suggest that the *grmA* transcript is translated in *M. purpurea*, as no experiments have been done to investigate this. GrmA specific antibodies could be used to detect protein being produced in *M. purpurea* using western blot analysis.

A different interpretation of the primer extension data (Chapter 4 Figures 4.1, 4.2 and 4.3) is that GrmA is methylating 16S rRNA at an alternative residue to G1389 in *M.*

purpurea. It seems unlikely that GrmA would methylate outside the decoding region because of the close association between the locations of aminoglycoside binding sites and the methylation giving resistance to those antibiotics in this region. However as discussed in Chapter 4 (section 4.4), there may be differences in the structure of 16S rRNA between *S. lividans* and *M. purpurea* which could result in GrmA methylating a different residue. Structural deviation in 16S rRNA may account for the variations in resistance phenotypes exhibited by different actinomycetes. The sisomicin-gentamicin resistance gene, *sgm*, was cloned in *M. melanosporea* and *S. lividans* (Kojic et al. 1992). Interestingly, these strains showed different resistance phenotypes. *M. melanosporea* became highly resistant to hygromycin B (which is a characteristic of aminoglycoside producers), whereas *S. lividans* remained sensitive to the antibiotic. It seemed that the expression of hygromycin B resistance determined by *sgm* was dependent on which organism it was cloned in. *Tetrahymena thermophila* displays hygromycin B resistance when its 16S rRNA is mutated at residue U1495 (Spangler and Blackburn 1985). It may be that differences in the conformation around this region of the 16S rRNA have arisen between *S. lividans* and *M. melanosporea* leading to different resistance patterns. These sorts of findings are worthy of further investigations into the comparisons of secondary and (more importantly) tertiary structure between different species of actinomycetes.

In previous studies, residues in the 16S rRNA methylated by products of other aminoglycoside resistance determinants were established once the genes were cloned in *E. coli* or *S. lividans* (Beauclerk and Cundliffe 1987, Holmes et al 1991). Further primer extension analysis should be performed to confirm the sites of action by these methyltransferases in their native hosts. It would be enlightening to find for example, that KgmB methylated at a site other than G1389 in *S. tenebrarius* 16S rRNA.

Further experiments would be needed to establish if GrmA does methylate the *M. purpurea* 16S rRNA, and where in the 16S rRNA this occurs. An approximate location of methylation could be determined. A strain of *M. purpurea* containing a disrupted copy of *grmA* would need to be generated first. The 16S rRNA would be extracted from this strain and radioactively methylated *in vitro* by cell extracts containing GrmA. Specific fragments of rDNA would be used to protect the methylated portion of the 16S rRNA

from nuclease digestion. RNA-DNA hybrids that protected the sites of methylation could be identified by fluorography (Beauclerk and Cundlife 1987).

6.1 Analysis of *grmA* transcript in *M. purpurea*

The possibility that *grmA* is silent in *M. purpurea* should not be ruled out. A lower level of *grmA* transcript was observed in *M. purpurea* than in *S. lividans* suggesting that transcription of *grmA* is controlled in *M. purpurea* but not in *S. lividans* (Chapter 3 Figures 3.8 and 3.9). Further work needs to be done to map the 5' and 3' ends of the *grmA* transcript in *M. purpurea* using SI nuclease protection. The possibility that *grmA* is regulated by transcriptional attenuation (Keleman et al. 1994) also needs to be ruled out, although the data presented here (Chapter 3, Figure 3.9) suggests that if the transcript is truncated in the uninduced state, it is only slightly shorter (by about 250 bp) than the full gene length. It would also be interesting to see how long the *grmA* transcript is in the strain of *M. purpurea* containing an extra copy of *grmA*, whilst comparing it to the *grmA* message produced in a clone of *S. lividans* carrying the gene.

6.2 Is *grmA* silent in *M. purpurea*?

It has been postulated that silent genes are often inactivated by a mutation that reduces the level of transcription or translation (Koch 1972). The mutation may revert back to the wt, resulting in the production of the protein. However, an accumulation of several mutations often leads to a potentially new protein that may have an alternative function. There is often another gene present in the genome which can carry out the function of the silent gene. However these two genes are not necessarily closely related in DNA or amino acid sequences (Burns and Beacham 1986). Cryptic genes on the other hand are not usually expressed but can revert back to a functional state in circumstances

when expression of the gene is required. The continued expression of these genes may be detrimental to the cell.

There are mechanisms (other than reversion of a mutation), by which silent genes can be reactivated. One extraordinary example, is the carbapenem resistance determinant, *cfiA* in *Bacteroides fragillis*. *cfiA* is present in the genome and is either silent or expressed at any one time. However in the active state, *cfiA* is transcribed from a promoter within insertion element IS1186 which inserts itself into the genome, upstream of the gene (Podglajen and Breuil 1994).

Examples of silent antibiotic biosynthetic genes have been found in *Streptomyces* spp. The phenoxazinone synthetase (PHS) gene, *phs*, is involved in the biosynthesis of actinomycin D. *S. lividans* also possesses a *phs* gene which is not expressed. It is interesting that *S. lividans* possesses *phs* because this organism does not synthesise actinomycin D or PHS (Madu and Jones 1989).

grmA may be regulated by a control mechanism similar to the ones described previously (Chapter 1, Section 11.11). However if the gene is silent according to the definitions and examples described here, *grmA* may be mutated so that the gene is not expressed in *M. purpurea*. Cloning *grmA* in *S. lividans* may cause activation of the gene by reversion of the mutation, or by some other mechanism.

grmA and *grmB* (from *M. rosea*) (Chapter 1 section 1.9) are very similar and could have been derived from a common ancestor. It would be interesting to establish if *grmB* is expressed in *M. rosea*. *M. purpurea* could have acquired *grmA* via a separate route from any gentamicin biosynthetic genes the organism might possess. There may already be been a functional gentamicin resistance gene in the biosynthetic cluster of this organism, leaving *grmA* redundant.

6.3 A novel gentamicin resistance gene in *M. purpurea*

There is evidence of another gentamicin resistance gene in *M. purpurea*. A novel gene similar to the fortimicin A resistance determinant, *fmrO*, originating in *M.*

olivasterospora, was claimed to have been cloned from *M. purpurea* (Ohta and Hasegawa 1993). During Southern analysis, a 12 kb *Sst*I fragment thought to contain the novel gene from *M. purpurea*, hybridised strongly with *fmrO* (Ohta and Hasegawa 1993) suggesting that the two genes may be similar. *fmrO* encodes a protein which shows aa sequence identities of 30.8% and 35.8% to GrmA and GrmB respectively, suggesting that *fmrO* may encode a methyltransferase that acts by modifying the 16S rRNA. It would be interesting to confirm this by primer extension analysis, and determine exactly where in the 16S rRNA the FmrO protein methylates. As with other aminoglycoside resistance genes, the site of methylation would be predicted to lie in the decoding region. If there is an *fmrO*-like gene in *M. purpurea*, its product may methylate at the same site as FmrO. Curiously though, no methylation-dependent pauses specific to *M. purpurea*, were observed in the decoding region of the 16S rRNA (Chapter 4 Figures 4.1, 4.2, 4.3).

Attempts were made to clone a resistance gene other than *grmA* from *M. purpurea*, however only *grmA* was found. Not much is known about gentamicin production in *M. purpurea*. Antibiotic resistance genes are commonly found in clusters with biosynthetic genes and regulatory genes in actinomycetes. It would be interesting to construct a cosmid library of the *M. purpurea* genome in order to study the biosynthetic cluster. This may also assist determination of the location of *grmA* and any other gentamicin resistance genes, relative to the cluster and give some insight into the way in which *grmA* is controlled.

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