An investigation of the molecular genetics of tetronasin biosynthesis and resistance in *Streptomyces longisporoflavus*.

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by

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The research reported in this thesis is my own work unless otherwise stated. No part of this thesis has been submitted for any other degree.

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October, 1989

CONTENTS

ABBREVIATIONS

ATP - adenosine 5'-triphosphate
BDH - British Drug Houses
bp - base pairs
BRL - Bethesda Research Laboratories Ltd.
CIAP - Calf Intestinal Alkaline Phosphatase
DEPC - diethyl pyrocarbonate
DMSO - dimethylsulphoxide
DMF - dimethylformamide
DNB - Difco Nutrient Broth
DTT - dithiothreitol
dNTP - deoxynucleotide 5'-triphosphate
dATP - deoxyadenosine 5'-triphosphate
dCTP - deoxycytidine 5'-triphosphate
dGTP - deoxyguanosine 5'-triphosphate
dTTP - deoxythymidine 5'-triphosphate
DNA - deoxyribonucleic acid
ssDNA - single stranded deoxyribonucleic acid
dH_2O - distilled water
dpm - disintegrations per minute
EDTA - ethylene diaminetetra-acetic acid (disodium salt)
EtBr - ethidium bromide
IPTG - isopropylthio-B-D-galactoside
kb - Kilo bases
NEN - New England Nuclear
NA - Difco Nutrient Agar
OD - Optical density
PEG - Polyethylene Glycol
PIPES - Piperazine-NN'-bis-2-ethane sulphonic acid
rRNA - ribonucleic acid
mRNA - messenger ribonucleic acid
rRNA - ribosomal ribonucleic acid
s.a. - specific activity
SDS - sodium dodecylsulphate
SNA - Soft Nutrient Agar
Tris - tris (hydroxymethyl) methylamine
TSB - Tripticase Soy Broth
XGal - 5-bromo-4-chloro-3-indolyl-B-galactoside
YEME - Yeast extract-malt extract

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SUMMARY

Streptomyces longisporoflavus makes tetronasin, a polyketide, polyether antibiotic. This thesis describes the isolation and preliminary characterisation of sequences from S. longisporoflavus which are probably involved in biosynthesis of, and/or resistance to tetronasin.

A DNA library representative of the genome of a high-producing mutant, S. longisporoflavus strain 83E6, was constructed in the positive selection vector pIJ699 and contained in S. lividans TK64. The sensitivity of S. lividans to tetronasin was increased in the presence of 0.2M NaCl enabling tetronasin resistance determinants to be selected directly in the primary cloning recipient. Two non-identical recombinants (tetR1 and tetR5) containing 5.0 and 5.5kb of DNA respectively, were found to increase the tolerance of S. lividans to tetronasin. TetR5 also conferred resistance upon a tetronasin-super-sensitive strain of S. albus to a level comparable with that found in the wild-type S. longisporoflavus 4584. Although the functions of these sequences in the parental strain remain undetermined, they may by virtue of their activity in heterologous hosts, represent the first examples of the isolation of resistance determinants to an ionophore antibiotic.

The 2.1kb actI fragment (Malpartida et al., 1987) which encodes components of the actinorhodin polyketide synthase, hybridized at low stringency to a 5kb BamHI fragment (AC36) from S. longisporoflavus. Paradoxically, a 4.3kb BamHI fragment from S. cinnamonensis (monI) with good sequence homology to actI (J. A. Robinson, pers. comm.) failed to hybridize with the 5kb fragment in Southern analysis of total DNA. However monI did anneal to a 7kb BamHI fragment (MB74). Both fragments were isolated from partial libraries of S. longisporoflavus DNA constructed in $pTZ18^R$ and contained in E. coli JM101.

Cross hybridization of AC36 and MB74 with total DNA from several Streptomyces sp. which were known to produce polyketide antibiotics was investigated. At 65°C in 0.5xSSC both probes failed to hybridize with DNA fragments which correlated with restriction fragments known to be involved in polyketide biosynthesis. MB74 also had greater sequence homology with an 11kb BamHI fragment from S. cinnamonensis

than the 4.3kb monI. Cross hybridization with isolated sequences involved in oxytetracycline (otcY), actinorhodin (actI) and putatively in monensin (monI) biosynthesis was tested. Not surprisingly, MB74 hybridized to a different restriction fragment from monI than actI and otcY. Interestingly, a fragment with a low level of homology to AC36 and otcY was discovered in MB74. It did not overlap with the sequences which hybridized to monI. A 2.8kb EcoRV/PvuII fragment from AC36 hybridized with actI and at 60°C, 2xSSC with monI. These data suggested that both AC36 and MB74 could be involved in polyketide biosynthesis but did not uniquely indicate sequences which might encode components of the tetronasin synthase.

Recombinant bacteriophages which contained flanking DNA of each of the clones (AC36, MB74, tetR1 and tetR5) were identified by hybridization of radiolabelled probe to a representative library of S. longisporoflavus DNA contained in the lambda replacement vector EMBL3. Close linkage between any of the four clones was not established. However, sequences with homology to tetR1 were found, overlapping with, and in the DNA flanking, MB74. On its own MB74 was unable to confer resistance to tetronasin upon S. lividans or S. albus.

Although S. longisporoflavus 4584 was shown to possess a photoreactivation system, conditions were defined for mutagenesis using short-wavelength UV light. Attempts to complement mutants which were deficient in the biosynthesis of tetronasin were inconclusive. Similarly, preliminary experiments designed to disrupt transcription of the endogenous copies of AC36 and MB74 in S. longisporoflavus 4584, require repitition. Therefore until otherwise established AC36, MB74 and sequences flanking the tetronasin resistance determinants must remain merely candidates for genes involved in tetronasin biosynthesis in S. longisporoflavus.

for mum and dad

CHAPTER 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

This thesis describes the isolation and preliminary characterisation of DNA sequences which could be involved in the biosynthesis of and resistance to the polyketide antibiotic tetronasin in S. longisporoflavus.

In this chapter a brief summary of the general biology of the streptomycetes is presented, followed by a more detailed, but necessarily selective, introduction to the large diversity of bioactive molecules produced by members of the genus. In particular the polyketide class of antibiotics is described with specific reference to the biosynthetic origins of the metabolites - which draws on comparisons with fatty acid metabolism.

Section 1.3.2 describes the structure and function of tetronasin and this is followed by a dicussion of the rationale behind the isolation of genes for the biosynthesis of antibiotics in general and tetronasin in particlular. Next the organisation of biosynthetic, resistance and regulatory genes which direct production of antibiotics is discussed in conjunction with the development of specialised strategies for their isolation.

The chapter is concluded by a review of recent advances which reveal the complexity of regulation of gene expression in these prokaryotes.

1.2 THE STREPTOMYCETES

First observed in the last century as plant and animal pathogens, the actinomycetes were classified, (until relatively recently) by their gross morphological growth habit - to adopt the three 'evolutionary levels', later described by Prauser (1978): coryneforms (alternating undifferentiated cocci and rod forms), nocardioforms (fragmenting mycelial forms), and sporoactinomycetes (true mycelial forms which produce specialised sporulating structures). Indeed until the 1950's, there remained doubt as to which kingdom they should occupy. However detailed observations of their fine structure, determination of their chemical composition , and the application of molecular genetics has delineated this order into a phylogeny comprising sixty different genera (Goodfellow and O'Donnel, 1989). Reclassification within the order is on-going and the application of new taxonomic techniques has resolved much

ambiguity.

The streptomycetes are classically described as aerobic, Gram-positive and differentiating soil bacteria, with a G + C content of 69 - 78% $mole^{-1}$ DNA. The most striking feature of the streptomycetes, and the reason for misclassification with the fungi, is their curious life cycle. A propagule (or spore), of these ubiquitous micro-organisms forms a system of branching, irregularly septate filaments which generally at some stage possess a capsule or extracellular surface coat of undetermined composition (this could contribute to tetronasin resistance as alluded to in Chapter 4.1.3). After a period of vegetative growth the substrate mycelia normally gives rise to specialised spore bearing structures - the aerial mycelia.

The success of the mycelial growth habit, despite being less prolific than simple fission, was related (Chater and Merrick, 1979), to the colonisation of a particular ecological niche. Streptomycetes are ecologically-important soil micro-organisms for recycling plant material. Plant cells are largely comprised of the complex polysaccharides, hemicellulose, pectin, and also cellulose, lignin and lignocellulose in higher plants, mannan and xylan in algae, and chitin in fungae. These present recalcitrant substrates for biodegradation, each requiring more than one extracellular enzymic activity to achieve complete degradation of the insoluble polymers. Chater and Merrick recognised the advantage of a growth habit which generated a coherent colony growing on and within its substrate, benefiting from the action of the secreted degradative enzymes. The ability to penetrate the food source should benefit those members of the genus unable to completely degrade the complex polysaccharides by increasing the availability of material which can be broken down.

Such a life style does not favour dispersal of the species. A successful species must be able to spread and colonise new food sources. This requirement is provided in streptomycetes by the growth of specialised sporulating structures into an aerial environment for dispersal by air, water or even adhered to arthropod cuticles. The aerial mycelium is structurally and morphologically distinct from the substrate mycelium (Kalatoutskii and Agre, 1976; Ensign, 1978). This cellular differentiation occurs at a period in

the life cycle when net dry weight accumulation and growth rate decrease - in other words during stationary phase. Formation of the aerial mycelia accompanies the break down of glycogen granules, which accumulate late in the growth phase, and lysis of the substrate mycelia. Having exhausted the food supply during vegetative growth the widely accepted scenario envisages a parasitic relationship between the aerial and substrate mycelia.

In contrast to the substrate mycelium, aerial mycelium is generally hydrophobic, septate and has fewer side branches. The compartmentalised units containing a single genome equivalent, round off and disarticulate from the hyphae to become individual spores or spore chains. The spore wall is thus derived from the wall layers of the parental hyphae and not synthesised *de novo* as in endospore development of other genera, although thickening can occur through deposition of additional material. The wall is surrounded by a fibrous sheath which can comprise lipid (hence the hydrophobicity of the spores), chitin, fatty acids and inorganic material. Streptomycete spores do not contain dipicolinic acid and are not particularly thermoresistant. They are, however, generally more tolerant of environmental changes than mycelia although the extent of the effect appears to be species-specific.

The molecular genetics of streptomycete differentiation is beginning to be unravelled. The efforts of Chater and co-workers have accumulated a number of *S. coelicolor* mutants blocked at different stages of development (Figure 1.1) indicating the involvement of at least 12 genes. DNA complementing two of the aforementioned mutations *viz.* *whiG* and *bldA* has been isolated and appears to encode a novel sigma factor and an infrequently used tRNA respectively - the implications of which are discussed in sections 1.8.2 and 1.8.3.

Teleologically the sporulating streptomycete must protect the degenerating substrate mycelium (food source) from scavenging micro-organisms, providing Chater and Merrick (1979), with their convincing view of the inter-relationship between differentiation and widespread antibiotic production within the genus. An alternative premise for the synthesis of secondary metabolites was aired by Hunter and Baumberg (1989). The authors suggest that secondary metabolites

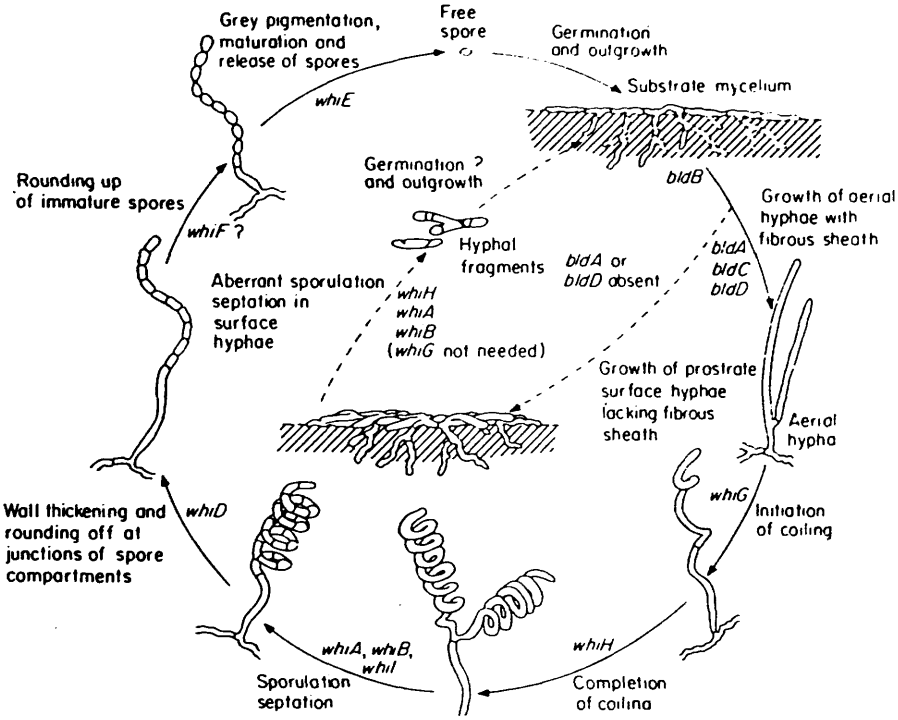


FIGURE 1.1; Differentiation Of S. coelicolor, Indicating The Phenotype Of Mutants Blocked At Various Stages Of Development, (After Chater And Merrick, 1979).

could have evolved as shunt products to alleviate the potentially toxic accumulation of metabolic intermediates when mycelial growth decelerates. However, loss of the ability to synthesise antibiotics is common (Cox et al., 1986; Hinterman et al., 1984), and such mutants are viable and can complete the life cycle, at least under laboratory conditions. The most plausible explanation may result from the combination of both this hypothesis and that of Chater and Merrick in a cumulative view of evolution. The initial driving force may have been the requirement to detoxify metabolite accumulation, but superseded, once a selective advantage over competing organisms was realised, in favour of accelerated evolution towards antibiotics.

However, in his book, "The Selfish Gene", Richard Dawkins most eloquently argues that the fundamental unit of evolution is the gene. When applied to clusters of antibiotic biosynthetic and resistance genes (see section 1.6) gene clustering can be viewed as sets of selfish genes which have evolved to function in a closely co-operative manner, and whose only "aim" is to increase their representation in the gene pool. Dispersal on the genome would lead to independent segregation of individual genes, and given their co-operative nature, could result in frequent loss of the selectable advantage conveyed upon the host cell. The close linkage of these genes may therefore function to minimise the size of the segregating unit for stable inheritance of the whole pathway.

1.3 SECONDARY METABOLITES

The discoveries of Selman A. Waksman, for which he received the Nobel prize in 1972, heralded a new era of industrial interest in the actinomycetes. The purification of actinomycin (Waksman and Woodruff, 1940), and streptomycin (Shatz et al, 1944), initiated the search for, and discovery of, a seemingly endless supply of useful products from the actinomycetes. The streptomycetes are undoubtedly the most valuable source of antibiotics (Goodfellow and O'Donnel, 1989), producing 60% of the 6 000 or so compounds now characterised (Omura, 1986). However only about 70 are used clinically having satisfied the high standard required for efficacy and tolerability. The most widely used antibacterial agents are sulphur-containing, bicyclic compounds

characterised by a Beta-lactam ring. 60% of the antibiotics made in industry are semi-synthetic derivatives of the naturally occurring Beta-lactam antibiotics penicillin G and cephalasporin C. The latter compound is produced both by, streptomycetes, in S. clavuligerus, and fungae, in Cephalosporium acremonium whereas biosynthesis of the former is restricted to the fungi. Much of this material is converted, via bioconversion using immobilised enzymes of bacterial origin, to 6-amino-penicillanic acid and 7-amino-cephalosporanic acid, both of which are starting materials for synthetic manipulations. The majority of the remainder of the antibiotic market is comprised of peptide, aminoglycoside and polyketide molecules.

Of course not all of the useful metabolites are anti-bacterial, others of practical importance which fall outwith this category or which are not used to control clinical bacterial infections include the following:

1. antihelmintics; the avermectin (Burg et al, 1979), and milbemycin (Pat. No. DT2329485), families of metabolites produced by S. avermitilis and Streptomyces sp. NRRL 5379, have potent activity against nematode worm infections of farm animals. The antibiotics have a wide spectrum of activity and appear to interfere with neurotransmission (see Chapter 4.1.1.2), so they may also find an insecticidal role.

2. coccidiostats; primarily monensin (Schumard and Callander, 1967), produced by S. cinnamonensis, and lasalocid (Mitrovic and Schildnecht, 1974), produced by S lasalensis, but also other polyether ionophores (Westley, 1977), were found to have a greater biological activity against the protozoan parasite of poultry Eimeria than any of the synthetic compounds in use. Included in this class of antibiotics is tetronasin, a product of S. longisporoflavus, and the subject of this study.

3. growth promotants; the polyethers - particularly monensin and tetronasin - are of major economic importance as growth promotants in ruminants. Many of the polyether ionophores when supplemented in the diet of cattle and sheep selectively inhibit methanogen bacteria and hydrogen and formate producing bacteria in the rumen. This improves feed utilisation by reducing wasteful methane production and increasing the production of volatile fatty acids for uptake by the ruminant (Chen and Wolin, 1979). The macrolide

antibiotic tylosin, a product of S. fradiae fermentation has also been reported to function as a growth promotant in swine (Cox et al, 1986).

4. herbicides; the tripeptide bialaphos, produced by S. hygroscopicus, acts as a potent herbicide. Bialaphos itself has no antibiotic activity, but when cleaved by peptidase in the plant cell, the phosphinothricin moiety inhibits glutamine synthetase (Bayer et al, 1972). Other streptomycete products with herbicidal activity include the herbimycins (Omura et al, 1979), and herbicidins (Takiguchi et al, 1979).

5. insecticides; the potential of avermectins and milbemycins as insecticidal agents has already been mentioned above. Nikkomycins produced by S. tendae, are nucleoside peptide antibiotics which act as competitive inhibitors of chitin synthetase. Chitin is a structural component of the fungal cell wall, so these compounds have been used as antifungal agents. Chitin is also the major constituent of arthropod exoskeletons and it has been shown that the nikkomycins are potent insecticides (Brillinger, 1979).

The streptomycetes are also a rich source of pharmacologically-active metabolites. Demain (1983), reviewed the application of microbial products and argued that the wealth of useful metabolites from streptomycetes was limited only by our ability to identify them. This is largely due to ethical and practical reasons against testing crude preparations in live animals. Indeed, some of the pharmacologically-active compounds in use today, were originally purified as antibiotics but never found a clinical use. These include anti-inflammatory (Ninomiya et al., 1980), vasodilator (Yoshia et al., 1982), and anti-cancer (Blum and Carter, 1974), agents.

In cases where aberrant enzymic activity is shown to be responsible for a physiological disorder, Umezawa (1972), reasoned that the testing of crude preparations by in vitro enzyme assays would be particularly useful. This, in part, can circumvent the problems of discovery and has directly led to streptomycete products with activity against alpha-amylase (Murau et al., 1977), protease (Meyn et al., 1977), and pepsin (Umezawa et al., 1973), which may find practical use in the treatment of diabetes, carcinogenesis and ulcers respectively.

1.4 THE BIOSYNTHESIS OF POLYKETIDES - IN COMPARISON TO FATTY ACID BIOSYNTHESIS

The diverse activities of products which can be obtained from the streptomycetes is paralleled by the heterogeneity of their chemical structures. These were classified by Berdy (1974), according to structure, into nine major families, each with several subdivisions. It is possible however, to adopt an alternative system and classify the metabolites by their biosynthetic origins (Turner and Aldridge, 1983). This identifies the following products of primary metabolism as important building blocks of secondary metabolites.

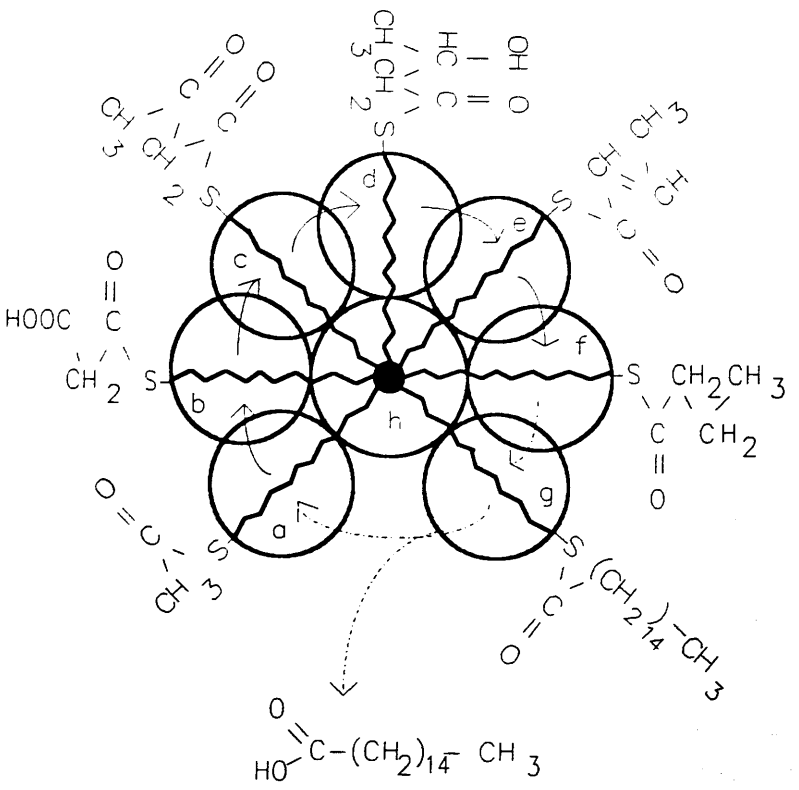
a. small molecular weight carboxylic acids.
b. amino acids and intermediates of the shikimate pathway.
c. nucleosides.
d. TCA cycle intermediates.

Particular metabolites can fall into more than one category, as for example, polyketide molecules become glycosylated or aminated.

The class of molecules which is important to this work is the polyketides. They are widespread in nature, occurring as plant pigments and flavourings, and as a huge diversity of fungal and actinomycete secondary metabolites which include such important classes as aflatoxins, anthracyclines, ansamycins, tetracyclines, macrolides, polyenes and polyethers.

The polymerisation of small molecular weight carboxylic acids - mainly acetate, propionate and butyrate - for the synthesis of the polyketide backbone parallels in many ways, the biosynthesis of fatty acids. Convincing evidence for the similarity of the reactions was found by the inhibition of both pathways by the antibiotic cerulenin (Arison and Omura, 1974). Yet more evidence was unearthed recently through the sequencing of DNA segments known to be involved in the early stages of polyketide biosynthesis (Bibb *et al.*, 1989; Sherman *et al.*, 1989). These genes have convincing homology to the Beta-ketoacyl synthase and Beta-ketoacyl-ACP reductase of fatty acid biosynthesis.

From an understanding of the biosynthesis of long chain fatty acids, the details of which have been known for some time (Birch and Donovan, 1953; Lynen *et al.*, 1978), an insight into the complexity of the biosynthesis of



Key;

a, acetyl transacylase
b, malonyl transacylase
c, Beta–ketoacyl synthase
d, Beta–ketoacyl–ACP reductase
e, enoyl–ACP dehydratase
f, crotonyl–ACP reductase
g, palmityl deacylase
h, acyl carrier protein (ACP)

= 4'–phosphopantethiene prosthetic group of ACP

FIGURE 1.2; The Biosynthesis Of Palmitic Acid By The Fatty Acid Synthetase Complex.

polyketides can be obtained.

Fatty acid biosynthesis proceeds as detailed in Figure 1.2. In higher animals and plants, fatty acids are synthesised from an excess of acetyl-CoA (the malonyl-CoA extender units are derived from acetyl-CoA via acetyl-CoA carboxylase), and stored ultimately as triglycerides. Palmitic acid, a primary product of fatty acid biosynthesis is also an important precursor for the synthesis of phosphoglycerides - the polar lipids found almost exclusively in biological membranes. Palmitic acid, a fully-saturated 16 carbon fatty acid, is synthesised from 1 acetyl-CoA and 7 malonyl-CoA units by an eight enzyme complex called the fatty acid synthetase (FAS). An acetyl transacylase transfers a single molecule of acetyl-CoA to a prosthetic group of a small molecular weight protein called the acyl carrier protein (ACP), on the FAS. The prosthetic group is 4-phosphopantetheine, which is covalently linked to the "active site" serine of the ACP. This serves to anchor the acyl intermediates via a thioester bond to the FAS and also as postulated by Lynen, rotate (as a "swinging arm"), the substrate from one enzyme to the next on the FAS.

This molecule of acetyl-S-ACP functions as a primer unit in fatty acid biosynthesis. Malonyl-CoA is then transferred to the FAS by the action of a malonyl transacylase. The malonyl-S-ACP and acetyl-S-ACP groups react in a condensation catalysed by the Beta-ketoacyl synthase, with the loss of CO_2 (the same CO_2 molecule used to carboxylate acetyl-CoA in the formation of malonyl-CoA). The pantetheine arm swings the Beta-ketoacyl-ACP intermediate from the condensing enzyme to the Beta-ketoacyl-ACP reductase, for the first reduction step, to form Beta-hydroxybutyryl-S-ACP. This molecule is then dehydrated to crotonyl-S-ACP by enoyl-ACP dehydratase. Crotonyl-S-ACP is fully reduced by crotonyl-ACP reductase to butyryl-S-ACP. Only now is the second malonyl-CoA extender unit added. The addition of the remaining malonyl-CoA units occurs in a reiterated cycle of reactions to form palmityl-S-ACP. Palmitic acid is released from the FAS complex in the final enymatic step catalysed by a hydrolytic deacylase.

This narrative has been written for the case where the FAS complex is comprised of eight independent dissociable proteins. This situation is found in E. coli and in the plastids of plants (Ohlrogge, 1982). In vertebrates the FAS

is a single but multi-domain polypeptide (Buckner and Kolattukudy, 1976). However S. cerevisiae represents an intermediate case with the FAS comprising two polypeptides, carrying three and five of the biosynthetic functions (Lin and Smith, 1978). According to the review by McCarthy and Hardie (1984), this represents protein evolution by gene fusion. The DNA sequence data from polyketide biosynthetic genes of several groups (Bibb et al., 1989; Sherman et al., 1989; C. Binnie, pers. comm.; J. A. Robinson, pers. comm.), indicates that the PKS for tetracenomycin C, granaticin, oxytetracycline, monensin and nonactin biosynthesis involves a multienzyme complex.

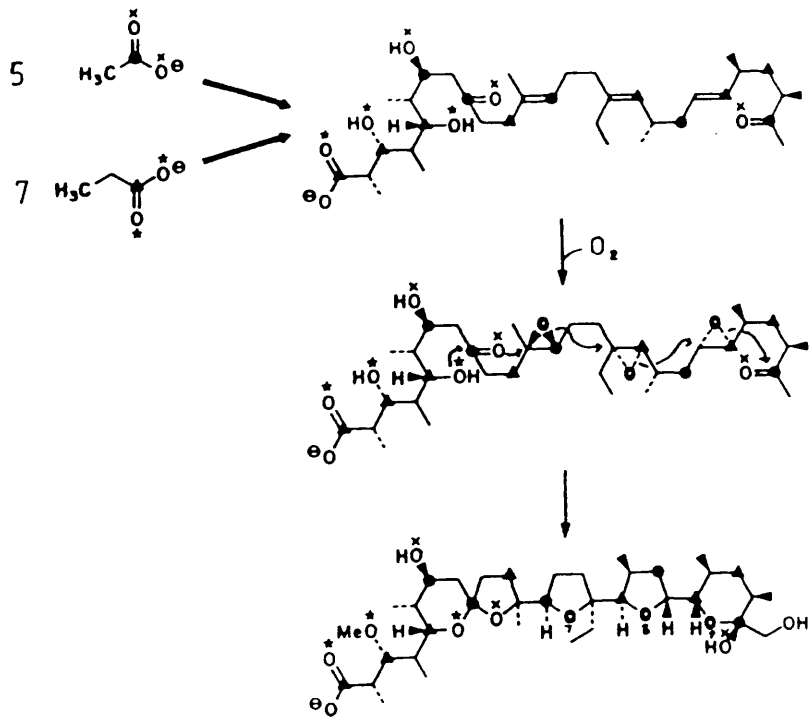
Despite the analogy between the biosynthetic origins of fatty acids and polyketides, they are certainly not identical. Obvious differences are apparent from the huge variety of polyketide structures generated. PKS's appear to be able to introduce complexity into the polyketide structure at five different levels. These are highlighted where possible using the polyketide polyether monensin A as a model. This example has been used by other authors (Sherman et al., 1988), and is convenient because investigation of its biogenesis through the incorporation of radiolabelled substrates (Cane et al., 1981 & 1982), has identified the biosynthetic precursors and led to the proposition of the mode of assembly involving particular intermediates (Cane et al., 1983), indicated in Figure 1.3.

The five levels of complexity are:

Choice of primer unit - although polyketide chain initiation with acetyl-CoA is common and also used for monensin A, it is not an absolute requirement for all polyketides e.g. propionate is used in erythromycin biosynthesis in Saccharopolyspora eythreae.

Choice of extender unit - unlike the FAS which is restricted to the use of malonyl-CoA as an extender unit, the PKS has evolved to allow incorporation of different units at each condensation step - acetate, propionate, methyl-/ethyl-/ propionate, and butyrate are common alternatives. The addition sequence of the extender units for monensin A is given in Figure 1.3.

Reduction of the keto group - the addition of each extender unit introduces a keto group into the growing acyl chain. In fatty acid biosynthesis this keto group is always fully



monensin A

primer unit, A, extender units, PPAPBAPAAPPP
biochemistry of each keto group, ZWXWXWXWZYYY

A = acetate, P = propionate, B = butyrate

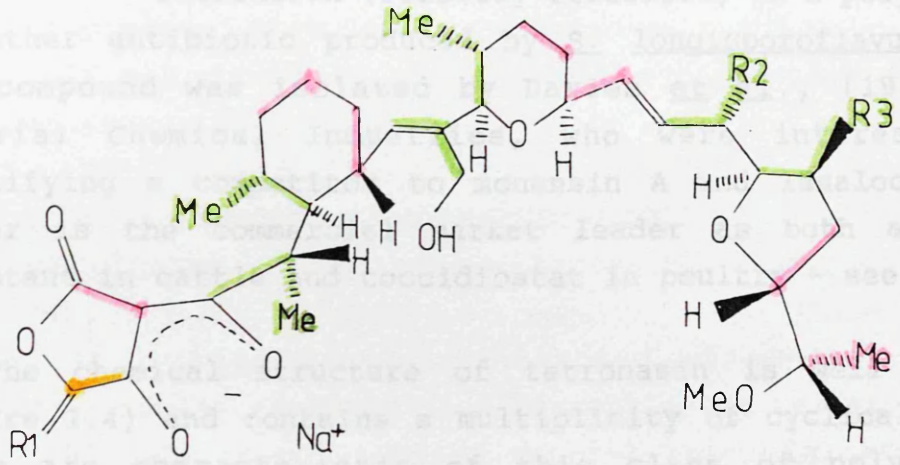
Z = condensation (C) only, resulting in a keto group
Y = C and first reduction (R1), resulting in a hydroxyl group
X = C, R1 and dehydration (D), resulting in an enoyl group
W = C, R1, D and second reduction resulting in an alky group

FIGURE 1.3; The Proposed Biosynthetic Route To Monensin A (Modified From Cane et al., 1983).

reduced by a series of reduction, dehydration, reduction reactions, before addition of the next extender unit. In polyketide biosynthesis it is clear that the keto group need not be fully reduced. The modification of the keto groups could take place immediately after condensation and prior to the addition of the next extender unit, or after formation of the complete polyketide backbone. Recently the incorporation of radiolabelled putative intermediates into the small molecular weight polyketide aspyrolle suggested that this compound is synthesized via the former, processive route in Aspergillus (J. Staunton, pers. comm.). The peculiar structure of the shunt product (with an incorrect ring arrangement) obtained from S. coelicolor mutants deficient in the dehydration step in actinorhodin biosynthesis confirms that the keto groups are modified prior to ring closure (Cole, 1986; H.G. Floss, cited in Sherman et al., 1988). The PKS is also selective in the number and choice of which keto groups are altered and to what extent (see Figure 1.3 for the likely biochemistry of monensin A biosynthesis). This regulation of enzyme activity is not understood.

Cyclisation and aromatisation - modifications to the tertiary structure of the polyketide includes ring closure of the carbon backbone of the molecule. These rings may be formed spontaneously or catalysed by a Beta-ketoacyl cyclase. The inability to identify mutants in the formation of, for example, macrolide, tetracycline or ether rings and the failure to purify any intermediates in ring formation has left this area open to speculation. What is clear from studies on the biosynthesis of monensin A is the origins of three of the cyclic ethers, oxygens O(7), O(8), and O(9), which are engineered by an epoxidase using molecular oxygen to epoxidise each of the double bonds of the proposed intermediate. The other oxygen atoms in the structure, including the two in the remaining cyclic ethers, are derived from their respective propionate or acetate extender units.

Additional modifications - this usually involves modification, or addition of side chains to the polyketide molecule. Examples include, oxidation and methylation of side chains of the tylactone ring (Baltz et al., 1981). The addition of unusual sugar molecules is found, in particular with macrolide antibiotics, but important anticancer-anthracyclines like duanorubicin and doxorubicin are also



= derived from acetate

= derived from propionate

= derived from glycollate

FIGURE 1.4; Chemical Structure Of Tetronasin And Tetronomycin, After Bulsing et al., (1984).

tetronasin, R1 = H_2, R2 = R3 = CH_3

tetronomycin, R1 = CH_2, R2 = R3 = H

stereochemistry shown is that for tetronasin

glycosylated. Once again tylosin biosynthesis provides a good specific example with the addition of the deoxysugars mycarose and mycinose and the deoxy-aminosugar mycaminose to the tylactone ring. More complex modifications can occur, for example in the bizarre structure of chlorothricin, a product of S. antibioticus. Chlorothricin is comprised of two polyketide rings, thought to be synthesised independently and subsequently linked by glycosylation (Lee et al., 1986). Similarly the isochromanequinone antibiotic actinorhodin which has a six ring structure with bilateral symmetry seems to be synthesized by combining two, three ring polyketides.

1.4.1 THE POLYKETIDE-POLYETHER-IONOPHORE TETRONASIN

Tetronasin (formerly ICI139603) is a polyketide-polyether antibiotic produced by S. longisporoflavus 4584. The compound was isolated by Davies et al., (1982), at Imperial Chemical Industries, who were interested in identifying a competitor to monensin A and lasalocid (the former is the commercial market leader as both a growth promotant in cattle and coccidiostat in poultry - see section 1.2).

The chemical structure of tetronasin is well defined (Figure 1.4) and contains a multiplicity of cyclical ethers which are characteristic of this class of polyketide. Isotopic labelling experiments identified the biosynthetic precursors to be 1 glycollate, 6 propionate and 7 acetate units (Figure 1.4; Bulsing et al., 1984; Dodrell et al., 1984; J. Staunton, pers. comm.). The structure is particularly unusual because it contains a cation-binding tetronic acid moeity (rather than the more common carboxylic acid group), and a six-membered carbocyclic ring. Furthermore it belongs in the acyl tetronic acid class of polyethers and therefore does not conform to the stereochemical model proposed by Cane et al., (1983), for structural similarity between the majority of polyketide polyether molecules. In this respect it is particularly intriguing that despite a strong similarity to tetronomycin, tetronasin has the opposite stereochemistry in all ten chiral centres (Figure 1.4).

The biological activity of polyethers is attributed to their ability to interfere with permeability barriers to ion transport across cell membranes (Pressman, 1976; discussed

further in Chapter 4.1.3). A study of the basis for the improved live weight gain and food conversion efficiency in cattle caused by tetronasin was conducted by Newbold *et al.*, (1988). They concluded that tetronasin and monensin A had broadly similar spectra of activity, but the increased potency of tetronasin and the differing abilities of some ruminal species to adapt to the drugs could be responsible for the greater efficacy of tetronasin.

The parental strain 4584, has undergone a classical mutational strain improvement programme, using several commonly used mutagens to isolate high-producing variants (D. Groves, pers. comm.). One of the products of this work, *S. longisporoflavus* strain 83E6, was used as a source of genomic DNA in gene cloning experiments. This strain was chosen because it produced high levels of tetronasin and as such may contain multiple copies of the antibiotic biosynthetic genes and/or resistance determinant(s).

1.5 WHY CLONE ANTIBIOTIC BIOSYNTHETIC GENES?

The cloning of antibiotic biosynthetic genes should permit both;

1. an investigation of the regulation of expression of secondary metabolic pathways in differentiating bacteria (see section 1.8). The formulation of general rules in this area of research is dependent upon a consensus being drawn from the data accumulated on a number of examples. With an increase in sample number a concluding hypothesis should more accurately reflect the natural situation. This field of research should therefore benefit from the cloning of more genes involved in antibiotic production and therefore also from genes involved in tetronasin biosynthesis.
2. a detailed examination of the biochemistry of the PKS. Enzymological studies of the PKS complex have been largely prevented by the inability, in most cases to achieve *in vitro* synthesis of polyketides. This has been partly due to the lability of the enzyme complex, but highly reactive poly-Beta-ketone intermediates have contributed to this difficulty (Harris and Harris, 1977). It is hoped that high level expression of the cloned polyketide biosynthetic genes can be engineered and purification of a substantial amount of protein achieved, so that the enzymes of the PKS can be characterised and their capabilities determined. This would

include the elucidation of the temporal sequence and regulation of the steps involved in polyketide biosynthesis. Accessibility to data accumulated on the biosynthesis of different antibiotics can only enhance the probability of a rapid solution to how the PKS regulates each of the "five levels of complexity" described in the previous section. An investigation of the biosynthesis of tetronasin could be especially interesting, if rewarded by an understanding of how the unusual stereochemistry of the molecule is attained. Precedence for the genetic control of stereochemical differences between polyketide metabolites was found by genetically engineering a stereochemical modification of dihydrogranaticin (see later in this section, under "mixing structural genes"). Enzymological studies of the tetronasin synthase could possibly define whether the chirality is genetically determined and if so whether novel components of the PKS are required.

DNA sequencing of cloned genes has already revealed that the PKS for tetracenomycin biosynthesis in S. glaucescens (Bibb et al., 1989) is a multi-enzyme complex, akin to the type II fatty acid synthase of most bacteria, including Sacharopolyspora (Hale et al., 1987) - a genera closely related to Streptomyces. This was in contrast to the largely circumstantial evidence for multi-domain proteins provided by the isolation of the PKS for 6-methylsalicylic acid from Penicillium spp. (Dimroth et al., 1978) and naringenin synthase from plants (Kreuzaler and Hahlbrock, 1975). In the former case all 12 enzymatic activities co-purified, and in the latter the 3 steps in the biosynthesis were encoded by a single mRNA.

Industry too, has, and should in the future, profit from the isolation of antibiotic biosynthetic genes. The biotechnologist, benefiting from an academic understanding of the biochemistry of polyketide biosynthesis will be able to manipulate the micro-organism in a more rational and directed manner to increase yields and to produce novel antibiotics. Some of the potential benefits which have been foreseen, and in some cases realised, are outlined below.

Improvement of antibiotic yield,

Commercially important antibiotic production strains are generally derived from the parental wild type strain by persistent sequential mutagenesis of the isolate with the

highest product yield. Sequential genetic selection in the past has often generated a thousand fold increase in production. Such a strategy is empirical, with large numbers of mutagenised clones assessed for yield improvement. However with increased knowledge of pathway regulation and the isolation of genes encoding the biosynthetic enzymes, more direct approaches may be applied. Increasing the copy number of structural genes concerned, or deregulation of enzymatic steps within the pathway may be postulated to increase the metabolic flux inducing antibiotic over-production, without altering the genetic background of the producing strain.

The efficacy of this approach for cephalosporin C biosynthesis in Cephalosporium acremonium was illustrated by Pratt, (1989). The ring expansion/hydroxylase (REXH) gene product which converts penicillin N to deacetylcephalosporin C was cloned and introduced into a production strain of C. acremonium, by integrative transformation. The resulting recombinant exhibited a 30-80% increase (over their best production strain) in cephalosporin C titre. Fermentation of this recombinant is being scaled up to full production volume at Eli Lilly & Co..

Beckman et al. (1988), also at Eli Lilly Research Laboratories, have demonstrated that this approach can work for antibiotic production is streptomycetes. Using an unstable SCP2 derived plasmid, part of the tylosin biosynthetic gene cluster was integrated into the genome of the tylosin production strain of S. fradiae. The increased gene dosage caused a 35% increase in the tylosin titre of the strain and correlated well with an increase in the activity of macrosin O-methyl transferase (MOMT). This enzyme catalyses a step in tylosin biosynthesis thought by the authors to be of regulatory importance. However the experiment was not repeatable with other plasmid constructs in which an increase in MOMT activity was engineered, whilst tylosin biosynthesis was actually decreased. The pertinent biochemistry of tylosin biosynthesis in these recombinants is not yet understood, and it may not be the case that deregulation of a single enzymatic step is responsible. It is also possible that the regulation of gene expression of all the genes for tylosin biosynthetic enzymes has effected an increase in tylosin production. This postulates the presence of a positively-acting regulatory gene. Although none has, as

yet, been found for tylosin production, it is not an unreasonable assumption when four positively-acting regulatory genes controlling the biosynthesis of their respective antibiotics viz. actinorhodin, bialaphos, streptomycin, and undecylprodigiosin, have been identified (Hopwood pers. comm.; Anzai et al., 1987; Ohnuki et al., 1985; D. A. Hopwood, pers. comm.) The relatively common occurrence of regulatory genes (5 out of the 12 gene clusters studied in any great depth, have regulatory genes including a negatively-acting element in methylenomycin biosynthesis), may represent a general mechanism by which an increase in antibiotic titre can be achieved.

There is an upper limit to the amount of antibiotic which can be produced by such an approach. One need only trace the biosynthetic origins of the polyketides back a further step in the physiology of the cell to imagine the supply of acetate or some other immediate precursor, to become limiting. This is where the control theory of Kacser and Burns (1973), complemented and popularised by Kell et al., (1989), is of importance to the biotechnology industry. The deregulation of secondary metabolic pathways can only increase product titre up to a point when the supply of immediate precursors becomes limiting. The important enzymes for further increasing polyketide biosynthesis in such a hypothetical streptomycete, would be glycolytic enzymes and other sources of acetyl CoA and any enzymes competing for this substrate.

This has been realised by both academia and industry, and funding for collaborative projects in streptomycete primary metabolism has been made available. This should redress the balance of work on streptomycete physiology and may ultimately lead to "designer strains" in which metabolic pathways to particular intermediates have been manipulated for maximal conversion into the desired product.

Economy of large scale production,

An alternative approach to economise large scale production of important antibiotics is currently being investigated in this lab. It involves the introduction of the complete biosynthetic pathway for an antibiotic from the mesophillic production strain, into a thermophilic fermentation host. It has been surmised that the main advantage would be a reduction of the cooling costs of

fermentation (Czaplewski, 1989).

Isolation of novel, natural antibiotics by cross hybridisation,

Currently the discovery of soil isolates capable of producing novel antibiotics is a laborious task. A pure culture must be obtained and products are assayed for antibiotic or therapeutic activity from spent culture broth or after diffusion into the surrounding agar if grown on solid media. Often the production of secondary metabolites by streptomycetes is dependent on media and growth conditions. It would therefore be advantageous to identify soil isolates with the genetic capability to synthesise potentially useful metabolites, without requiring the phenotypic expression of the genotype in the discovery phase. It is possible to identify, by Southern analysis using cloned genes as probes, strains which can synthesise related structures. Further manipulation of these strains (genetic manipulation, or modification of the growth media), may induce the production of a novel and useful metabolite from the isolate.

The first example of the identification of a strain carrying cryptic genes for polyketide biosynthesis was demonstrated by Malpartida et al., (1987). S. parvalus, known to produce the peptide antibiotic actinomycin, was shown to contain DNA homologues of both the condensing enzyme and Beta-ketoacyl-ACP reductase involved in actinorhodin biosynthesis. The strain was later found to be competent to produce the polyether polyketide nonactin (U. Keller, cited in Malpartida et al., 1987). Similarly, cross hybridization of the act genes with DNA from S. venezualae indicated the presence of homologous sequences in this strain which was thought to only produce chloramphenicol. Again the strain has since been shown to be capable of polyketide biosynthesis (C. Stuttard, pers. comm.).

Novel antibiotics; (a) Gene activation,

While trying to isolate the structural gene for phenoxazinone synthase (an enzyme involved in actinomycin biosynthesis), Jones and Hopwood, (1984), observed that small fragments of DNA cloned from the producing species, Streptomyces antibioticus induced in the recipient host S. lividans the expression of the hitherto "silent" gene for phenoxazinone synthase. The cloned DNA fragment was shown to contain no structural information for the enzyme, but was

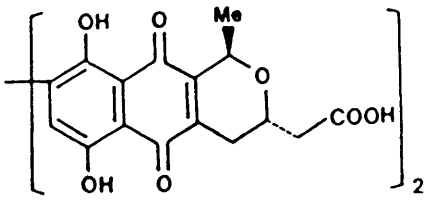
thought to be present on the same, approximately 20kb SphI fragment as the coding sequences in the S. antibioticus genome. This result suggests that the cloned DNA gene product, functions in trans to activate the silent gene in S. lividans. It was postulated that cloning antibiotic biosynthetic genes from a producing strain into a non-producing recipient, might induce the recipient to unmask "silent genes" modifying the antibiotic structure and thus altering its activity.

(b) Mixing structural genes

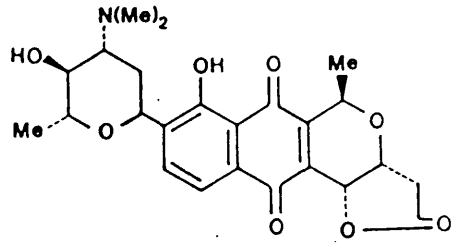
A more direct method for generating hybrid antibiotics was first effectively demonstrated by Hopwood et al., (1985b), with the isochromanequinone antibiotics actinorhodin and medermycin. By introducing part of the cloned S. coelicolor actinorhodin gene cluster, which included sequences which complemented the class V act mutants (defective in a late step in actinorhodin biosynthesis), into the producer of medermycin (Streptomyces sp. AM-7161) a hydroxylated derivative of medermycin was generated and given the name mederrhodin A, (Figure 1.5). Later data of Malpartida and Hopwood, (1986), indicates that the act V gene may have been under the control of a vector promoter and the gene product present at elevated levels. The importance of this is not known.

Intriguingly, transfer of the whole actinorhodin gene cluster resulted in the production of the chromogenic actinorhodin (red in acid, blue in base), and medermycin (yellow in acid, brown in base) alone, and the purple mederrhodin A was only produced when the class V act clone was present by itself, or in the absence of actinorhodin biosynthesis.

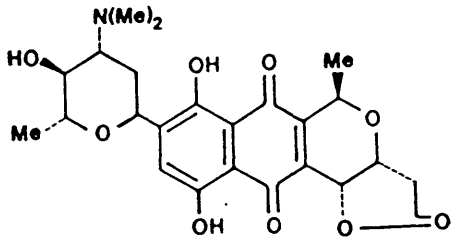
Another example of production of a hybrid antibiotic was provided by S. Omura (1986), using cerulenin (see section 1.4) to disrupt the biosynthesis of the endogenous antibiotic. S. ambofaciens was used to bioconvert the tylactone ring (purified from a mutant of S. fradiae, blocked in the first glycosylation step of tylosin biosynthesis). This produced chimeramycin, a molecule composed of the tylactone ring modified by glycosylation with the saccharides normally reserved for the endogenous S. ambofaciens antibiotic, spiramycin. This bioconversion required that the S. ambofaciens strain be treated with cerulenin to inhibit



actinorhodin



medermycin



mederrhodin A

Figure 1.5; Generation of a hybrid antibiotic (Hopwood, et al. 1985).

the synthesis of the spiramycin polyketide backbone. This seems to imply that the perturbation of at least one of the antibiotic biosynthetic pathways might be a general prerequisite for introducing novel combinations of enzymic activities which make a new product. This is not an unbending hypothesis however, because S. violaceoruber Tu22 transformed with pIJ2303 (Hopwood et al., 1985b), contains a complete set of genes for the production of both dihydrogranaticin and actinorhodin, yet produced, almost exclusively the recombinant molecule dihydrogranatirhodin (a variant of dihydrogranaticin, with the strereochemistry of actinorhodin in one chiral centre). Another important consequence of the Omura experiment was the demonstration that the glycosylation enzymes would modify the different lactone rings.

For other examples of genetically engineered novel antibiotics the reader is directed to;
McAlpine et al., (1987) - the production of 2-norerythromycins by a Saccharropolspora erythraea mutant of erythromycin biosynthesis, transformed with DNA, shotgun cloned, from the oleandomycin producer, S. antibioticus.
Schoner et al., (1988) - production of an iso-valeryl derivative of spiramycin, by S. ambofaciens transformed with the carE gene from S. thermotolerans. CarE encodes an acylase which acylates the mycarose residue of carbomycin in S. thermotolerans, and catalyses the same modification in the recombinant spiramycin producing strain.

In all but one of the above examples involving polyketide metabolites a substituent group of the polyketide molecule is modified - the catalysis of which is likely to occur after polyketide chain assembly. Indeed S. lividans carrying the carE gene will convert exogenous spiramycin to its iso-valeryl derivative. Therefore, other than for the production of dihydrogranatirhodin (a strereochemical substitution), and some examples of shunt products from blocked mutants, the early biochemical functions of the PKS have not been altered. In the Beta-lactam antibiotics industry there exists more scope, because the isopenecillin N synthase will convert other tripeptides into novel antibiotics. This low substrate specificity has been capitalised on, by feeding unusual synthetic precursors to the strains for bioconversion into new Beta-lactams (Baldwin

et al., 1986; and reviewed by Pratt, 1989).

Any antibiotic biosynthetic pathway might be amenable to manipulation by the strategies described above. It is therefore hoped that by the isolation of tetronasin production genes biotechnological advances will be made. In particular the genetic engineering of hybrid antibiotics although still in its infancy might benefit from the isolation of tetronasin synthase genes, especially if the unusual features of the chemical structure (section 1.3.1) are genetically determined. This being the case, the pertinent DNA could offer alternative capabilities in the construction of future hybrids.

1.6 STRATEGIES FOR CLONING ANTIBIOTIC BIOSYNTHETIC GENES

Some special features of antibiotic biosynthesis, pertinent to the development of cloning strategies must be considered. Antibiotics fall into the category of secondary metabolites, purely because they are not absolutely required for vegetative growth and are generally produced after most of the cellular growth has occurred (Weinberg, 1970). This makes direct selection of antibiotic producing clones virtually impossible. In most cases, laborious bioassays, or chromatographical tests must be performed in order to identify antibiotic producing isolates, (although a few antibiotics are pigmented allowing straightforward clone recognition). Furthermore, secondary metabolites are generally not produced until the onset of stationary phase (chloramphenicol biosynthesis in S. venezuelae is monophasic and so constitutes a notable exception), introducing a temporal consideration in screening systems.

More importantly antibiotics are generally synthesised from a complex biochemical pathway involving a number of enzymatic steps. The majority of metabolic intermediates in the pathway often have no biological activity, thus before activity can be established in a heterologous host the whole pathway, perhaps some 10 to 20 genes may have to be cloned. One redeeming feature is that, all the examples studied to date, indicate that the antibiotic biosynthetic genes for a particular pathway are clustered in the genome, and a number of cloning strategies

have been developed to exploit this feature.

The basic procedures of the seven cloning strategies are outlined below with examples in Table 1.1.

1.6.1 COMPLEMENTATION OF MUTANTS DEFICIENT IN ANTIBIOTIC BIOSYNTHESIS

Ideally, this approach requires the isolation of a large number of mutants blocked at the various steps in antibiotic biosynthesis, which can be transformed efficiently with plasmid DNA. The genes directing the biosynthesis of the isochromanequinone polyketide, actinorhodin were cloned from Streptomyces coelicolor by Malpartida and Hopwood, (1984), and represented the first example of the cloning of an entire antibiotic biosynthetic pathway. A large number of mutants blocked in actinorhodin biosynthesis were isolated with relative ease by following the pigmentation profile of colonies arising from mutagenised spores (Rudd and Hopwood, 1979). It was also possible to categorise the mutants into seven distinct classes by; 1. differences in pigmentation, and 2. cross feeding experiments between mutants leading to restoration of the wild type colour. The system was thus also blessed with freely diffusible biosynthetic intermediates of the later steps in the pathway. All mutants mapped to the same segment of DNA on the S. coelicolor chromosome, indicating that the genes were likely to be closely linked. This was confirmed when two overlapping clones, isolated by complementation of act mutants, were found to complement all seven classes. A 33kb DNA fragment constructed in vitro in a low copy number vector complemented all classes of mutation. When introduced into S. parvalus this gene cluster caused production of actinorhodin, providing strong evidence that the 33kb fragment carried all the information necessary to synthesise actinorhodin and confer resistance on the recipient (see strategy 7).

1.6.2 CLONING ANTIBIOTIC RESISTANCE GENES, AND ANALYSIS OF LINKED DNA FOR GENES INVOVED IN ANTIBIOTIC BIOSYNTHESIS

Gene cloning, genetic mapping, and transcriptional analysis has established that genes encoding antibiotic biosynthetic enzymes are tightly clustered within the DNA and very often closely linked to the resistance gene(s). This knowledge has prompted the development of an effective

TABLE 1.1; Examples Of Cloning Strategies Used To Isolate Genes For Antibiotic Biosynthesis

metabolite	class	producing organsim	cloning strategy	genes included	DNA cloned (kb)	reference
actinorhodin	polyketide	*S. coelicolor*	C,D	1,2,3	26	Malpartida & Hopwood, (1984)
methylenomycin-A	epoxy-cyclopentane	*S. parvalus*	M	1,2,3	17	Chater & Bruton, (1983)
undecylprodigiosin	tripyrrole	*S. coelicolor*	C	1,3	21	Feitelson & Hopwood, (1983)
cephamycin C	B-lactam	*S. cattleya*	D	1,	29	Chen *et al.*, (1986)
tetracenomycin	polyketide	*S. glaucescens*	C	1,2	24	Motamedi & Hutchinson, (1987)
oxytetracycline	polyketide	*S. rimosus*	R,C, O,D	1,2	34	Butler *et al.*, (1989) Binnie *et al.*, (1989)
erythromycin	polyketide	*Sac. erythraea*	R,D	1?@	35	Stanzak *et al.*, (1986)
bialaphos	tripeptide	*S. hygroscopicus*	R,C	2,3	16	Thompson & Anzai, (1986)
streptomycin	aminoglycoside	*S. griseus*	C,R	2,3	16	Distler *et al.*, (1985a & b) Distler & Piepersberg, (1985)
tylosin	polyketide	*S. fradiae*	O,C,R	2	70	Fishman *et al.*, (1987)
clavulanic acid	B-lactam	*S. clavuligerus*	C		9.1	Bailey *et al.*, (1984)
candicidin	polyketide	*S. griseus*	P		4.5	Gil *et al.*, (1983)
actinomycin	peptide	*S. antibioticus*	P		2.4	Jones & Hopwood, (1984)
granaticin	polyketide	*S. violaceoruber*	H	3?*	28	Malpartida *et al.*, (1987)
milbemycin	polyketide	*S. hygroscopicus*	H	3?*	27	Malpartida *et al.*, (1987)
carbamycin	polyketide	*S. thermotolerans*	R,C	2	70	Schoner *et al.*, (1988)
spiramycin	polyketide	*S. ambofaciens*	R,T	2	40	Nagaraja Rao *et al.*, (1988)
avermectin	polyketide	*S. avermitilis*	C		65	Streicher *et al.*, (1988)
cephalosporin C	B-lactmam	*S. clavuligerus*	O,C		20	Leskiw *et al.*, (1988) Jensen *et al.*, (1988)

@see text, *by virtue of functional complementation of *act*II mutants of *S. coelicolor*

Key; C = complementation of blocked mutants
D = direct cloning of the whole antibiotic pathway
O = probing with synthetic oligonucleotides
H = cross hybridization
M = mutational cloning
R = isolation of resistance gene
P = assay for expression of a single gene product
T = transposon mutagenesis

1 = gene cluster known to contain a compete set of genes for the biosynthesis of the antibiotic
2 = gene cluster known to contain an antibiotic resistance gene
3 = gene cluster known to contain a regulatory gene

strategy for the isolation of genes for antibiotic production. The basic approach is outlined using the cloning of genes involved in the biosynthesis of, and resistance to, the macrolide antibiotic erythromycin as an example - this also allows the qualification registered in Table 1.1 to be explained. Genetic mapping of mutants of erythromycin A biosynthesis had shown that the genes involved were likely to be tightly linked (Weber *et al.*, 1985). The *ermE* gene was cloned from *Saccharopolyspora erythraea*, formerly *Streptomyces erythraeus*, and found to confer resistance to macrolide, lincosamide and streptogramin B antibiotics by N6-dimethylation of a single adenine residue in the 23S rRNA (Thompson *et al.*, 1982; Uchiyama and Weisblum, 1985). From a genomic library of *Sac. erythraea* made in the *E. coli*/*Streptomyces* bifunctional cosmid vector pKC462a and contained in *E. coli*, Stanzak *et al.*, (1986) isolated, by DNA-DNA hybridization, clones containing the *ermE* gene. One clone, pKC488, conferring erythromycin resistance on *S. lividans* generally caused production of an antibiotic in the recipient that was indistinguishable from erythromycin A in chromatographic tests. pKC488 was also shown to complement three independent mutants of early and late steps in erythromycin biosynthesis in *Sac. erythraea*, indicating that most if not all the genes involved, had been cloned. However the instability of the construct in *S. lividans* prevented further analysis of the DNA and the chemical composition of the antibiotic was not determined due to the poor titre obtained from the recombinant. The finding therefore remains to be confirmed, but the technique has proved to be of general application, with several recent examples in Table 1.1. The researcher should be aware however of the possibility of resistance genes unlinked to gene clusters for antibiotic biosynthesis or the presence of multiple resistance genes (examples include strains with three resistance genes for the one antibiotic *viz*. tylosin [Seno and Baltz, 1988], oxytetracycline [Ohnuki *et al.*, 1985a; Hunter and Baumberg, 1989], and spiramycin [Nagaraja Roa *et al.*, 1988], one or more of which may be encoded outwith the cluster of genes for biosynthesis).

1.6.3 MUTATIONAL CLONING

Genes for the biosynthesis of the epoxy-cyclopentane

antibiotic methylenomycin A are encoded by the large, low copy number plasmid SCP1 of S. coelicolor. The copy number of SCP1 is elevated 27 times in S. parvalus, and may also be higher in S. lividans. These features were exploited by Chater and Bruton (1983) when the genes for methylenomycin A were cloned. Their work also introduced the strategy of mutational cloning for the isolation and identification of streptomycete genes. Small fragments (2-6kb) of S. parvalus SCP1$^+$ total DNA, were cloned into an attachment site deleted temperate phage oKC400 (a derivative of oC31) and used to transfect S. lividans. These plaques were replica plated onto a lawn of spores from S. lividans SCP1$^+$. Lysogeny of the recipient would only be possible by integration into the DNA by Campbell type recombination at a site of homology between the cloned DNA in the recombinant phage and host DNA. 14% of phage in the primary library and 39% of the recombinant phage population were competent to lysogenise S. lividans SCP1$^+$ but not S. parvalus SCP1$^-$. These recombinants also formed lysogens with S. coelicolor strain NF JI507 which contains a single copy of SCP1 DNA integrated into the genome. S. coelicolor and S. parvalus are only distantly related species, thus most of the lysogens were likely to carry SCP1 DNA. If the cloned piece of DNA is completely internal to a transcription unit, a single cross over event should disrupt the gene function by insertion of the phage vector DNA. 270 S. coelicolor NF ϕKC400 lysogens were screened for mutations in methylenomycin A biosynthesis. 9 recombinant phage, carrying fragments of DNA involved in methylenomycin A biosynthesis (mmy), spanning 7kb of SCP1 DNA were recovered from lysogens with such a phenotype. This DNA was shown to be closely linked to a previously cloned methylenomycin A resistance gene (Bibb et al., 1980) by Southern analysis. ϕKC400::mmy lysogens of S. coelicolor were used to isolate more DNA concerned with methylenomycin A biosynthesis by shotgun cloning into E. coli plasmid vectors and selection of the viomycin resistance carried by the phage DNA. In this way 17kb of SCP1 DNA was cloned and shown to contain genes for the biosynthesis and regulation of, and resistance to, methylenomycin A.

This technique avoids both transformation of the producing strain, and the need for a collection of classically generated blocked mutants. The approach is

considered to be applicable to any system where the strain is sensitive to infection by oC31, although substantially more lysogens will have to be screened when the primary library cannot be biased in favour of common DNA between two distinct species.

1.6.4 SYNTHESIS OF OLIGONUCLEOTIDES FOR PROBING GENE BANKS

A general strategy for isolating genes is to probe a gene bank with a synthetic oligonucleotide which has been constructed to complement the coding DNA, determined from the partial amino acid sequence of a purified protein.

The first example of this strategy used to isolate genes for antibiotic biosynthesis from Streptomyces was reported by Fishman et al. (1987). Purification of macrocin O-methyltransferase (MOMT), an enzyme known to catalyse the final step in tylosin biosynthesis in S. fradiae, allowed the amino acid sequence of the N-terminal of the protein to be determined. From this data 64 oligonucleotides, 44 bases in length, were synthesised covering all possible codons for the amino acids from position 20 to position 33 and including 2 nucleotides of the codon for the 34^{th} amino acid from the amino terminal. Determining the base composition of the coding DNA from the amino acid sequence is aided by the strong codon bias of streptomycete DNA which has a 93% GC content in the third base position (Hopwood et al. 1986, Bibb et al. 1984). The synthetic oligonucleotides constructed were found to consistently hybridize to the same S. fradiae DNA fragment in Southern blot experiments. By hybridization to bacteriophage and cosmid based genomic libraries, ten recombinant phage and four recombinant cosmids were isolated with homology to the probe. This gave 58kb of contiguous S. fradiae DNA which was found to complement nine distinct mutations in tylosin biosynthesis, including tylF - the structural gene for MOMT.

1.6.5 ISOLATION OF INDIVIDUAL GENES BY SPECIFIC ASSAY OF THEIR PRODUCTS

Two examples of the use of this strategy in the isolation of genes involved in antibiotic biosynthesis have been reported. Gil and Hopwood (1983), and Jones and Hopwood (1984), cloned the p-aminobenzoic acid (PABA) synthase gene (pabS), from S. griseus and phenoxazinone synthase gene from

S. antibioticus respectively. The basic technique involved shotgun cloning DNA from the producing strain into a non-producing recipient and by some enzymatic, chemical or other test, identifying clones producing the particular gene product.

In the case of PABA synthase, an enzyme involved in candicidin biosynthesis in S. griseus, selection for sulphonamide resistance or PABA prototrophy was used to isolate the coding gene.

Jones, however, was required to perform a much more laborious biochemical test to isolate the phenoxazinone synthase gene involved in actinomycin biosynthesis in S. antibioticus. Using "sib selection", a technique whereby pools of recombinants of decreasing size were successively assayed until the individual clone was isolated, the number of tests performed was substantially reduced.

1.6.6 CROSS HYBRIDIZATION WITH GENES ENCODING THE BIOSYNTHESIS OF RELATED ANTIBIOTICS

As discussed previously (section 1.4.1) the shared precursors and biosynthetic origins of metabolites within the different structural classes of antibiotic, suggests that the diversity of individual metabolites formed within each class has evolved from a common ancestral pathway. It is therefore reasonable to assume that the conserved function of the enzymes for early steps in the biosynthetic pathway of a particular class is maintained by conservation of the primary amino acid sequence of the polypeptides and will be reflected in the nucleotide sequence of the genes. It follows therefore that the isolation of the first genes involved in Beta-lactam, aminoglycoside or polyketide biosynthesis should facilitate the cloning of their counterparts from other strains by DNA-DNA cross hybridization.

The efficacy of this strategy for the identification and isolation of genes for polyketide biosynthesis has already been demonstrated in a collaborative effort between researchers from the John Innes Inst., the University of Wisconsin, Pfizer Ltd. and Apcel Ltd. (Malpartida et al., 1987). Cross hybridization of DNA involved in the early biosynthetic steps of actinorhodin, tetracenomycin and oxytetracycline identified homologues from each strain (S. coelicolor, S. glaucescens and S. rimosus respectively),

thought to encode the B-ketoacyl-ACP synthase and B-ketoacyl-ACP reductase. Homology to the actIII gene, encoding the B-ketoacyl-ACP reductase in the actinorhodin biosynthetic pathway, was absent from the tetracenomycin gene cluster, but biosynthesis of this polyketide does not require a reductive step. Cross hybridization between actI (encoding the B-ketoacyl-ACP synthase for actinorhodin biosynthesis), and DNA from 15 of 18 strains known to produce polyketides, was demonstrated in Southern blot analysis. Of the three strains in which homology was undetected Sac. erythraea has been shown to have weak homology to actI (Weber et al., 1988). The authors went on to isolate closely linked sequences with homology to actI and actIII from the milbemycin producer viz. S. hygroscopicus ssp. aureolacrimosus and the granaticin producer viz. S. violaceoruber Tu22. The cloned DNA was shown to functionally complement some act mutants of S. coelicolor - actII & III by fragments from S. hygroscopicus, actI, II, III, IV & VII by fragments from S. violaceoruber. Isolation of cross-hybridizing DNA sequences does not necessarily confirm the involvement of the DNA in granaticin or milbemycin production in the respective strains, as signified by a recent publication by Stutzman-Engwall and Hutchinson (1989). They reported the isolation of five different types of cosmid clone from S. peucetius (the producer of duanorubicin), by homology with tcmIa (the B-ketoacyl-ACP synthase for tetracenomycin), actI and actIII. The five classes of clone were not closely linked in the S. peucetius genome and exhibited different patterns and degrees of homology to the three probes. Group II also contained a resistance determinant to duanorubicin. Sequence similarity between the groups was tested by hybridization and no homology was detected under conditions of high stringency - calculated to disrupt any hybrids with less than 79% homology. The four groups (I-IV) with homology to both B-ketoacyl-ACP synthases were able to direct or influence the production of polyketide metabolites in S. peucetius mutants of duanorubicin biosynthesis or S. lividans. However only group IV clones, which directed the synthesis of E-Rhodomycinone, an intermediate in duanorubicin biosynthesis, were able to restore duanorubicin biosynthesis in a S. griseus mutant, blocked at an early step in the pathway. The role of these DNA fragments in duanorubicin biosynthesis in

S. peucetius remains unclear, and although they appear to alter the polyketide production profile in recombinant strains, it seems unlikely that all will be involved in the biosynthesis of duanorubicin in the wild type strain. Thus it is possible to identify clones by cross hybridization, which by implication are homologous to a B-ketoacyl-ACP synthase and can be shown to have a function in polyketide biosynthesis, but are not necessarily involved in the production of the metabolite under investigation in the wild type strain. It was therefore important for Malpartida and co-workers to confirm, by gene disruption, that the clones obtained from S. hygroscopicus and S. violaceoruber were involved in milbemycin and granaticin biosynthesis respectively. Although insertional inactivation of the actIII homologue from S. hygroscopicus has not been acheived, mutants derived from this approach have deletions of DNA surrounding this locus and fail to synthesize milbemycin.

1.6.7 DIRECT CLONING OF THE ENTIRE GENE CLUSTER FOR THE BIOSYNTHESIS OF AN ANTIBIOTIC, AND ESTABLISHING PRODUCTION IN A HETEROLOGOUS HOST

The biosynthesis of cephamycin C is thought to require nine catalytic steps in S. cattleya. Including sequences with regulatory and resistance functions the number of genes required for the complete synthesis of the antibiotic would not be expected to be very large and encoded, possibly, by as little as 20kb of DNA. Assuming clustering of the genes involved Chen et al., (1986), cloned large fragments (20-40kb) of genomic DNA from S. cattleya into the low copy number vector pIJ943 and screened for recombinants in S. lividans with novel production of the Beta-lactam antibiotic. One positive clone (pIF900) among 30 000 recombinants was identified which directed the biosynthesis of a compound with activity against a Beta-lactam-supersensitive culture, Comamonas terrigena M2. pIF900 was shown to contain a 29.3kb insert and carried all the information required to synthesise cephamycin C.

Clearly this approach would be most suited to the isolation of the smaller gene clusters for antibiotic biosynthesis, and so for example, would not be applicable to the isolation of genes involved in macrolide biosynthesis - of the compounds listed in Table 1.1, erythromycin, tylosin,

carbamycin, and spiramycin, are macrolides and their production (with the possible exception of erythromycin) appears to require large amounts of DNA. Furthermore, a cloning recipient which does not have the genetic capacity to synthesise related metabolites would also be helpful, because interference between antibiotic biosynthetic pathways in recombinant strains has already been demonstrated and may complicate interpretation of the results (see section 1.4).

1.7 STRATEGIES ADOPTED FOR THE ISOLATION OF GENES INVOLVED IN TETRONASIN BIOSYNTHESIS IN S. longisporoflavus AND PROJECT AIMS

The strategies described above are not all applicable to the problem in question. The expedience of each approach must be tempered by the characteristics of the biosynthetic pathway, the product itself and the proposed cloning recipient.

Although the chemical structure and biosynthetic precursors of tetronasin have been determined (section 1.4.1) the lack of knowledge of the biochemistry of its biosynthesis, immediately undermined the propriety, if not excluded the identification of individual enzymes in the pathway - essential to strategies 4 and 5, described in section 1.6.

Of the remaining approaches, number 7, requiring the whole biosynthetic pathway and resistance determinant(s) to be expressed in a cloning recipient, appeared to be impractical. The aliphatic, polyether and macrolide antibiotics are structurally closely related (the former are mainly branched-chain, polyoxygenated carboxylic acids and the latter are branched-chain, polyoxygenated carboxylic lactones). This formal resemblance might reflect similarities in the biosynthesis of the two classes and with large stretches of DNA required to direct the synthesis of macrolide structures (section 1.6.7) the possibility of large gene clusters for polyether biosynthesis cannot be overlooked. Furthermore to overcome the technical difficulties of cloning large DNA fragments in a low copy number vector, efficient transformation of the cloning recipient is necessary. Very few strains have been developed for this purpose and the most widely used host (S. lividans), produces the polyketide actinorhodin. Combined with the commonly encountered

instability of large recombinant molecules in Streptomyces, particularly when the construct includes genes for polyketide biosynthesis (Stanzak et al., 1986; Motamedi and Hutchinson, 1987), this approach was perceived to be speculative.

The aim of this project was to identify and to isolate (not necessarily in that order), genes for the biosynthesis of tetronasin in S. longisporoflavus. At the outset of this work, no genes involved in the biosynthesis of any polyether-ionophore antibiotic had been isolated. So without precedent, the remaining four strategies were investigated empirically for a pragmatic solution. Nevertheless, due to the nature of the antibiotic, some special difficulties might be incurred, which one was best to be aware of from the start.

Analysis of DNA closely linked to a resistant determinant, for genes involved in tetronasin biosynthesis obviously requires, first, the isolation of one or more tetronasin resistance gene(s). Naively this appears to be a straightforward route, however the bioactivity of tetronasin is targeted against the cell membrane, and it is difficult to conceptualise a simple solution to the problem of self-immunity in S. longisporoflavus (this point is discussed further in Chapter 4). The strategy was pursued in the hope that the micro-organism had solved the conundrum in a manner suitable for exploitation by the technique.

No special problems in the use of mutational cloning (see Chapter 7.3) and cross hybridization (Chapter 5) for the isolation of genes involved in tetronasin biosynthesis in S. longisporoflavus could be pre-judged without trial. Even if unsuccessful in the primary goal, the attachment-site deleted vectors derived from oC31 might prove to be useful for identifying and confirming the role of DNA isolated in other ways.

As mentioned previously, the complementation of S. longisporoflavus mutants in tetronasin biosynthesis, requires efficient plasmid transformation of the host strain and ideally would benefit from a large collection of well-characterised strains blocked at different steps in the pathway. Clearly this route had to be explored (Chapter 3), and again, even if unfruitful for the isolation of primary clones, blocked mutants and competent protoplasts might be obtained which could be used to help confirm the involvement of genes, isolated by alternative strategies, in tetronasin

biosynthesis.

1.8 AN INTRODUCTION TO REGULATION OF GENE EXPRESSION IN *Streptomyces*

The streptomycetes, as detailed at the beginning of this chapter, undergo a complex cellular differentiation resulting in the formation of aerial mycelia and spores, which differ markedly in both morphology and physiology from the substrate mycelia. The appearance of many gene products has been associated with different stages of the life cycle. Most antibiotics are synthesized late in the life cyle and their appearance regulated by a number of factors (see later in this section). The recent development of promoter probe vectors using bacterial luciferase to report expression both temporally and spatially within the colony (Guijano *et al.*, 1988), and the analysis of mRNA abundance in time course experiments, has identified many genes whose expression, controlled at the level of transcription, is restricted to, or altered by, different stages of the life cycle. The disparity is therefore achieved through global changes in gene expression.

1.8.1 GLOBAL REGULATION

The co-ordinate regulation of many genes is most easily imagined to be under the control of a regulatory network of the type proposed by Horinouchi and Beppu, (1987).

Mutations in genes acting early in the cascade would be expected to have pleiotropic effects and indeed these are observed. The best-characterised examples are mutant in A-factor biosynthesis - a small molecular weight "bacterial hormone". These are easily isolated in *S. griseus* and result in loss of sporulation, streptomycin biosynthesis and resistance. Perhaps an important consequence of the secretion of this metabolite is the synchrony of antibiotic resistance throughout the culture prior to antibiotic production. The molecule was first isolated from cultures of *S. griseus* by Khokhlov *et al.*, (1967). Horinouchi *et al.*, (1984), cloned the 1.2kb DNA fragment (*afs*A), required to restore biosynthesis in A-factor-deficient mutants of *S. bikiniensis* and *S. griseus*. By Southern hybridization the gene was shown to be widespread in streptomycetes although its biological role may differ in other species - mutants of *S. coelicolor*

deficient in A-factor biosynthesis sporulated normally and continued to produce the same range of antibiotics as the parental strain (Horinouchi et al., 1986). Two additional closely-linked loci were identified by genetical analysis of these A-factor-deficient mutants of S. coelicolor, which also functioned in the regulatory network (Hara et al., 1983). Rescue of the afsB mutation in S. lividans was provided by a 2kb DNA fragment cloned from S. coelicolor. The nucleotide sequence of the putative afsB gene indicated that the gene product was significantly homologous to known DNA binding proteins of the lambda CI family (Horinouchi and Beppu, 1987). The afsC gene, closely linked to the putative afsB, was found to stimulate the function of the putative afsB in trans. Based on the available data Horinouchi and Beppu proposed the regulatory network shown in Figure 1.6.

Stein and Cohen, (1989) cloned a DNA fragment from S. lividans which was structurally and functionally equivalent to the DNA designated afsB by Horinouchi and Beppu. However it did not map to the afsB locus of S. coelicolor. Their evidence suggests that the gene when cloned in high copy numbers, can suppress several developmental mutations in S. lividans by pleiotropic regulatory effects. They propose that it should be renamed afsR. The precise function of these genes in single copy in the natural situation still remains to be determined.

1.8.2 INITIATION OF TRANSCRIPTION

How the regulatory functions of the identified genes of the cascade are mediated is unknown, but there is a plethora of evidence which points to the importance of transcription initiation catalysed by different RNA polymerase holoenzymes at promoter elements of marked sequence heterogeneity. The presence of multiple forms of RNA polymerase holoenzyme, differing in the σ factor component which specifies selectivity on the core enzyme for different promoters, was first demonstrated by Westpheling et al., (1985) using developmentally-regulated promoters (veg and ctc) from B. subtilis in "run off" experiments to identify the specificity of purified RNA polymerase from S. coelicolor.

This work has been extended to an investigation of gene expression of the galactose utilisation operon of S. lividans

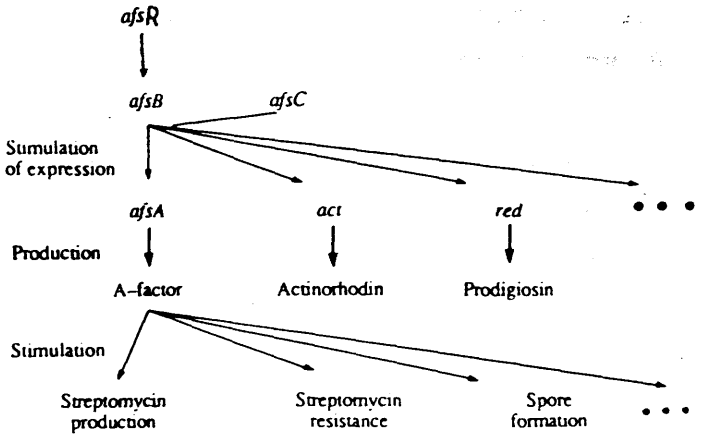


FIGURE 1.6; Proposed Regulatory Cascade Controlling The Expression Of Developmentally Regulated Genes (Modified From Horinouchi And Beppu, 1987)

(Westpheling and Brawner, 1989) which is regulated by two promoters galP1 and galP2, and the agarase gene of S. coelicolor (Buttner et al., 1988) which is under the control of four promoters, dagAP1-4.

For the galactose operon, galP1 is induced to high levels by galactose, repressed by glucose and is transcribed by a RNA polymerase holoenzyme thought to be associated with a 28-kDa σ species (σ^{28}). galP2 is a low level constitutive promoter which is likely to be specified by σ^{35}, the veg-transcribing activity described in 1985.

For the agarase gene, by adding partially purified σ factor to RNA polymerase core enzyme in reconstitution experiments, three RNA polymerase holoenzymes were positively identified which each initiated transcription from a single, different promoter. Thus dagP2 was transcribed by RNA polymerase containing σ^{28}, dagP3 by σ^{52} (thought to be equivalent to the σ factor specific for ctc transcription, estimated by Westpheling and Brawner to be 49kDa in size), and dagP4 by σ^{35}. Transcription of dagP1 was observed in "run off" experiments - however the σ factor responsible was never purified.

Further evidence for the occurrence of many different σ factors in streptomycetes was presented in Tokyo 1988. Takahashi et al., (1988), isolated four non-identical fragments of DNA from S. coelicolor by conserved homology with rpoD from E. coli. The family of genes, hrdA, B, C, and D, likely to be of the σ^{35} type, are very similar to each other at the nucleotide level and to rpoD homologues from B. subtilis and E. coli at the amino acid level. Buttner (cited by M. J. Bibb, 1989), independently isolated hrdB and C by homology to rpoD from Myxococcus. The result of gene disruption experiments showed that hrdC was inessential in S. coelicolor, but the apparent inability to generate viable mutants of hrdB (an attachment-site deleted derivative of ϕC31 carrying the DNA from gyl and hrdB was used to transduce S. coelicolor, but only lysogens with phage integrated at the glycerol operon were recovered), by implication indicated an absolute requirement for a functional hrdB gene product. Westpheling and Brawner, (1989) speculate that the role of closely related families of σ factor may be to independently regulate similar promoters with minor sequence or spacing differences in the individual promoter elements.

The obvious hypothesis from the balance of this data is for global regulation of genes mediated through the presence or absence of σ factors required for transcription. This implies a further stage in the regulatory cascade, and one might imagine the expression of genes encoding σ factors to be regulated by a component of the proposed regulatory network. Perhaps surprisingly, there is little data on the σ specificity of genes involved in antibiotic biosynthesis so the hypothesis remains to be tested. However evidence in the form of a pleiotropic mutation, whiG, of S. coelicolor implicates the importance of a specific σ factor. whiG is one of six distinct mutations which prevent sporulation of the aerial mycelia. It functions early in sporulation, at the initiation of hyphal coiling. The complementing gene was cloned and sequenced, and significant homology to σ^{28} from B. subtilis was noted (Chater et al., 1989). Furthermore when introduced into S. coelicolor in high copy numbers, σ^{whiG} increased the level of antibiotics produced and induced sporulation of the substrate mycelia (Chater et al., 1989). It seems likely therefore, that whiG is tightly regulated to limit expression of the gene to the aerial mycelium.

1.8.3 TRANSLATIONAL CONTROL

bldA mutants of S. coelicolor are deficient in antibiotic production and aerial mycelia formation (although this phenotype can be rescued by growth on mannitol or maltose - Chater and Merrick, 1979). The bldA gene was cloned by Piret and Chater, (1985), and five different bldA mutant alleles were identified as single base pair substitutions localised in 16bp of sequence within a potential tRNA gene (Lawlor et al., 1987). The predicted tRNA specifies a leucyl tRNA with the AAU anticodon (one of the mutant alleles mapped to the second base position of the anticodon). The complementary UUA codon is particularly rare in streptomycete genes (frequency of use is 0.2×10^{-3}). The two cases identified with UUA codons (carB from S. thermotolerans) and hyg from S. hygroscopicus) function poorly in a bldA background - erythromycin and hygromycin resistance is respectively eliminated and greatly reduced. Furthermore genes containing TTA codons can only be expressed efficiently after alteration of the coding sequence to replace the TTA codons with codons for more abundant leucyl-tRNAs (Lawlor et

al., cited in Chater, 1989). This argues, at least in this case, that the bldA gene product plays a translational role. Expression of the bldA gene was determined by dot blot analysis and the tRNA transcript was found to accumulate late in the growth phase. Since UUA codons are absent in many genes for antibiotic biosynthesis, the working hypothesis is for some gene(s) of the regulatory network to contain UUA codon(s), such that the presence or absence of the bldA gene product might influence translation of the transcript from a regulatory gene. However the possibility of a non-translational role of the bldA gene product cannot be ruled out.

1.8.4 GLYCOGEN METABOLISM

The synthesis and degradation of carbon storage compounds correlates well with key stages in the differentiation process. The accumulation of glycogen granules begins immediately prior to aerial mycelium formation (Wildermuth, 1970). Depolymerisation is then observed concommitant with the extension of aerial hyphae, before glycogen is accumulated for a second time prior to sporulation (Hardisson et al., 1986), and finally degraded after septation. This oscillation in the intracellular levels of glycogen prompted Chater, (1989), to propose a model for the osmoregulation of differentiation. The anabolism and catabolism of glycogen would cause fluctuations in the osmotic potential of the hyphae. Increased turgor pressure as glycogen is degraded may be relieved, in the first instance by hyphal extension, and in the second by "rounding off" the spore compartments. Similarly the decrease in turgor pressure which would follow the second phase of accumulation of glyocogen may cause a reduction in hyphal extension and promote the formation of septa. Whether such fluctuations in the abundance of storage compounds are regulatory or merely the consequence of oscillating supply and demand for cell wall precursors is open to speculation. However the model provides an attractive control point for the action of bld genes which can be overridden by nutritional factors.

1.8.5 STRINGENT RESPONSE

The occurence of the stringent response in Streptomyces sp. was demonstrated by Ochi, (1986). Relaxed

mutants were obtained which failed to accumulate ppGpp and as a consequence continued to synthesise rRNA and tRNA in the absence of protein synthesis (which was inhibited by thiopeptin). These rel mutants were pleiotropic and in addition to the stringent response they were impaired in antibiotic biosynthesis and had reduced formation of aerial mycelium. This coupling of guanine phosphate metabolism with antibiotic biosynthesis and sporulation may prove to be linked by bldA (Chater, 1989). With expression of tRNA genes regulated by the level of ppGpp it will be interesting to discover how expression of bldA responds to changes in the guanine nucleotide pools, because a positive regulatory effect, in contrast to negative regulation likely to be found with other tRNA genes, would be the expectation.

1.8.6 CATABOLITE REPRESSION

Differentiation and the synthesis of secondary metabolites are subject to catabolite repression, although often in a species or pathway specific manner. Carbon, nitrogen, phosphate and oxygen levels of the growth medium have all been shown to have regulatory effects on antibiotic biosynthesis and sporulation (Martin and Demain, 1980; Campbell, 1986).

An investigation of the regulation of primary carbon source metabolism in S. coelicolor (Hodgson, 1982) indicated that the active transport systems for arabinose and glycerol and the catabolic enzymes for galactose and fructose metabolism were repressed by glucose. Derepressed mutants unable to metabolise glucose were isolated by growth on glycerol in the presence of 2-deoxyglucose. Physiological studies pointed to a loss of glucose kinase activity. Genetic evidence and reversion experiments suggested that accumulation of glucose-6-phosphate or glucose kinase activity per se was involved in control of gene expression within the regulon. This apparently differs from the regulatory circuit operating in enteric bacteria as no evidence for the involvement of cAMP was found. Unfortunately none of the three antibiotics produced by S. coelicolor (actinorhodin, undecylprodigiosin and methylenomycin) are glucose repressed and thus precluded an investigation of a possible interaction between the regulation of primary carbon metabolism and secondary metobolite production.

In contrast, the biosynthesis of cephalosporin C in S. clavuligerus is depressed by ammonium and the effect of mutants deregulated in primary nitrogen metabolism has been investigated (Bascaran et al., 1989b). A system regulating the expression of four genes involved in primary nitrogen metabolism was identified (Bascaran et al., 1989a). Synthesis of glutamine synthetase (GS), urease, arginase and ornithine aminotransferase were all found to be repressed by growth on ammonium. Several mutants derepressed for all four enzymes were found to contain a temperature sensitive GS, which strongly suggested that the defect lay in glnA - the structural gene for GS. This in turn, implied that intracellular glutamine levels may regulate nitrogen repression and indeed growth on glutamine suppressed the mutant phenotype. This evidence allied to the observation that GS was covalently modified upon ammonium shock implied that GS had an important role in the regulation of primary nitrogen metabolism. With regard to secondary metabolism, all of the mutants retained the ability to synthesize cephalosporins and production remained regulated by ammonium. It appears therefore, that depression of cephalosporin biosynthesis by ammonium in S. clavuligerus is mediated by a different regulatory circuit to the one operating in primary metabolism.

In the single case, where the molecular genetics of catabolite depression of antibiotic biosynthesis has been closely investigated Gil et al., (1985) and Martin et al., (1988) reported that the promoter for the PABA synthase gene (pabS) was sensitive to phosphate levels. Martin et al. suggest that repression is mediated through the binding of a protein to a putative regulatory element in the promoter region thus altering transcription of the gene.

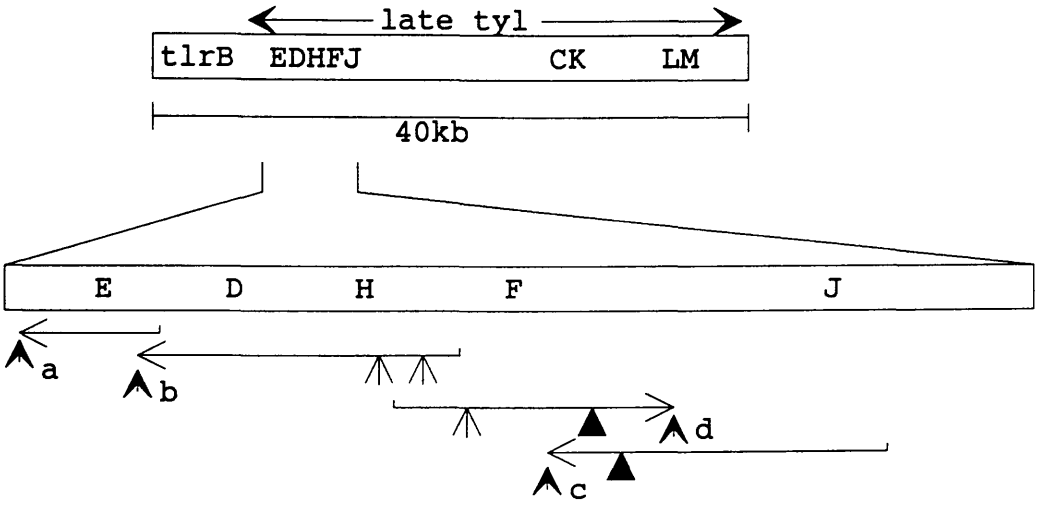
1.8.7 SPECIFIC CONTROL OF GENE CLUSTERS FOR ANTIBIOTIC BIOSYNTHESIS AND RESISTANCE

The absence of gene clustering for other "common pathway" genes in streptomycetes, including those for anabolic pathways of primary metabolism and for morphological differentiation argues against a simple advantage in intra-pathway regulation to explain the clustering of genes for antibiotic biosynthesis and resistance. Nevertheless, this characteristic may be important for the co-ordinate

regulation of gene expression and/or to facilitate the assembly of the antibiotic synthase complexes (an alternative view of gene clustering is presented in Chapter 1.2 which should not be ruled out).

The transcriptional maps of the gene clusters for methylenomycin A, defined by gene disruption (Chater and Bruton, 1985), and actinorhodin, defined by gene disruption and S1 mapping (Malpartida and Hopwood, 1986) have been published. These are the only clusters in which all the genes have been cloned and a study of the gross transcriptional organisation has been made. However the evaluation of a transcriptional map of the act cluster by gene disruption is not consistent with the S1 nuclease mapping results, and the DNA sequence of mmr indicates that it is transcribed in the opposite direction to the surrounding mmy genes, in contrast to the results of gene disruption. Despite the inconsistency of results it seems clear that the gene clusters are transcribed in a mixture of large and small units, and for the act gene cluster the larger transcripts correspond roughly to the subclustering of genes for early and late steps in the pathway. This feature has been reported elsewhere - the DNA for tylosin (Seno and Baltz, 1988) and tetracenomycin (Motamedi and Hutchinson, 1987) biosynthesis exhibits subclustering - which prompted Motamedi and Hutchinson to propose that subclustering may allow temporal regulation of genes for sequential steps in the pathway.

Divergent and convergent transcription is another common feature of antibiotic gene clusters. Data presented by Hershberger at the Harden conference in 1989, indicated a complex transcriptional and translational arrangement of genes involved in tylosin biosynthesis (tyl) from S. fradiae (Figure 1.7). Although the 3' end of tylH,D overlaps with the 5' end of tylE, transcription of tylH,D terminates at the nucleotide prior to the translational start codon of tylE. Similarly the 3' ends of the tylF and tylJ transcripts overlap but in this case both terminate within the ORF of the convergent transcript. However translational stop codons in both transcripts may prevent an overlap of the translational frames. Where the 5' ends of two transcripts overlap, RNA polymerase must continue to transcribe each gene through the first transcriptional terminator reached, in order to generate a full length transcript of both converging genes. A



Key; → = transcription unit and direction of transcription

▲ = transcriptional terminator

⋀ = potential translational start

▼ = potential translational stop codon

FIGURE 1.7; Transcriptional And Translational Pattern Of Part Of The Tylosin Biosynthetic Gene Cluster

novel role for transcriptional and translational coupling in transcriptional termination was postulated. RNA polymerase transcribing tylF must "read through" transcriptional terminator (c) to synthesise the full length tylF transcript, and RNA polymerase transcribing tylJ must do likewise with terminator (d). The transcripts differ biologically at the two terminators, because each transcript is being translated at the first (c for tylF, d for tylJ) but not the second transcriptional terminator (d for tylF, c for tylJ) and this distinction may be important for termination at the correct signal. A similar hypothesis could also be proposed for transcription of tylE through terminator (b).

However, alternative explanations, including unidirectional terminators (not applicable for terminator b) and anti-termination systems, cannot be discounted. Transcriptional termination signals also feature in regulation of part of the streptomycin gene cluster (Distler et al., 1988). In this case the authors favour an anti-termination system, mediated by the strR gene product (regulatory gene), for control of gene expression (see later in this section).

The 5' end of the transcript from the tylH,D region overlaps the 5' untranslated region of the tylF gene. The tylH,D transcript contains two putative translational starts, one outwith and one within the overlapping region. Transcription of this region is further complicated by seven putative transcriptional start sites spanning eight nucleotides in the tylF 5' region. tylH,D may also have a tandem promoter. The biological significance of these observations for tylosin biosynthesis is unknown, however multiple transcriptional start sites for streptomycete genes are common (see earlier in this section) and have been investigated for some antibiotic resistance genes located within gene clusters for antibiotic biosynthesis.

The ermE gene of S. fradiae has two promoters ermEp1 and p2 which overlap with three promoters (orfp1-3) for divergent transcripts of undetermined function. Analysis of deletions causing a decrease in the activity of ermEp1 appeared to have an inverse effect on orfp3 (Bibb and Jansen, 1987).

In contrast, a 3bp deletion in the -35 region and a point mutation in the -10 region of aphp1 (one of the twin

promoters of the neomycin phosphotransferase gene of S. fradiae), indicated a direct relationship in the activity of this promoter and two (p1A and p2A respectively for the two mutations) of five divergent promoters (Bibb and Jansen, 1987).

Interestingly this may represent a general form of control, with reports of divergent transcription from the promoter regions of resistance genes for streptomycin (Distler et al., 1987), methylenomycin A (Neal and Chater, 1987), and oxytetracycline (Doyle, et al., 1988).

Pathway-specific regulatory genes are another widespread feature of the control of gene expression in gene clusters for antibiotic biosynthesis. Table 1.1 contains seven examples of clusters known or thought to contain regulatory genes. The first example of such a pathway specific element was discovered by analysis of mutants deficient in actinorhodin biosynthesis in S. coelicolor (Rudd and Hopwood, 1979). Class II mutants were unable to co-synthesise actinorhodin with any other mutant so were suspected to be either polar mutations or deficient in a positive regulatory gene. Recombinants containing plasmid borne copies of actII overproduced actinorhodin (D. A. Hopwood pers. comm.) supporting the latter hypothesis. Similarly Guthrie, (1989) was able to achieve increased production of undecylprodigiosin (red) by introducing a fragment of the red gene cluster in high copy number into S. coelicolor. Interestingly this fragment which contains redD (the proposed positive-regulatory gene) was able to activate the rest of the red gene cluster in a bldA mutant, implying that the structural genes for the pathway do not contain a TTA codon (see earlier in this section).

In two cases listed in Table 1.1 sequences adjacent to genes involved in the biosynthesis of milbemycin and granaticin were thought to represent positive regulatory elements by virtue of functional complementation of actII mutants of S. coelicolor (Malpartida et al., 1987).

Anzai et al., (1987) were also able to demonstrate in S. hygroscopicus the positive regulation of the bialaphos gene cluster by re-introduction of a 1kb DNA fragment (brpA). brpA has been shown to be a trans-acting transcriptional activator of several genes involved in the biosynthesis of bialaphos.

The existence of negatively-acting regulatory elements has also been postulated. Supporting evidence comes from the disruption of a region of the methylenomycin biosynthetic gene cluster - by insertional inactivation (Chater and Bruton, 1985) or deletion (Hopwood *et al.*, 1986) - which caused an increase in the production of the antibiotic.

The final example concerns streptomycin biosynthesis in *S. griseus* in which evidence for both positive and negative regulation of gene expression was found. The sequence of the regulatory *strR* gene, predicted a product with significant homology to the lambda Q protein to allow an antitermination function to be proposed. Northern analysis of transcripts from *strR* and adjacent genes (*aphD* and *strB1*) has indicated putative sites for the action of this positive regulator of transcription (Distler *et al.*, 1988). Sequence analysis of the region between *aphD* and *strB1* also revealed an open reading frame encoding a 40kD protein which was subsequently purified and which has been implacated in negative regulation of the aminoglycoside-phosphotransferase gene *aphD*.

The regulation of gene expression in stretomycetes and in particular of genes involved in antibiotic metabolism is perhaps more complex than first assumed. However, the recent rate of accumulation of data on the subject bodes well for a clearer understanding of the interactions of the various levels of control in the near future. It is hoped that the isolation of genes involved in tetronasin production will augment our understanding of this area of research by providing examples of developmentally regulated sequences. These could be used as probes to monitor expression levels in test cases where regulation is thought to be perturbed or equally the sequences can be analysed for responsive elements in more detailed studies of the molecular genetics of gene expression.

CHAPTER 2
MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

All strains used are listed below:

Name	Genotype	Reference/Source
E. coli strains		
JM109	*sup*E44,*thi*, (*lacpro* A, B)/F' *tra*D36,*pro*A,B,*lac*Iq,ZΔM15, *rec*A1,*end*A1,*gyr*A96,*rel*A1, *hsd*R17	J. Messing cited in Hanahan, (1983)
DS941	*rec*F143,*pro*A7,*str*31,*thr*1 *leu*6,*tsx*33,*mtl*2,*his*4,*arg*E3 *lac*Y^+,*lac*Z M15,*lac*I,*gal*K, *ara*14,*sup*E44,*xyl*5	D. J. Sherratt (pers. comm.)
1400	*sup*E,*sup*F,*hsd*S^-,*rec*A56 *met*$^-$,*thi*$^-$, L512	Cami and Kourilsky, (1985)
NM621	F^-,*thi*-1,*thr*-1,*leu*B6,*lac*Y1, *ton*A21,*sup*E44,*mcr*A,*mcr*B, *hsd*R,*rec*D1009,*pho*R::Tn10	Whittaker *et al.*, (1986)
LE392	F^-,*hsd*R514(r_k^-,m_k^-),*sup*E44, *sup*F58,*lac*Y1 or (*lac*IZY)6, *gal*K2,*gal*T22,*met*B1,*trp*R55	L. Enquist, sited Maniatis *et al.*, (1982)
P2392	P2 lysogen of LE392	
S.lividans strains		
TK24	*str*6	Hopwood *et al.*, (1983)
TK54	*his*2, *leu*2, *spc*1	Hopwood *et al.*, (1985)
TK64	*pro*2, *str*6	Hopwood *et al.*, (1985)
S. longisporoflavus strains		
4584	wild type	I.C.I.
83E6	high level tetronasin producer	I.C.I.
S. rimosus strain		
G7	wild type	Pfizer Ltd.
S. coelicolor strain		
G94	wild type	I. S. Hunter (p.c.)
S. albus strain		
G153	r^-m^+ for *Sal*GI	I. S. Hunter (p.c.)

2.2 PLASMIDS AND BACTERIOPHAGES

The plasmids and bacteriophages used in this study, including those whose construction are described in this Thesis, are listed in Tables 2.1 to 2.4.

TABLE 2.1; High Copy Number Plasmids Used Or Constructed In This Study For Propagation In *E. coli*

Plasmid	Description	Source/Reference
pTZ18^{R}	cloning vector derived from colE1 and M13 offering chromogenic identification of recombinants	Pharmacia
pLorist6	cloning vector derived from phage lambda	Gibson *et al.*, (1987)
pIJ2330	pBR329 containing 8kb of the *act* cluster from *S. coelicolor*	F. Malpartida (pers. comm.)
pMonI	pBR325 containing a 4.3kb insert carrying the putative monensin synthase from *S. cinnamonensis*	J. A. Robinson (pers. comm.)
pPZ508	pUC8 containing part of the *otc* synthase from *S. rimosus*	Pfizer Ltd.
pPZ510	pUC8 containing part of the *otc* synthase from *S. rimosus*	Pfizer Ltd.
ptetR21	pTZ18^{R} containing tetR1	Chapter 4
ptetR51	pTZ18^{R} containing tetR5	Chapter 4
pLoractI	pLorist6 containing a 2.1kb BamHI *act*I fragment subcloned from pIJ2330	Chapter 5
pLormonI	pLorist6 containing a 4.3kb BamHI *mon*I fragment subcloned from pMonI	Chapter 5
pAC36	pTZ18^{R} containing AC36: a 5kb BamHI insert from *S. longisporoflavus* with homology to *act*I	Chapter 5
pMB74	pTZ18^{R} containing MB74: a 7kb BamHI insert from *S. longisporoflavus* with homology to *mon*I	Chapter 5

TABLE 2.2; Bacteriophage Of *E. coli* Used And Constructed During This Work

Bacteriophage	Description	Source/Reference
lambda EMBL3	Spi^+, gene replacement cloning vector derived bacteriophage lambda	Frischhauf *et al.*, (1983)
phage 4	EMBL3 arms containing a 15.6kb insert carrying part of AC36 from *S. longisporoflavus*	Chapter 6
phage 5B	EMBL3 arms containing a 19.5kb insert carrying all or part tetR1 from *S. lonigsporoflavus*	Chapter 6
phage 13A	EMBL3 arms containing a 19.4kb insert carrying part of MB74 from *S. longisporoflavus*	Chapter 6
phage 13B	EMBL3 arms containing a 19.4kb insert carrying part of tetR1 from *S. lonigsporoflavus*	Chapter 6
phage 20	EMBL3 arms containing a 16.1kb insert carrying part of MB74 from *S. longisporoflavus*	Chapter 6
phage 22	EMBL3 arms containing an 11.8kb insert carrying all or part of tetR5 from *S. longispororlfavus*	Chapter 6
phage 24	EMBL3 arms containing an 18.0kb insert carrying all or part of MB74 from *S. longisporoflavus*	Chapter 6
phage 31A	EMBL3 arms containing an insert carrying part of tetR1 from *S. lonigsporoflavus*	Chapter 6
phage 31B	EMBL3 arms containing a 15.9kb insert carrying all or part of tetR1 from *S. lonigsporoflavus*	Chapter 6
phage 31C	EMBL3 arms containing a 14.0kb insert from *S. longisporoflavus*	Chapter 6
phage 55	EMBL3 arms containing a 14.1kb insert spanning MB74 from *S. longisporoflavus*	Chapter 6
phage 56	EMBL3 arms containing a 20.8kb insert from *S. longisporoflavus*	Chapter 6
phage 57	EMBL3 arms containing a 19.3kb insert carrying all or part of AC36 from *S. longisporoflavus*	Chapter 6

TABLE 2.3; Actinophage Which Were Used During This Work

Actinophage	Description	Source/Reference
øC31	a temperate actinophage which infects around two thirds of streptomycetes	Lomovskaya *et al.*, (1980)
øKC515	an *att* site deleted, c^+ cloning vector derived from øC31	Rodicio *et al.*, (1985)
R4	a temperate actinophage isolated by infection of *S. albus*	Chater and Carter, (1979)

TABLE 2.4; High Copy Number Plasmids Used Or Constructed During This Work For Propagation In *Streptomyces*

Plasmid	Description	Source/Reference
pIJ702	a 5.8kb vector which carries the *mel* fragment for chromogenic identification of recombinants	Katz *et al.*, (1983)
pIJ680	a 6.0kb vector which carries the *aph*I gene encoding neomycin resistance	Hopwood *et al.*, (1985a)
pIJ385	a 6.0kb vector which carries the *aph*I gene encoding neomycin resistance	Hopwood *et al.*, (1985a)
pIJ699	a 9.6kb positive-selection cloning vector	Kieser and Melton, (1988)
paa/-	pIJ702 containing DNA fragments from *SS. longisporoflavus* which interfere with endogenous antibiotic production in *S. lividans*	Chapter 4
pPro/-	pIJ699 containing DNA from *S. longisporoflavus* which complements the *pro* mutation in *S. lividans* TK64	Chapter 4
ptetR1	pIJ699 containing the tetronasin resistance gene tetR1	Chapter 4
ptetR5	pIJ699 containing the tetronasin resistance gene tetR5	Chapter 4
ptetR11	pIJ385 containing the tetronasin resistance gene tetR1	Chapter 4
ptetR51	pIJ385 containing the tetronasin resistance gene tetR5	Chapter 4
pKJL1	pIJ702 containing AC36	Chapter 5
pKJL2	pIJ702 containing MB74	Chapter 5
pXE_13	bifunctional *E. coli*/*Streptomyces* vector derived from pUC8 and SCP2 (low copy-number replicon in *Streptomyces*) which contains a promoter-less *xyl*E gene	T. Clayton (pers. comm.)

All vectors used for propagation in *Streptomyces* carried the *tsr* gene from *S. azureus* and their presence was maintained by selection for thiostrepton resistance. Unless otherwise stated, all were derivatives of the natural pIJ101 replicon and as such were high copy-number vectors.

2.3 CHEMICALS

Chemicals	Source
General Chemicals	BDH, Hopkins and Williams, Koch-light Laboratories, May and Baker, Formachem.
Ultrapure Chemicals (Gold Label)	Aldrich Chemical Co. Ltd.
Media	Difco, Oxoid.
General Biochemicals	Sigma, Pharmacia, BRL.
Agarose	BRL
Radiochemicals	NEN
Antibiotics	Sigma, Squibb and Sons

2.4 CULTURE MEDIA

2.4.1 FOR PROPAGATION OF E. coli

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, made up to 1 litre in distilled water and adjusted to pH 7.0 with NaOH.

L-Agar: As L-broth without glucose and the addition of 15g/l agar.

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g $(NH_4)_2SO_4$, 0.25g trisodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1 litre with distilled water.

Supplements: When required, supplements were added to the above minimal medium at the following concentrations:
glucose 2mg/ml thiamine vitamin B1 20ug/ml
amino acids 40ug/ml

SOB: 20g bacto tryptone, 5g yeast extract, 0.6g NaCl, 0.2g KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, pH 7.0. $MgCl_2$ was prepared separately and filtered sterilised.

SOC: As SOB but with 20mM glucose.

2.4.2 FOR PROPAGATION OF BACTERIOPHAGE LAMBA IN E. coli

L-Agar: As L-Agar above with the addition of 1g/l maltose

Top agar: 1g bactotryptone, 0.5g bacto-yeast extract, 0.5g NaCl, 0.25g $MgSO_4$, 1g bacto-agar - adjust to pH7 and made up to 100ml.

Top agarose: As top agar except 1g/l agarose was substituted for bacto-agar.

2.4.3 FOR PROPAGATION OF Streptomyces

Tryptone Soya Broth (TSB): 30g Oxiod tryptone soya broth powder (CM129) per litre.

Yeast extract-malt extract (YEME): 3g Difco yeast extract, 5g Difco bacto peptone, 3g Oxoid malt extract, 10g glucose, 340g sucrose made up to 1 litre in distilled water. After autoclaving 1/20 volume of 100mM $MgCl_2$ was added. For the preparation of protoplasts 1/20 volume of 10% (w/v) glycine was also added.

Pre-germination Medium: (double strength) 10g Difco yeast extract, 10g Difco casaminoacids, 0.01M $CaCl_2$ (autoclaved separately), made up to 1 litre with distilled water. 1x pregermination medium was made by the mixing of equal parts of the above and TES buffer (0.05M, pH8)

R2 Medium: R2A- 44g agar, 0.5g K_2SO_4, 20.2g $MgCl_2.6H_20$, 5.9g $CaCl_2.2H_2O$, 20g glucose, 6g proline, 0.2g casamino acids, 4ml trace elements solution (Hopwood et al., 1985), made up to 1 litre in distilled water.

R2B- 11.5g MOPS, 10g yeast extract, 203g sucrose, adjusted to pH 7.4 with NaOH, made up to 1 litre in distilled water.

Equal volumes of R2A (melted and cooled to 55°C) and R2B plus 1ml of 1% (w/v) KH_2PO_4 were combined prior to use.

Soft R2: Prepared by mixing equal volumes of R2 and medium P.

R9 Medium: R9A- 44g agar, 0.5g K_2SO_4, 8.2g $MgCl_2.6H_20$, 4.7g $CaCl_2.2H_2O$, 4g $NaNO_3$, 1g KCl, 0.4g $MgSO_4$, 20g glucose, 0.8g casamino acids, 4ml trace elements solution (Hopwood et al., 1985), 2ml $FeSO_4$ (1% [w/v] solution), made up to 1 litre in distilled water.

R9B- 11.5g MOPS, 10g yeast extract, 410g sucrose, adjusted to pH7.4 with NaOH, made up to 1 litre in distilled water.

Equal volumes of R9A (melted and cooled to 55°C) and R9B plus 1ml of 1% (w/v) KH_2PO_4 were combined prior to use.

Emersons agar: Emersons agar was purchased as a powder from Difco and was used as directed by the manufacturers.

Difco Nutrient Agar (DNA): DNA was purchased as a powder from Difco and was used as directed by the manufacturers.

Difco Nutrient Broth (DNB): DNB was purchased as a powder from Difco and was used as directed by the manufacturers.

Soft Nutrient Agar (SNA): 8g DNB powder, 3g agar, made up to 1 litre in distilled water.

Hopwood's Minimal Medium (HMM): 0.5g L-asparagine, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 10g glucose, 10g

agar, made up to 1 litre with distilled water.

Ludox Minimal Medium (LMM): 2.5ml of sterile 10xHMM (with agar omitted), 13.2ml Ludox (Dupont, dialysed against 0.05M K_2HPO_4 at 30°C over-night followed by dialysis against H_2O over-night with one water change), 3.75ml sterile 0.1M K_2HPO_4, 5.5ml distilled H_2O, just prior to pouring 10ml 2M NaCl was added to 25ml of Ludox medium and the resulting mixture was allowed to solidify for 24hrs.

Supplements: when required, amino acids were added to a concentration of 40ug/ml.

2.5 STERILIZATION

All growth media were sterilised by autoclaving at 120°C for 15 mins; supplements and buffer solutions at 108°C and $CaCl_2$ at 114°C for 10 mins. Amino acids and other heat sensitive solutions were sterilized by filtration through 0.22um filters.

2.6 BUFFERS AND SOLUTIONS

2.6.1 FOR NEUTRAL GEL-ELECTROPHORESIS

10X TBE Buffer pH 8.3: 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1 litre in distilled water, pH to 8.3.

10X TAE Buffer pH8.2: 48.4g Tris, 16.4g Na acetate, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with glacial acetic acid.

Agarose gel loading buffer: 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) SDS, 50mM EDTA.

RNA gel loading buffer: 50% (w/v) glycerol, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol, 1mM EDTA.

2.6.2 FOR ALKALINE AGAROSE GEL-ELECTROPHORESIS

Agarose gel solution: 50mM NaCl, 1mM EDTA.

Electrophoresis buffer: 30mM NaOH, 1mM EDTA

Alkaline loading buffer: 50mM NaCl, 1mM EDTA, 2.5% Ficoll, 0.025% bromocresol

2.6.3 FOR DNA PREPARATION AND MANIPULATION

10x Core buffer: 500mM Tris-HCl (pH8.0), 100mM $MgCl_2$, 500mM NaCl. Stored at 4°C

10x KGB buffer: 1M K-Glutamate, 250mM Tris-acetate pH7.6, 10mM Mg-Acetate, 500ug/ml BSA, 5mM 2-mercaptoethanol.

10x CIP buffer: 0.2M Tris.Cl, pH8, 10mM $MgCl_2$, 10mM $ZnCl_2$, 0.5mg/ml BSA.

5X BRL Ligation Buffer: 250mM Tris-HCl (pH 7.6), 50mM MgCl, 5nM ATP 5mM DTT 25% (w/v) PEG. Stored at -20°C.

TE Buffer: 10mM Tris-HCl, 1mM EDTA, pH 8.0.

TES Buffer: 20mM Tris-HCl (pH 7.5), 70mM $MgCl_2$, 500mM NaCl. Stored at -20°C.

Phenol: All phenol used in the purification of DNA or RNA contained 0.1% (w/v) 8-hydroxyquinoline and was buffered against 0.5M Tris-HCl (pH 8.0).

Phenol/Chloroform: 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol.

Birnboim Doly I (BDI): 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA. Lysozyme was added to 1mg/ml immediately before use, if necessary.

Birnboim Doly II (BDII): 0.2M NaOH, 1% (w/v) SDS stored in a plastic container.

Birnboim Doly III (BDIII): 5M KOAc (pH 4.8); To prepare this equal volumes of 3M CH_3COOK and 2M CH_3COOH were mixed. The pH was checked at 4.8.

STET Buffer: 8% (w/v) sucrose, 5% (w/v) Triton X-100, 50mM EDTA, 50mM Tris-HCl (pH 8.0).

5x SCG Buffer: 10% (w/v) ficoll, 5% (w/v) SDS, 0.25% (w/v) orange C, 0.05% (w/v) bromophenol blue. Diluted 1 in 5 in TBE for 1x using strength with 1mg ml^{-1} RNase added.

2.6.4 FOR RNA PREPARATION AND MANIPULATION

All glass and plastic-ware were submerged overnight in dH_2O containing DEPC prior to autoclaving and all solutions were prepared in DEPC-treated dH_2O. DEPC was added to dH_2O to 0.1% (v/v), the solution was shaken vigorously and left overnight at room temperature before autoclaving at 120°C for 15min.

Kirby mixture: 1g tri-isopropylnapthalene sulphonate, 6g 4-amino salycilate (Na salt), 50mM Tris-HCl (pH 8.3), 6ml phenol, made up to 100ml in distilled water.

10X MOPS: 0.2M Morpholinopropanesulphonic acid, 0.05M sodium acetate, 0.01M EDTA.

10X DNase RQ buffer: 400mM Tris-HCl, pH 7.9, 100mM NaCl, 60mM $MgCl_2$.

Caesium Chloride cushion: 5.7M CsCl, 10mM EDTA.
S1 Nuclease Mapping Solutions;
hybridization buffer: 40mM Pipes adjusted to pH6.4 with NaOH, 400mM NaCl, 1mM EDTA, 80% (v/v) formamide (de-ionized with mixed-bed resin). Stored at -70°C.
(5X) S1 nuclease digestion buffer: 1.4M NaCl, 150mM $NaCH_3COO$, pH4.4, 22.5mM $Zn(CH_3COO)_2$. Stored at -70°C.
S1 nuclease stop buffer: 2.5M NH_4CH_3COO, 50mM EDTA. Stored at 4°C.

2.6.5 FOR SOUTHERN TRANSFER AND PLAQUE/COLONY LIFTS
Denaturing solution: 1.5M NaCl, 0.5M NaOH.
Southern transfer solution: 1.5M NaCl, 0.25M NaOH.
Neutralising solution: 3M Na-Acetate ph7, 1.5M NaCl.
100X Denhardt's solution: 10g Ficoll, 10g polyvinylpyrolidone, 10g bovine serum albumin. Made up to 500ml with dH_2O.
20X SSC: 3M NaCl, 0.3M Na_3 Citrate.
Dextran sulphate stock solution: 50% (w/v) dextran sulphate.

2.6.6 FOR TRANSFORMATION OF E. coli
FSB: 10mM KOAc, 100mM KCl, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $HACOCl_3$, 10% redistilled glycerol, pH to 6.4 with HCl, stored at 4°C.

2.6.7 FOR TRANSFORMATION OF Streptomyces
Transformation mix (T-mix): 2.5% (w/v) sucrose, 100mM $CaCl_2$, 2.5mM K_2SO_4, 1 ml trace elements, 50mM maleic acid, adjust to pH 8.0 with 1M Tris. Add 25% (w/v) PEG 1000 before use.
Medium P: 5.73g TES, 103g sucrose, 2.93g $MgCl_2.7H_2O$, 0.5g K_2SO_4, 3.68g $CaCl_2.2H_2O$, 2ml trace element solution. Adjust to pH 7.4 with NaOH and make up to 1 litre in distilled water.
Lysozyme solution: 10% (w/v) sucrose, 25mM TES buffer (pH 7.2), 2.5mM K_2SO_4, 2ml trace elements, 2.5mM $MgCl_2$, 2.5mM $CaCl_2$. 0.3mg/ml lysozyme and 0.5ml of 1% (w/v) KH_2PO_4/100ml solution were added immediately before use.
PEG Solution: 20% (w/v) PEG 8000. 2.5M NaCl.

2.6.8 FOR BACTERIOPHAGE MANIPULATION
SM Buffer: 5.8g NaCl, 2g $MgSO_4.7H_2O$, 5ml 1M Tris HCl pH7.5, 5ml 2% gelatin dissolved in 800ml water with volume adjusted

to 1 litre and sterilized.

Divalent cation concentrations: 10mM $MgSO_4$, with 4mM (low salt) or 24mM (high salt) $Ca(NO_3)_2$.

2.7 ANTIBIOTICS AND INDICATORS

The antibiotic concentrations used throughout for both liquid and plate selection were as follows:

name	selective concentration	stock solution	storage temperature
Ampicillin	50ug/ml	20mg/ml (water)	-20°C
Neomycin	10ug/ml	10mg/ml (water)	4°C
Thiostrepton	25ug/ml	10mg/ml (DMSO)	4°C
Viomycin	30ug/ml	30mg/ml (water)	4°C

Stock solutions were added to molten agar, cooled to 55°C.

X-gal (5-bromo-4-chloro-3-indolyl-B-galactosidase) was used in conjunction with the host strains JM109 and DS941 and the $pTZ18^R$ vector, providing a screen for plasmids with inserts in the polylinker region. Recombinants containing inserts were generally white while those lacking inserts were blue. X-gal was stored at a concentration of 20mg/ml in DMF at -20°C and added to L-agar plates to a final concentration of 20ug/ml.

2.8 GROWTH AND MAINTENANCE OF STRAINS

2.8.1 E. coli

Liquid cultures for transformation and DNA preparations were grown routinely in L-broth at 37°C with orbital shaking at 250rpm. L-agar or minimal media was used as a solid substrates. Supplements and antibiotics were added as required. Plates contained 25mls of agar solution and were incubated overnight. All dilutions were carried out in L-broth. Strains were stored in 20% (v/v) glycerol and 1% (w/v) peptone at -70°C. Inocula from these stocks were streaked out on L-agar plates and incubated overnight from which single colonies were re-streaked on selective plates.

2.8.2 Streptomyces

2.8.2.1 Making a Streptomyces spore suspension:

A concentrated spore suspension was required for inoculating liquid cultures of Streptomyces spp.. For species used during this work the protocol described by Hopwood et al., (1985) was followed with minor modifications.

The solid growth medium which resulted in good sporulation was species-dependent. For S. longisporflavus and S. lividans a boiling tube containing a slant of R2 agar (produced by pouring about 15ml of molten agar into the tube and allowing it to solidify with the tube placed 5° to the horizontal) was inoculated with 150ul of a spore or mycelial fragment suspension and incubated at 30°C. After several days incubation, the culture was covered in a dark grey mass of spores. The slant was frozen at -20°C; spores could then be harvested immediately or stored indefinitely.

To harvest the spores, the frozen surface of the slant was rubbed with a 5ml pipette containing 5ml of sterile water. When the surface of the slant was scraped clean of spores the 5ml of water was used to wash the slant and resuspend the spores. The spore suspension thus produced was dark grey in colour and contained little agar or mycelial fragments. Any of the latter were removed by passage through a cotton wool filter as described in Hopwood et al., (1985). The filtered spore suspension was aliquoted and frozen at -20°C. The aliquots were thawed and vortexed prior to use. The titre of the spore suspension was determined after storage at -20°C overnight and was found to be constant over the short term (3 months).

Spore suspensions of S. albus and S. rimosus were prepared from cultures grown on TSB agar.

2.8.2.2 Pre-germination of Streptomyces spores:

The method described by Hopwood et al., 1985 was used to pre-germinate S. longisporoflavus spores for infection by ϕKC515. Around 10^8 spores, suspended in 5ml of TES buffer, were heat shocked for 10min at 50°C, and cooled under a cold water tap. An equal volume of double strength pre-germination medium was added and the suspension was incubated for 3hrs at 30°C with orbital shaking at 250rpm. The pre-germinated spores were recovered by centrifugation (12000g for 10min) and resuspended in 2ml of sterile water. For infection by actinophage, 100ul of pre-germinated spores were added to

0.8ml SNA (melted and cooled to 45°C) and poured onto DNA plates on which the phage suspension has been spread.

2.8.2.3 Growth of *Streptomyces* mycelium:

The methods described for the growth of mycelium by Hopwood *et al.*, (1985) were used with minor modification. Dispersed growth of *S. longisporoflavus* was achieved in YEME liquid media and the addition of glycine (to 5mM) and $MgCl_2$ (to 0.5% [w/v]) to the culture broth was used to grow *S. longisporoflavus* mycelium for the preparation of protoplasts. *S. albus* was grown in TSB (glycine was omitted from the culture broth) and *S. lividans* in YEME. For the formation of protoplasts all streptomycetes were incubated at 30°C unless otherwise stated. Routinely 100ul of a dense spore suspension was used to inoculate 100ml of broth. The volume of the culture grown depended on what the mycelium was to be used for. The formation of protoplasts required 25ml while for plasmid isolation 200ml or multiples of this were generally used. The flask volume was at least five times the volume of the broth to allow good aeration when shaken at about 250 rpm.

2.8.3 PRESERVATION OF BACTERIOPHAGE

Bacteriophage were stored by filter sterilising (0.22um filter) high titre phage preparations. An air tight container was used to stop evaporation and the phage suspensions were kept at 4°C.

2.9 *E. coli in vivo* TECHNIQUES

2.9.1 TRANSFORMATION WITH PLASMID DNA

Plasmids were introduced into *E. coli* by genetic transformation. Two methods were employed;

2.9.1.1 $CaCl_2$ method:

For routine introduction of plasmid DNA into *E. coli*.

Preparation of competent cells,

An overnight culture of the recipient was diluted 1 in 100 into 20ml L-broth and grown to a density of approximately 10^8 cells/ml (until $O.D._{600}$ = 0.5, about 90 min - 2 hours). The cells were harvested (12000g, 5 min, 4°C) and resuspended in 10ml of cold 50mM $CaCl_2$. The cells were pelleted again, resuspended in 1ml of cold 50mM $CaCl_2$ and kept on ice for at least 15 min before use. At this stage

the competent cells could be divided into 200ul aliquots and stored at -70°C in 20% glycerol for several months without loss of transformation efficiency.

Transformation with plasmid DNA,

200ul aliquots of the competent cells were added to the plasmid DNA, mixed gently and left on ice for 30min. The cells were heat shocked (2 min, 42°C) before an equal volume of L-broth was added. The culture was incubated at 37°C for 1 hour to allow expression of the plasmid resistance genes. The cells were then spread on L-agar containing the apropriate antibiotic.

2.9.1.2 Hanahan, (1983) method:

Used for high efficiency transformation of E. coli. For this proceedure to be efficacious it was necessary that all solutions were freshly prepared from ultrapure Gold Label chemicals.

Preparation of competent cells,

A 50ml culture of the recipient was grown in SOB from an inoculum of 5 single colonies from a fresh minimal media plate. The culture was incubated at 37°C in an orbital shaker (250 rpm) until the OD_{600} reached 0.5. The cells were dispensed into new falcon polypropylene centrifuge tubes (prerinsed in dH_2O and prechilled) and maintained on ice for 15min. The cells were harvested by centrifugation (4000g, 4°C for 15min) resuspended in 18ml TFB and incubated on ice for 15min. The cells were recovered by centrifugation as before and resuspended in 4ml TFB. 140ul DMSO was added and the solution was left on ice for 10min then a further 140ul DMSO was added followed by incubation on ice for 5min. 210ul aliquots were placed in pre-chilled Nunc tubes and flash frozen in liquid N_2. The competent cells could be stored at -70°C for several months without loss of transformation efficiency.

Transformation with plasmid DNA,

Frozen competent cells were thawed in at room temperature and returned to incubate on ice for 10min. DNA was added in a volume less than 10ul and the mixture was incubated on ice for 30min. The sample was heat pulsed for 90secs at 42°C without agitation. The cells were then incubated on ice for 2min. 800ul of SOC was added and the cells were incubated at 37°C with orbital shaking (225rpm) for 1hr. Dilutions were made in SOB before spreading the

sample on L-agar plates containing suitable antibiotic.

2.9.2 INFECTION WITH BACTERIOPHAGE LAMBDA

The recipient cells were prepared from an overnight culture grown in 50ml L-broth supplemented with 0.2% maltose. The cells were harvested by centrifugation at 4000g for 10 minutes at room temperature and resuspended in ice cold 10mM $MgSO_4$ to a concentration of $1.5x10^9$cells/ml (OD_{600} = 2, roughly 0.4x the original volume). The plating bacteria were stored at 4°C and for best results, used within 3 days. To infect with phage lambda, 100ul of the plating bacteria were added to 100ul of phage suspended in SM buffer. The mixture was incubated at 37°C for 15 minutes before 4ml of liquid top agar (45°C) was added and the contents poured onto prewarmed L-agar plates. The cultures were incubated at 37°C overnight.

2.10 Streptomyces in vivo TECHNIQUES

2.10.1 TRANSFORMATION WITH PLASMID DNA

Plasmids were introduced into Streptomyces by genetic transformation using the PEG-mediated method described by Hunter (1985).

2.10.1.1 Preparation of protoplasts:

25ml cultures were grown in the appropriate medium at 30°C. The time to harvest the mycelia for recovery of the most competent protoplasts was strain-dependent and the optimal time for recovery of S. lividans, S.albus and S. longisporoflavus mycelia was determined to be 65hrs, 24hrs and 100hrs respectively. The mycelia was pelleted at 12000g for 10 minutes and washed twice in 10.3% (w/v) sucrose. It was then resuspended in 4ml of lysozyme solution (containing 1mg ml^{-1} lysozyme) and incubated at 37°C for 15-30 minutes. The sample was examined microscopically to monitor protoplast formation. To terminate the reaction 5ml of P medium was added, triturated twice then the protoplasts were filtered through cotton wool. The protoplasts were pelleted at 12000g for 10 minutes and washed twice in P medium. Finally they were resuspended in 2ml of medium P, dispensed into 200ul aliquots and frozen at -70°C.

2.10.1.2 Transformation of protoplasts:

The protoplasts were thawed on ice. DNA was added

in a volume of less than 10ul and the mixture was incubated on ice for 10min 0.5ml of PEG solution was added and the solution returned to incubate on ice for 1min. 2ml of medium P was added and all subsequent dilutions were made in medium P.

2.10.1.3 Regeneration of transformed protoplasts;

The method of preparation of regeneration medium for protoplasts was standardised. The media were stored in two parts (A and B); the former solid and the latter liquid. Both were steamed until portion A melted. They were incubated at 50°C for about 30min and the B portion was poured into A and mixed by swirling. 1ml of a 1% KH_2PO_4 was then added. The mixture was swirled and poured into 9 cm diameter petri dishes. 200 ml of medium was sufficient for 8 plates on average. The plates were left half open to the air in a laminar flow hood for 45min when they were turned 180° and their position in the hood altered so that those at the front were positioned toward the back of hood. After a further 45min the plates were removed to 30°C where they were left overnight. The next day any contaminated plates upon which colonies had developed were removed and the rest were used for the regeneration of protoplasts.

The only drug resistance used for plasmid selection in Streptomyces was thiostrepton (provided by E. R. Squibb, New Jersey, U.S.A.). It was dissolved in DMSO to make a 1% (w/v) stock solution. Transformed protoplasts were selected after 16-22hrs non-selective growth at 30°C by overlaying the protoplast regeneration plates with 1ml of a 220ug ml^{-1} thiostrepton solution in a 10.3% (w/v) sucrose solution.

S. lividans protoplasts were regenerated on R2 agar plates. S. albus and S. longisporoflavus were regenerated on R9 agar.

2.10.2 TRANSFECTION OF PROTOPLASTS WITH ØKC515 DNA

Transfection of the protoplast preparations was performed similar to the transformation proceedure except that the DNA was mixed with 100ul of DNA-free liposomes (Rodicio and Chater, 1982), prior to mixing with the protoplasts. After spreading the mixture on R2 agar an overlay of soft R2 containing at least $1x10^7$ S. lividans spores was added.

2.10.3 INFECTION WITH ϕKC515

0.1ml of a phage suspension in DNB was pipetted on to a 5cm Petri dish containing DNA with 0.5% glucose and the appropriate divalent cations. 0.8ml melted and cooled (to 45°C) SNA containing 100ul of a dense spore suspension (spores of S. longisporoflavus were pre-germinated to increase the efficiency of phage infection) was added and the plate was incubated at 30°C until plaques could be counted. The concentration of divalent cations that were added to the growth medium was strain dependent. For S. longisporoflavus, S. lividans and S. coelicolor high salt media was used and for S. albus low salt media was used.

2.10.4 MUTAGENESIS OF Streptomyces

2.10.4.1 Mutagenesis by short wavelength UV-light:

This proceedure is described in Chapter 3.3.2

2.10.4.2 Mutagenesis with NTG:

This proceedure is described in Chapter 3.3.1

2.10.5 ASSAY FOR TETRONASIN PRODUCTION

This proceedure is described in Chapter 4.2.7

2.11 PREPARATION OF NUCLEIC ACIDS

2.11.1 PREPARATION OF PLASMID DNA

Three methods were used to obtain DNA from cells.

2.11.1.1 Alkaline lysis modified from Birnboim & Doly (1979):

200ml cultures of stationary phase cells were harvested by centrifugation (12000g, 5min at 4°C). The pellet was resuspended in 4ml of Birnboim-Doly I solution and incubated on ice for 5min for E. coli cultures and incubated at 37°C for 15min with 100ug/ml lysozyme for Streptomyces cultures. 8ml of Birnboim-Doly II solution was added and the solution was left on ice for 5min before 6ml of cold Birnboim-Doly III solution was added. The solution was gently mixed and left on ice for a further 5 min. The cell debris and most chromosomal DNA was removed by centrifugation (32000g, 5min at 4°C) and the remaining nucleic acid was precipitated by addition of an equal volume of isopropanol followed by centrifugation at 39200g for 15 min. The plasmid DNA was further purified by centrifugation to equilibrium in

a CsCl/EtBr gradient. The DNA was resuspended in 4.5ml TE and 4.5g CsCl was added. 500ul ethidium bromide solution (15mg ml^{-1}) was added to give a density of 1.58g ml^{-1}. The solution was centrifuged in a Beckman VTi65 vertical rotor at 289,000g for 5hrs at 20°C. Two bands were visible with UV illumination; the lower represented supercoiled ccc plasmid DNA and the upper, chromosomal and relaxed plasmid DNA. The lower band was recovered using a 1ml syringe and the EtBr removed by repeated butanol extractions (using water saturated butanol). After dilution with 2 volumes of TE, 6 volumes of absolute ethanol were added. The precipitated plasmid DNA was pelleted by centrifugation (27000g, 4°C, 15min). The pellet was washed twice with 70% (v/v) ethanol, dried briefly in a vaccum dessicator before resuspension in 1ml TE. This preparation yields large amounts of pure plasmid DNA (up to 1mg from *E. coli* and 100ug from *Streptomyces* cultures containing multy-copy number vectors) suitable for all *in vitro* manipulations.

2.11.1.2 STET preparation, mini DNA preparation from *E. coli* using the method of Holmes and Quigley, (1981):

1.5ml of an overnight *E. coli* culture containing the plasmid of interest was harvested by centrifugation in a 1.5ml eppendorf tube and resuspended in 350ul of STET buffer. 25ul of STET buffer containing lysozyme (10mg/ml) was added and the contents vortexed briefly. This solution was boiled for 40sec and centrifuged in an eppendorf microfuge for 15 min at 4°C. The pellet was discarded using a toothpick and 40ul of 3M $NaCH_3COO$ and 400ul of cold isopropanol were added, followed by microcentrifugation for 7min to precipitate the nucleic acid. The pellet was washed twice in 70% (v/v) ethanol and dried briefly in a vacuum drier before being resuspended in 20-50ul 1xTE. This method yields aroumd 5ug of plasmid DNA suitable for most *in vitro* manipulations.

2.11.1.3 Single Colony Plasmid DNA Preparation from *E. coli*:

Cells were scraped from a patch (0.5cm x 0.5cm) of *E. coli* using a sterile tooth pick and resuspended in 200ul SCG buffer. The suspension was left at room temperature for 15min. It was then centrifuged in an eppendorf microfuge for 30min to pellet the cell debris - including the genome. 50ul of the suspension (avoiding the pellet) was then loaded onto an agarose gel for electrophoresis. This method was useful for identifying *E. coli* cells which contained the

desired plasmid construct when chromogenic selection was not possible.

2.11.2 ISOLATION OF TOTAL DNA FROM *Streptomyces*

The method of Hunter (1985) was followed. Mycelia was harvested from a 200ml culture by centrifugation (22100g, 4°C for 10min). The pellet was resuspended in 10ml TE containing 10mg ml^{-1} lysozyme and incubated at 30°C for 15min. 1ml of 20% (w/v) SDS was added and the suspension was gently mixed for 15secs then 10ml phenol and 1.5ml NaCl (5M solution) were added and the tube was repeatedly and gently inverted at room temperature for 20min. The phases were partitioned by centrifugation in a swing out rotor at 3000g for 10min at room temperature. The aqueous phase was recovered using a wide bore pipette and extracted twice with an equal volume of chloroform. An equal volume of isopropanol was added to form two phases and the DNA precipitating at the interface was spooled onto a pasteur pipette. The DNA was redissolved in 2ml TE. RNase was added to a concentration of 20ug ml^{-1} and the solution was incubated for 1hr at 50°C. Protease K was added to a final concentration of 100ug ml^{-1} followed by NaCl to 100mM and SDS to 0.4% (w/v). The solution was incubated at 37°C for 1hr after which it was extracted with phenol and chloroform before precipitation with ethanol and resuspension in 2ml TE.

2.11.3 PREPARATION OF TOTAL RNA (AFTER HOPWOOD *et al.*, 1985)

RNase is a very persistent enzyme and precautions were taken against contamination of equipment and solutions. All glassware was incubated overnight at 300°C. Distilled water was treated with DEPC (0.1% [v/v] of total volume) overnight and autoclaved. All solutions were made using DEPC-treated water and with fresh chemicals.

A 100ml culture of *Streptomyces* mycelia was harvested quickly by centrifugation (22100g, 4°C, 5min) in pre-chilled centrifuge tubes. The mycelia was resuspended in 5mls of Kirby mix with 12g of 0.45mm glass beads, vortexed for 2min and centrifuged at 6000g for 5min. The supernatant was poured off into an equal volume of phenol/chloroform, vortexed and centrifuged at 6000g for 5min. The aqueous phase was recovered and extracted as before with phenol/chloroform. Again the aqueous phase was recovered and the nucleic acid

precipitated by addition of 1/10 volume of 3M $NaCH_3COO$ and an equal volume of isopropanol. The nucleic acid was pelleted by centrifugation at 12100g for 5min and resuspended in 1ml DEPC-treated dH_2O.

To remove DNA from the preparation the nucleic acid solution could either be treated with RNase free DNase or the RNA pelleted through a dense CsCl cushion.

Treatment with DNase RQ (Promega),

DNase was added to 50U/ml. The reaction mixture was incubated at 37°C for 20-30 min then extracted with an equal volume of phenol/chloroform solution. The remaining nucleic acid in the aqueous phase was precipitated by addition of 1/10 volume of sodium acetate and an equal volume of isopropanol. After centrifugation the pellet was washed in 70% (v/v) EtOH, dried briefly in a vaccum dessicator and redissolved in DEPC-treated dH_2O.

Pelleting the RNA through a dense CsCl cushion,

A dense CsCl cushion was prepared and a 1.2ml CsCl cushion was placed in a 6ml SW50 tube. 1g CsCl was added per ml of nucleic acid preparation and this was layered onto the cushion. The tubes were centrifuged at 35000rpm for 12 hours at 20°C using an SW50.1 rotor. After centrifugation the supernate was removed using a pasteur pipette. The tube was then inverted to drain away the remaining fluid. Using a fresh scalpel blade the centrifuge tube was cut to isolate the bottom in the form of a small cup. The pellet was washed with 80% (v/v) ice cold ethanol, and dried very briefly under vacuum. It was then resuspended in 0.6ml TE in which the RNA could be stored at -70°C.

2.11.4 PREPARATION OF BACTERIOPHAGE LAMBDA DNA

Phage were obtained by liquid culture lysis of sensitive *E. coli*. To 20ml exponentially growing culture (OD_{600} = 0.5) phage soaked from a single plaque were added and the mixture was incubated overnight or until lysis occurred. The culture was transferred to a centrifuge tube and the cell debris was pelleted by centrifugation at 22100g for 10min. The supernatant was decanted into a new tube and DNase and RNAase were added to final concentrations of 1ug/ml and 2ug/ml respectively. The solution was incubated at room temperature for 1 hour. The supernatant was adjusted to 1M NaCl and centrifuged at 32000g for 2.5hrs. The phage pellet

was resuspended in 2ml SM buffer, transferred to a Sarstedt tube and SDS and protease K were added to a final concentration of 0.1% (w/v) and 100ug/ml respectively. After incubation at 65°C for 1hr the solution was twice extracted with phenol/chloroform by slowly roll-mixing for twenty minutes followed by extraction with chloroform/iso-amyl alcohol. 0.4 volumes 5M ammonium acetate were added and 1 volume isopropanol. After 1hr at 4°C the DNA was pelleted by centrifugation at 27000g for 20min. The supernatant was discarded and the DNA pellet washed with 70% (v/v) ethanol and briefly dried in a vacuum dessicator before resuspending in TE.

2.11.5 PREPARATION OF BACTERIOPHAGE ØKC515 DNA

ØKC515 DNA was prepared following the proceedure described by Hopwood et al., (1985). Phage were obtained by plate lysis of S. coelicolor G94. Phage were grown in 2x70ml top layers containing a high titre of S. coelicolor spores infected with $1.5x10^5$pfu. The top layers were recovered and soaked in 200ml DNB for 2hrs. The suspension was centrifuged at 16000g for 10min at 4°C. The supernatant was recovered and centrifuged at 34000g at 4°C overnight. The phage pellet was resuspended in 7ml SM buffer and 5.95g of CsCl was added and the solution was centrifuged at 85000g for 18hr at 20°C. The blue phage band was recovered through a 1ml syringe and dialysed against 2x100ml SM buffer for 4hrs at room temperature. 1/20 of a volume of 20xSSC was added and the mixture heated to 60°C for 10min before addition of 1/20 of a volume of 5M NaCl. The phage preparation was then extracted three times with phenol, once with chloroform and twice with ether before precipitating the DNA with ethanol. The pellet was recovered by centrifugation (22100g, 4°C, 15min), washed once with 70% (v/v) ethanol, dried in a vacuum dessicator and dissolved in 1ml TE. The DNA in this form was stored at 4°C.

2.11.6 QUANTIFICATION OF RECOVERED NUCLEIC ACID

The concentration and purity of the nucleic acid was determined by spectrophotometry; an OD_{260} of 1 is equivalent to 20ug ml^{-1} RNA and 50ug ml^{-1} dsDNA. Pure preparations of RNA and DNA have an $OD_{260/280}$ of 1.8 and 2.0 respectively. Contaminating protein or phenol significantly lowers these values.

2.12 PACKAGING OF BACTERIOPHAGE LAMBDA DNA

Packaging extracts were purchased from Stratagene (Gigapack Gold Cat. No. 200216) and the manufacturers recommendations were followed without modification.

2.13 ETHANOL OR ISOPROPANOL PRECIPITATION OF NUCLEIC ACID

1/10 of a volume of 3M NaOAc was added to the DNA solution and 2 volumes of cold absolute ethanol (1 volume of isopropanol). After mixing, the DNA was pelleted by centrifugation (27000g, 15 min, $4^{o}C$ for large volumes or 12000g, 15 min, $4^{o}C$ for small volumes in eppendorf centrifuge tubes). The pellet was washed in 70% (v/v) ethanol and dried briefly in a vacuum drier.

2.14 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

Restriction enzyme digests were usually performed as directed by the supplier. Where convenient, multiple digests were carried out in KGB buffer. Both potassium and glutamate are effective buffering ions and most enzymes are tolerant of their varying concentrations. McClelland et al., (1988) was referred to for the precise conditions suitable for digestion of DNA with different combinations of restriction enzymes.

2.15 LIGATION OF DNA FRAGMENTS

Unless otherwise stated ligation of DNA fragments was carried out at a DNA concentration of 20mg ml^{-1} with a 2:1 molar ratio of insert fragment to vector, where the vector DNA has been treated with phosphatase or a 10:1 molar ratio when treatment with phosphatase was omitted. Ligations were performed in 1x BRL ligation buffer with 0.1U of T4 DNA ligase ug^{-1} DNA.

2.16 PREPARATION OF RADIOLABELLED PROBES

2.16.1 RANDOM PRIMED DNA LABELLING METHOD

Labelling of DNA fragments with ^{32}P followed the procedure of Feinberg and Vogelstein (1983 and 1984) using a Boehringer Mannheim "Multiprime" kit. The labelling reaction was set up in the following way:

25-50ng of denatured DNA fragment in a volume less than 10ul (denatured by heating for 10min at $95^{o}C$ with subsequent

cooling on ice).
1ul of each unlabelled dNTP, from 0.5mM stocks.
2ul of reaction mixture (containing hexanucleotide mix and 10X concentrated reaction buffer).
5ul (50uCi) of [^{32}P] dCTP (3000 Ci/mmol).
1ul (2U) of Klenow enzyme
made up to 20ul total volume with dH_2O.

The reaction mixture was incubated at 37°C for 30 minutes, and stopped by heating to 65° for 10 minutes. The labelled DNA fragments (and template DNA) was purified from the unincorporated dNTP's by sephadex-G50 column chromatography.

2.16.2 SEPHADEX G50 COLUMN CHROMATOGRAPHY

5ul of dextran blue (50mg/ml) and 5ul of phenol red (50mg/ml) were added to the reaction mixture after the labelling reactions had been completed. The samples were then loaded onto Sephadex-G50 (20 x 0.5cm dimension gravity column) equilibrated with column buffer (100mM NaCl, 10mM Tris HCl, pH 7.5, 1mM EDTA). Fractions of approximately 500ul were collected. The dextran blue co-eluted with the DNA fragments and all other aliquots were discarded. 1ul of this fraction was spotted onto a Whatman glass microfibre filter and subjected to Cherenkov scintillation counting. The specific activity (s.a.) of the probe was calculated by the following formula:

$$\frac{\text{incorporated radioactivity} \times 10^3}{(\text{input DNA} + \text{newly synthesized DNA})} \quad \text{in dpm ug}^{-1}$$

where the amount of incorporated radioactivity could be calculated by;

$$\text{uCi dNTP} \times 2.2\text{x}10^4 \times \% \text{ incorporation}$$

and the amount of newly synthesized DNA by;

$$\frac{\text{uCi dNTP} \times 13.2 \times \% \text{ incorporation}}{\text{specific activity dNTP (Ci/mmol)}}$$

The labelled DNA sample was boiled for 5min prior to use.

2.17 GEL ELECTROPHORESIS

Both DNA and RNA were visualized on horizontal neutral agarose gels. 0.8% (w/v) gels were most commonly used. However 0.4% agarose to 2.0% agarose gels were used when large (>15kb) and small (<1kb) DNA fragments respectively had to be sized accurately. Alkaline agarose gel electrophoresis was performed as neutral agarose gel electrphoresis except for the substituition of alkaline solutions for preparation of the gel and electrphoresis buffer. Ethidium bromide was also omitted from alkaline gels which were stained after soaking for 1hr in neutral electrphoresis buffer.

2.17.1 MINI GELS

BRL model H6 gel kits were used for rapid analysis of DNA digested with restriction enzymes or after precipitation steps. 0.16g agarose was added to 20ml of TBE (TAE), boiled then cooled to 60^{o}C. EtBr was added to 200ng/ml and the molten agarose poured into a 7.6cm X 5.1cm gel caster with an 8 well slot former (4.1 X 0.8mm wells). After the gel had set, the slot former was removed and the gel placed in the tank with 500ml of TBE (TAE). The gel was electrophoresed for 30-45 mins with an applied voltage between 2-10V cm^{-1} depending on the time available and the level of resolution required.

2.17.2 LARGE GELS

200ml gels were also used to ensure good separation of DNA fragments for accurate sizing and/or Southern analysis. They were made by pouring 200ml of molten agar containing 200ug EtBr, into a 16.5 X 23cm gel former with a 20 space slot former. The gels were run overnight at 20V in TAE or TBE buffer in gel tanks with a capacity of 3 litres.

DNA samples were mixed with 1/5 volume of 5xFSB before loading onto the gel.

2.17.3 PHOTOGRAPHY OF AGAROSE GELS

Gels stained with ethidium bromide were viewed on a 302nm UV transilluminator and photographed using Polaroid type 67 land film or using a Pentax 35mm SLR loaded with Ilford HP5 film. Both cameras were fitted with Kodak Wratten filters (No. 23A).

2.18 RECOVERY OF DNA FROM AGAROSE GELS

DNA fragments were recovered from TAE agarose gels (borate can influence some enzyme reactions and Gene Clean did not give high yields of recovery with TBE so use of TBE was avoided) using two methods. Use of a Gene Cean kit was suitable for fragments less than 10kb in size. Larger fragments were susceptible to shear by this process and were recovered by electro-elution.

2.18.1 GENE CLEAN

A Gene Clean kit was purchased from Bio 101. The TAE agarose gel was placed on a 302nm UV transilluminator and a small portion ($<1cm^2$) of the agarose gel containing the DNA fragments of interest was excised. The agarose was diced and 3 volumes of NaI solution were added. The suspension was heated at 55^oC for 5 minutes or until the agarose had completely dissolved. 5ul of "glassmilk" suspension was added the solution mixed and placed on ice for 5min. The mixture was centrifuged (5 seconds in an eppendorf microfuge) and the supernatant discarded. The pellet was washed three times in ice-cold NEW solution by resuspending the pellet 500ul, centrifuging as before and discarding the supernatant. After the final wash, care was taken to remove all traces of NEW solution. The DNA was recovered from the glass beads by adding 20ul TE (pH 8.0) and incubating at 55^oC for 5min. The glass beads were pelleted by spinning for 30 seconds then the supernatant was recovered. The exact composition of the NaI, Glassmilk and NEW solutions were undisclosed by the manufacturers.

2.18.2 ELECTRO-ELUTION

The TAE agarose gel was placed on a 302nm UV transilluminator and a trough measuring 0.5cm wide (the length and depth was determined by the length of slot and thickness of gel respectively) was excised from the TAE agarose gel immediately in front (with respect to the direction of migration) of the DNA fragments of interest. The distal edge of the trough (with respect to the DNA) was lined with dialysis tubing (pre-treated by bioling in 2% (w/v) sodium bicarbonate, 1mM EDTA) and electrophoresis was continued until the desired DNA had eluted from the agarose

and migrated across the trough to be stopped by the dialysis tubing. The polarity of the current was then reversed momentarily before switching off, and the DNA was pipetted from the surface of the dialysis tubing. The recovered DNA was then extracted with phenol/chloroform and precipatated with ethanol. To recover relatively undamaged DNA agarose gel electrophoresis was performed in the absence of ethidium bromide, with afterwards only a small "reference" strip of the gel stained to monitor the migration of the DNA into the trough.

2.19 LOW RESOLUTION S1 NUCLEASE MAPPING

After isopropanol precipitation of the test DNA together with the RNA, the washed (80% [v/v] EtOH) and dried pellet was dissolved in 20ul of hybrization buffer. The tubes were incubated at 85^{o}C for 10min before slowly equilibrating to the hybridization temperature (determined empirically - see Chapter 5) and incubating for a further 4hr. 300ul of chilled S1 digestion buffer containing 100U of S1 nuclease were added to each of the tubes which were immediately vortexed and placed on ice. The samples were incubated at 37^{o}C for 45min, before terminating the reaction by adding 75ul of S1 termination buffer. 10ug carrier tRNA were added and 400ul isopropanol. The precipatated nucleic acid was pelleted by microcentrifugation (eppendorf microfuge), washed in 80% EtOH and dried briefly in a vacuum dessicator. Each pellet was dissolved in 20ul alkaline loading dye and electrophoresed through an alkaline gel. The protected DNA fragments were identified by Southern analysis.

2.20 REMOVAL OF THE 5' PHOSPHATE FROM DNA

Calf Intestinal Alkaline Phosphatase (CIAP) was used for this purpose. Around 5pmoles of 5' terminal phosphoryl DNA with 5' protruding ends (roughly 7ug of a 5kb molecule) in 1xCIAP buffer were incubated with 0.1U of CIAP at 37^{o}C for 30min. The reaction was terminated by heating to 65^{o}C in 1x gel loading buffer for 10min prior to purification of the vector DNA by recovery of the desired fragment from an agarose gel after electrophoresis.

2.21 COLONY AND PLAQUE TRANSFER TO AMERSHAM HYBOND-N

Top agarose was used to prepare double layer

phage plates from which plaque lifts were to be taken, otherwise the procedures for colony and plaque blotting were very similar. Culture plates were chilled for 1hr at 4°C before placing Hybond-N nylon membrane on the plate surface. After 30 seconds the membrane was removed and placed (plaque/colony side up) on Whatman 3MM soaked in denaturing solution for 7 minutes and then transferred to neutralizing solution for 2x 3min. The membrane was then allowed to air dry on Whatman 3MM prior to cross-linking the DNA to the membrane by UV illumination (5min of illumination from a 302nm wavelength source). With transfer of bacteriophage lambda, replica filters were prepared from the same plate in the same way however the phage were transferred for an extra 30 seconds with each replicate. Replica filters from colony blots were best prepared by preparing a single blot from each of two identical plates.

2.22 SOUTHERN ANALYSIS

2.22.1 SOUTHERN BLOTTING

After electrophoresis and photography, agarose gels were blotted under alkaline conditions to Amersham Hybond-N. The gel was soaked in denaturing solution for 30 minutes. DNA was transferred to the nylon membrane in Southern transfer solution by capillary action (nappies cut to the size of the gel proved particularly useful as an absorbent material used to drive the process). After transfer for at least 4hrs the membrane was washed for 1min in 2xSSC and air dried.

2.22.2 AQUEOUS PRE-HYBRIDIZATION AND HYBRIDIZATION CONDITIONS

The procedures which follow were adapted from Southern, (1975) and apply to DNA immobilized on filters from plaque/colony lifts and Southern transfer. The stringency of hybridization, determined by temperature and SSC concentration, was defined empirically (see Chapter 5). Unless otherwise stated the pre-hybridization and hybridization solutions contained 5x Denhardts solution, 10% (w/v) dextran sulphate, 100ug ml^{-1} sheared and denatured Salmon sperm DNA, with the SSC concentration and temperature of incubation varied as described in the text.

2.22.3 WASHING OF MEMBRANES AFTER HYBRIDIZATION

The nylon membrane was washed in aqueous conditions (4x 100ml for 20min each) at the same stringency as used for hybridization (unless otherwise stated).

The probe DNA could be stripped from the filter (provided that the filter was always maintained wet after hybridization) by washing in 0.4M NaOH at 45^{o}C for 30min. The blot was then washed in 2xSSC for 15min after which pre-hybridization could begin.

CHAPTER 3

AN ATTEMPT TO CLONE DNA INVOLVED IN THE BIOSYNTHESIS OF TETRONASIN BY COMPLEMENTATION OF MUTANTS DEFICIENT IN TETRONASIN BIOSYNTHESIS

3.1 INTRODUCTION

The main aim of this work was to determine the feasibility of using mutation rescue for the isolation of genes involved in tetronasin biosynthesis. In order to achieve this a number of mutants blocked at different steps in the tetronasin pathway would have to be isolated and the plasmid transformation efficiency of each strain determined.

3.1.2 RATIONALE FOR MUTAGENESIS OF *Streptomyces longisporoflavus*

The isolation of *S. longisporoflavus* mutants, deficient in the biosynthesis of tetronasin would be useful in several experimental strategies.

a. complementation of antibiotic non-producing mutants for identification and localisation of sequences involved in tetronasin biosynthesis.

b. mutant phenotypes characterised by co-synthesis experiments and/or accumulation of pathway intermediates can simplify identification of the function of DNA cloned (Motamedi *et al*, 1986; Rhodes *et al*., 1981; Rudd and Hopwood, 1979; Baltz *et al*., 1981; Chater and Bruton, 1983; Distler *et al*., 1985).

c. mutants blocked in tetronasin biosynthesis may prove useful in the generation of novel, hybrid antibiotics by either, providing suitable intermediates for use as substrates for conversion by antibiotic biosynthetic genes from other streptomycetes or deregulation of the tetronasin synthase complex, releasing individual components to modify other antibiotics (see Chapter 1.5).

3.2 AN OVERVIEW OF MUTAGENESIS OF *Streptomyces*

Two of the most commonly used mutagens, N-methyl-N-nitro-N-nitrosoguanidine (NTG) and short wavelength ultraviolet light (UV) were employed to generate mutants of *S. longisporoflavus*.

NTG, $CH_3N(NO)C(NH)NHNO_2$, is a member of the class of biological alkylating agents which transfers its methyl group to DNA (Auerbach, 1976). Mutations are thought to arise because the modified base has a greater tendency to mispair in replication, giving rise to a transition (substitution of a purine-pyrimidine base pair by another purine-pyrimidine base pair), or transversion (substitution

of a purine-pyrimidine base pair by a pyrimidine-purine base pair) event. N-7 methylation of guanine results in a weakening of the bond between the sugar phosphate backbone and the modified base leading to loss of the guanine residue and eventually a single base pair deletion/frameshift mutation in the DNA.

Mutagenesis using short wavelength UV light is more complicated and is dependent upon the error-prone repair of photo lesions in the DNA. The majority of photo-products in dsDNA are covalently-linked dimers formed from neighbouring pyrimidines. Of the three possible types (C:C, C:T, T:T), thymine dimers are the most frequent and stable. The amount of pyrimidine dimers formed is dose-dependent, and some strains of E. coli have been estimated to survive as many as 1000 per cell (Setlow and Setlow, 1972). Cell death is avoided because a number of survival systems have evolved to remove or repair the lethal damage. The distinction between error-proof and error-prone repair was first made by Witkin (1967).

Error-proof repair systems, which repair photo lesions with high fidelity, do not generate many mutations. Examples include, 1) Photo-repair, which enzymatically resolves pyrimidine dimers into monomers using energy from short wavelength visible light. The remaining repair systems are collectively known as dark repair because they do not use light energy to drive the reaction. They include: 2) Excision repair, which involves excision of the pyrimidine dimer and accurate repair of the single strand gap against the complementary strand. 3) Post replicative recombinational repair which employs recombination between sister double helices to repair dsDNA damage, caused by replication gaps, resulting from the inability of DNA polymerase to pair nucleotide triphosphates with pyrimidine dimers.

Error-prone repair appears to be another form of gap repair (either excision or post replication gap) which can insert the wrong base into the DNA sequence being repaired and generates several types of error, namely transitions, transversions and frameshifts.

Both mutagens have been used individually in Streptomyces to good effect (Harold, 1969; Baltz, 1978; Hara and Beppu, 1982), and also in combination, increasing the mutation frequency obtained with UV irradiation after a

sensitising dose of NTG (Motamedi et al, 1986). The rationale behind the use of mutagens in pairwise combination is not clearly understood. However it seems likely that a low level dose of alkylating agent acts to inhibit a repair mechanism for UV-induced damage (Auerbach, 1976).

3.3 EVALUATION OF METHODOLOGY FOR MUTAGENESIS OF S. longisporoflavus

3.3.1 NTG MUTAGENESIS, after the method of Hopwood et al, (1985)

Around $1x10^9$ spores of S. longisporoflavus 4584 were harvested from one 9cm diameter agar plate, washed in sterile water and pelleted at 10 000g for 10 minutes. The spores were resuspended in 9ml TM buffer at pH8. The suspension was prewarmed to 30°C, then 26.1mg of NTG was added and the mixture was vortexed for two minutes. The suspension was returned to incubate at 30°C with occasional agitation to keep the spores in suspension. 1ml aliquots were taken after 0, 1, 2, 4, 8, 16, 32, 64, and 128 minutes exposure to NTG. To terminate the reaction, 1ml of the spore suspension was added to a precooled 2ml screw cap Nunc tube and spun at 1500rpm at 8°C for 2min. The supernatant was discarded into 1M NaOH. The spores were washed in 1ml sterile water, pelleted as before, and finally resuspended in 1ml sterile water.

Viable spore counts calculated for each sample showed that NTG under the conditions used caused no cell death. This result was unfortunate and precluded a comparison of the mutagenicity of the different treatments used individually or in combination. Reasons for the failure to mutagenise S. longisporoflavus with NTG were not investigated. However it is possible that the spore wall was largely impermeable to NTG, or its more mutagenic derivative, diazomethane. The treatment of protoplast preparations has been shown to improve results in S. fradiae (Fillipini et al., 1986). Furthermore the use of TM buffer at pH9 - rather than pH8 - causes rapid decomposition of NTG to the diazomethane increasing the mutagenicity of the treatment (Delic et al., 1970).

3.3.2 SHORT WAVELENGTH UV MUTAGENESIS, after the method of Hopwood et al., (1985)

Around $1x10^9$ spores were harvested from a well-sporulated 9cm diameter lawn of S. longisporoflavus 4584, washed once with sterile water and pelleted at 10 000g for 10 minutes. The spores were resuspended in 35ml sterile water and dispensed into seven 5ml aliquots. One aliquot was retained as the control with zero illumination and the other six were treated identically except for the duration of exposure to UV. Each 5ml aliquot was placed in a sterile 9cm glass petridish and exposed under constant agitation to a 250nm wavelength UV source delivering 120uW cm^{-2} at a distance of 28cm. After a timed exposure to UV the sample was pipetted from the petridish into a 10ml screw cap sterilin test tube. The petridish was washed with 5ml sterile water, which was retained and the pooled spore suspension made up to 10ml with sterile water (thus allowing for some observed evaporation of water under longer illumination).

3.3.2.1 Sensitivity of S. longisporoflavus to short wavelength UV light and photoreactivation

The viable spore titre of the UV-irradiated samples was determined by serial dilution of each sample in 10 fold steps to $1x10^{-5}$. 500ul of each dilution was added to 20ml of cool (45^oC) Emersons agar and poured into 9cm petridishes. The viable cells were titred in duplicate at 30^oC, with one plate from each dilution incubated in light impervious canisters (35cm x 15cm), whereas the other was incubated under visible light.

After 96hrs the number of colonies growing on plates incubated in the light were counted. Upon examination it was evident that the colonies grown in dark conditions had not achieved an advanced stage of growth and were returned to 30^oC in the light for a further 24hrs. The slow growth rate exhibited by the dark-grown cells could most likely be attributed to a drop in metabolic rate because of low oxygen tension in the enclosed environment of the canisters. The experimental results are presented in Table 3.1 and are plotted as survival curves in Figure 3.1. Curiously all S. longisporoflavus strains were observed to be growing in a horizontal plane: colony growth may therefore be geotropic. It was apparent from the data that S. longisporoflavus is highly sensitive to UV light at the dosage levels used.

TABLE 3.1; UV Mutagenesis And Photoreactivation in *S. longisporoflavus*

dose (min)	incubation	dilution	colonies counted	viable cell number/ml	percentage survival	log percentage survival
0	LIGHT	0.0001	185	4.1E+7	100	2.00
1	LIGHT	0.001	175	3.8E+6	9.2	0.96
2	LIGHT	0.01	1608	3.5E+6	8.5	0.93
4	LIGHT	0	756	1.7E+5	0.041	-1.39
8	LIGHT	0	5	110	0.00024	-3.62
16	LIGHT	0	1	22	-	-
32	LIGHT	0	1	22	-	-
0	DARK 72hrs	0.001	2840	6.2E+7	100	2.00
1	DARK 72hrs	0.01	896	2.0E+6	3.2	0.50
2	DARK 72hrs	0	642	1.4E+4	0.022	-1.66
4	DARK 72hrs	0	104	2.3E+3	0.0037	-2.43
8	DARK 72hrs	0	5	110	0.00018	-3.74
16	DARK 72hrs	0	0	0	-	-
32	DARK 72hrs	0	0	0	-	-

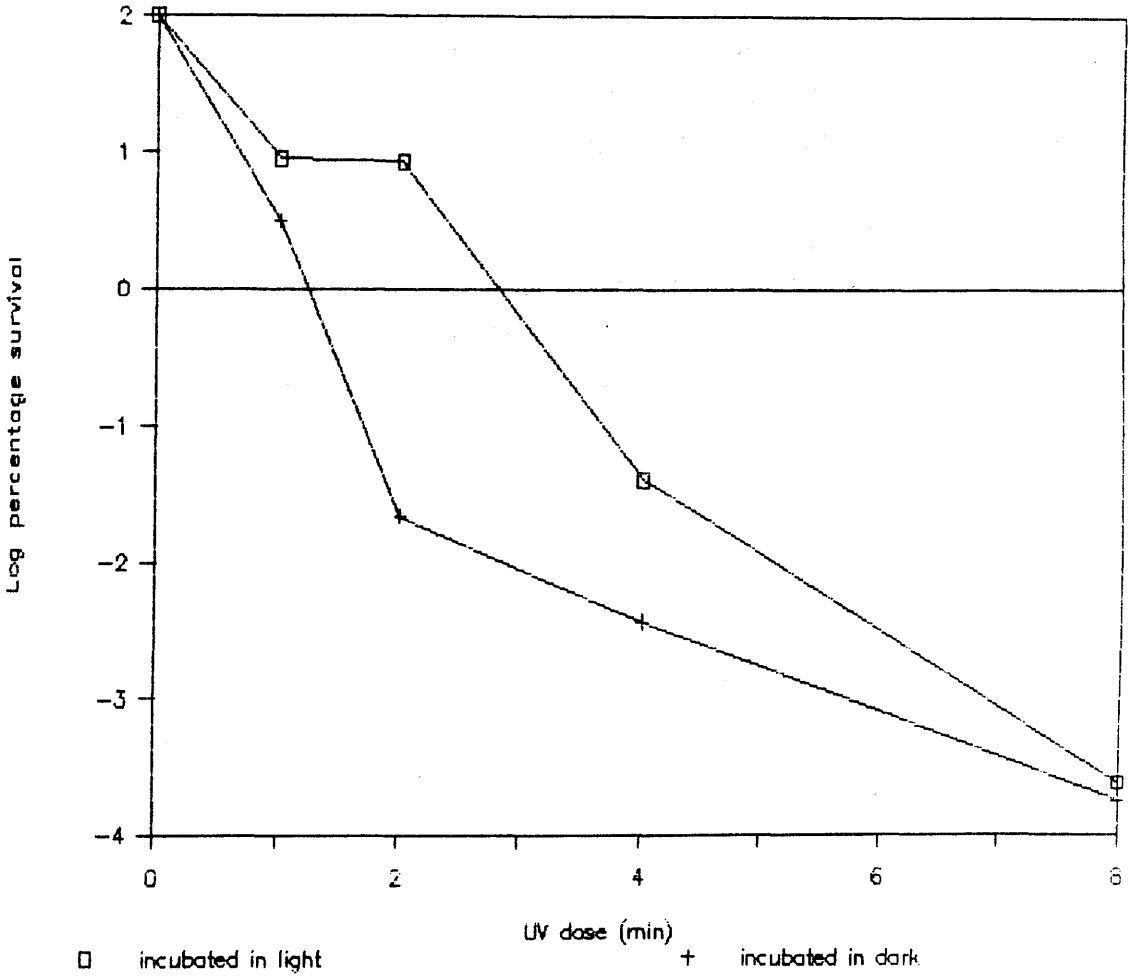


FIGURE 3.1; UV Dose v's Log Percentage Survival

Exposure to 1 minute and 2 minutes of UV light followed by incubation in the dark, spans the desired level of survival (around 1%) at which single mutants in antibiotic biosynthesis may be expected (Motamedi et al., 1986).

From the comparison of spore outgrowth in the presence or absence of visible light, it appears that the strain harbours a photoreactivation system, which as previously mentioned is likely to rescue cells from UV damage in an error-proof manner. The length of the photoreactivity period was investigated empirically. Spores given a 1 and 2 minute UV dose were grown in Emersons agar as before. However, samples of both spore populations were subjected to one of the following treatments; incubation of the culture plates in visible light, incubation in darkness for 12hrs or 72hrs then visible light until the survivors could be counted (Table 3.2). After 12hrs incubation of plates in darkness at 30^{o}C, surviving cells still retained the ability to photo-repair lesions. The photoreactive period of the treated spores was expected to have lapsed after 12hrs growth on complex media. However, the duration of this period was likely to be influenced by the metabolic state of the spores - in particular the length of time between completion of irradiation and the first post-treatment genome replication is important. This phenomenon was first recognised in E. coli by Roberts and Aldous, (1949), and termed "liquid holding recovery" or LHR. LHR relies on the delay of post-treatment DNA replication allowing more time for pre-replication excision repair (Ganesan and Smith, 1969). The possibility that LHR is an important factor in S. longisporoflavus survival after UV irradiation is not entirely improbable given the long generation time of the strain. Although this was not measured it has been noted that cultures of S. longisporoflavus take around 8 days to grow and sporulate on complex media, almost twice as long as S. lividans. This factor was likely accentuated by the very slow growth rate observed in the light controlled incubation system, however the canisters remained the most convenient method of maintaining the growing cultures in darkness. This whole scenario may explain the apparently anomalous survival of 117% of spores given a 1 minute UV dose then incubated in the dark for 12hrs; If DNA replication is delayed for over 12hrs by incubation in the canisters, this sample of spores may

TABLE 3.2; Photoreactivation In S. longisporoflavus

dose (min)	incubation	VIABLE cell number/ml	percentage survival
0	LIGHT	5.2E+7	100
1	LIGHT	9.2E+6	18.0
1	DARK 12hrs	6.1E+7	117.3
1	DARK 72hrs	3.6E+6	6.9
2	LIGHT	5.0E+6	9.6
2	DARK 12hrs	1.5E+6	2.9
2	DARK 72hrs	7.4E+4	0.14

benefit from both LHR and photo-repair. A similar situation would pertain for the spores treated for 2 minutes with UV light, and incubated under the same conditions. However the increased number of potentially lethal photo-lesions may overload the capacity of the repair mechanisms, explaining the absence of a dramatic increase in the survival rate. The low level of survival after 72hrs incubation in the canisters would be explained by the inability of the dark-repair systems, by themselves, to remove all the photo-damage, and for DNA replication to occur between 12hrs and 72hrs incubation.

3.3.2.2 Expression of UV-induced mutations

In unicellular haploid organisms a mutation in one strand of the DNA helix will be expressed in one daughter cell after one round of genome replication and cell division. Clearly the mycelial nature of streptomycete growth complicates the analysis of the mutated spore sample because the daughter cells of any cell division remain closely associated. The mutant phenotype will therefore not be expressed in the resultant colony unless it is dominant or there exists a powerful selection against the wild-type phenotype. In fact the likely outcome is a colony with a mosaic of two cell types derived from either DNA strand of the mutated spore. However, the complex life cycle of streptomycetes provides a solution to this problem. The spores produced as the conclusion of differentation are haploid. Therefore if mutated spores are germinated and allowed to complete their life cycle under non selective conditions they will give rise to a population of "expressed" spores, each containing a single genome comprised of homogeneous strands.

To obtain a population of "expressed" S. longisporoflavus mutants, the spores from twenty 9cm petridishes were harvested. Each plate contained around 10 000 colonies, grown from spores UV-irradiated for either 1 or 2 minutes. The viable spore titres of both the 1 minute and 2 minute UV-treated and mutationally-expressed spore samples were determined to be around $1x10^{8}$ ml^{-1}.

3.4 DEVELOPMENT OF A BIOLOGICAL ASSAY FOR TETRONASIN PRODUCTION

Of four micro-organisms tested, Saccharomyces

cerevisiae, Escherichia coli, Streptococcus faecalis and Bacillus subtilis, both S. faecalis and B. subtilis were sensitive to tetronasin. Two streptomycetes, namely S. albus G153 and S. rimosus G7 were also sensitive to tetronasin (see chapter 4.2.1) and could have been adopted for the bioassay. However B. subtilis was chosen as the test organism because of its rapid growth rate and lack of pathogenicity. The extent of sensitivity of B. subtilis to tetronasin was tested by streaking colonies of the bacterium on L agar plates with increasing concentrations of tetronasin and comparing the results with growth in the absence of the drug. B. subtilis was found to be resistant to 0.1ug ml^{-1} but sensitive to 1ug ml^{-1} tetronasin.

S. longisporoflavus will produce tetronasin on Emersons agar after seven days, concomitant with aerial mycelium formation.

These discoveries formed the basis of a biological assay for production of tetronasin by S. longisporoflavus: To an Emersons agar plate containing 8 day old colonies of S. longisporoflavus, a 500ul suspension of B. subtilis (5ul of an overnight culture of B. subtilis per 1ml L broth) was added. The plate was then held at an angle of 45^{o} and fully rotated to ensure the suspension covered the entire surface area of the plate. After an overnight incubation at $30^{o}C$, nine day old S. longisporoflavus 4584 colonies were routinely encompassed by an area of inhibition of growth of B. subtilis, measuring 10-20mm from the edge of the colony.

Other bioassay methods with agar plugs used for oxytetracycline (I. S. Hunter, pers. comm.) and tetracenomycin C (Motamedi and Hutchinson 1986) biosynthesis, offer the advantage of standardization, by growth of the test organism on an agar plug of fixed dimensions. However, such systems can lack sensitivity requiring the diffusion of antibiotic through ~1cm of agar before reaching the test organism.

3.5 IDENTIFICATION OF S. longisporoflavus MUTANTS DEFICIENT IN TETRONASIN PRODUCTION

The expressed spores of the 1 minute and 2 minute UV-irradiated samples (section 3.3.2.2) were grown on Emersons agar to give a density on average, of one colony per $3cm^2$ (around 20 colonies per 9cm diameter plate). After eight

days the plates were overlaid with a B. subtilis suspension to assay for production of tetronasin. From around 820 colonies of the 1 minute UV irradiated spores and around 300 colonies of the 2 minute UV irradiated spores, 47 and 35 colonies respectively, were selected for further analysis based on their inability to inhibit the growth of B. subtilis. Only colonies with normal morphology were selected.

Each colony, which was potentially impaired only in the biosynthesis of tetronasin, was streaked out on R2 agar containing 1ug ml^{-1} tetronasin to enable spore samples to be prepared free of B. subtilis. This strategy may have eliminated any mutants in tetronasin biosynthesis with concomitant loss of tetronasin resistance. If these double mutants were desired it would be a simple task to first replica plate onto Emersons before bioassay, retaining the master plate as an uncontaminated source of material. In retrospect this fear was unfounded as all 82 colonies proved to be resistant to tetronasin. Spore suspensions prepared from the R2 plates were maintained at -20°C in 20% (v/v) glycerol. From this source of inoculum, single colonies were obtained on Emersons agar plates for each putative mutant impaired in tetronasin production. After sporulation of the colonies the plates were bioassayed for the presence of tetronasin. Following three subsequent bioassay tests, six stable mutants were identified (Table 3.3). Hereafter the mutants are referred to as Tetronasin Non Producer (TNP) mutants. The mutation frequency was observed to be quite high, i.e. 0.4% mutants per 3% survivors for the the 1 minute UV dose, and (as expected) a higher frequency for the 2 minute UV dose, i.e. 1% mutants per 0.02% survivors. The high frequency of mutation probably reflects the large number of biosynthetic steps and therefore potential targets for mutagenesis of tetronasin biosynthesis.

Of the six mutants, four were completely deficient in detectable tetronasin production on Emersons agar plates and two, TNP38 and TNP44, were leaky. Leaky mutants in antibiotic biosynthesis are not uncommon (Rudd and Hopwood, 1979; Motamedi et al., 1986). In this case, under the conditions of the bioassay, TNP38 and TNP44 inhibited growth of B. subtilis only 1-2mm from the colony edge.

Several plausible explanations exist for the low level production of a bioactive compound from TNP38 and TNP44 or

TABLE 3.3; Tetronasin Production Levels Of *S. longisporoflavus* Strains

STRAIN	UV DOSE (min)	TETRONASIN PRODUCTION
4584	0	HIGH
TNP12	1	ABSENT
TNP38	1	LOW
TNP44	1	LOW
TNP56	2	ABSENT
TNP61	2	ABSENT
TNP62	2	ABSENT

indeed any other leaky antibiotic biosynthetic mutant.

a. Down regulation of antibiotic biosynthesis leading to a reduction in the antibiotic titre. The discovery of positively-acting regulatory elements in several gene clusters for antibiotic biosynthesis (see Chapter 1.5) provides a potential site of non-pleiotropic regulatory mutant.

b. Mutants blocked, usually in a late stage of antibiotic biosynthesis may still generate an intermediate or shunt product with some biological activity.

c. It is possible that polyketide synthase mutants may be partially complemented by recruitment of fatty acid synthetase or additional polyketide synthase enzymes or even the use of "silent" genes whose existence has been demonstrated by Jones and Hopwood, (1986).

3.6 ANALYSIS OF CO-SYNTHESIS BETWEEN MUTANTS BLOCKED IN TETRONASIN BIOSYNTHESIS

The co-synthesis of a product by two mutants blocked in different steps of a biosynthetic pathway requires that the pathway intermediate or biosynthetic enzyme be freely diffusible between the strains. Even with so few mutants this experiment was attempted in the hope that individual mutants could be classified as blocked early (converters) or late (secretors) in the biosynthesis of tetronasin.

The adopted strategy involved streaking on Emersons agar plates, from dense spore suspensions, an inoculum measuring 2cm by 0.5cm. Individual mutants and all pairwise combinations were tested, separating the pairs by 0.5cm and overlapping by 1cm. The cultures were incubated at 30^{o}C for eight days, then bioassayed for tetronasin production.

In all pairwise tests except one, no cross feeding between mutants could be discerned. In one case TNP38 (secretor), a low level producer of a bioactive compound, caused production of a bioactive compound when grown pairwise with TNP62 (converter). This was indicated by inhibition of growth of B. subtilis between the two areas of streptomycete growth, extending beyond the end of TNP38 growth along TNP62. There was also an area of inhibition of B. subtilis growth on the distal side, of TNP62 with respect to TNP38.

There are two possible explanations of this result. Either tetronasin production was induced in TNP62 by a diffusible substance produced by TNP38, or the bioconversion of a pathway intermediate from TNP38 by TNP62. It would seem unlikely that TNP62 induced an increase in tetronasin production by TNP38 as the increased bioactivity was clearly associated with TNP62. Whichever the correct explanation, it seemed reasonable that tetronasin biosynthesis in the two mutants was likely to be interrupted at different points.

The absence of co-synthesis in any other pairwise combination of mutants could imply that TNP- 12, 44, 56, 61 and 62 were deficient in the same step. Precedence suggests that early mutations (likely to be deficient in the formation of the polyketide backbone), are more commonly obtained than mutations of later steps in the biosynthesis of antibiotics (Motamedi et al., 1986; Butler et al., 1989). Other plausible explanations include regulatory or polar mutants, neither of which would be expected to synthesise tetronasin in combination with other mutants blocked in the pathway. Also the lack of availability of pathway intermediates from the mutant strains could explain the absence of co-synthesis. The absence of secretor strains could be attributed to one of several possibilities.

a. Poor diffusion of the compound between strains.

b. Chemical reactivity of the intermediate, polyketide antibiotic intermediates, particularly if they contain a high number of keto groups, may be very unstable.

c. Enzyme bound intermediates. Perhaps related to the latter possibility, it is thought that polyketide antibiotic intermediates may remain bound to the PKS complex and may be closely associated with the cell membrane. If this is the case, then it is unlikely that the enzyme-substrate complex is freely diffusible from the cell, particularly if the enzymes are, as postulated, closely associated with the cell membrane. With over 100 S. cinnamonensis mutants of monensin biosynthesis and no evidence of co-synthesis, this is the explanation favoured by Donovan et al., (1988).

The inability to achieve co-synthesis on solid media may be overcome in liquid culture, by alleviating low diffusion problems or allowing conversion of enzyme-bound intermediates when released into the culture medium after lysis of the mycelia. To test this, a mixed culture method

based on growth in YEME liquid media was developed. A 70ml culture of each mutant was grown in YEME from an inoculum of 50ul dense spore suspension. After 3 days growth 10ml of each culture was used as a seed inoculum in 20ml cultures of each pairwise combination of all six mutants. The remaining 20ml of pure culture was treated in the same way as the mixed mutant cultures to provide a negative control, along with YEME itself. Similarly a 70ml culture of S. longisporoflavus 4584 was reduced to 20ml to give a positive control. The 20ml cultures were returned to 30°C with vigorous shaking for a further 5 days, before 5ul of culture supernate was spotted onto a seeded lawn of B. subtilis for bioassay. Filter discs (1cm diameter Whatman 3MM) soaked in culture supernate and allowed to dry, were also used to deliver drug for bioassay.

Under these conditions all mutants, except TNP12, synthesised as much bioactive compound inhibitory to growth of B. subtilis as wild type S. longisporoflavus 4584. Supernatant from any TNP12 culture, whether pure or mixed, inhibited only a small area of B. subtilis growth, just 1-2mm from the edge of a filter disc compared to 1-2cm for the wild type strain. The inhibition by TNP12 supernate was enhanced by the addition to the plates of NaCl to 0.2M (supplementing the growth medium with NaCl was shown in Chapter 4.4 to increase the sensitivity of S. lividans to tetronasin). This increase in bioactivity was reproducible for all mutants indicating the likely production of tetronasin or a closely related metabolite, as the bioactive compound. It is not clear why the TNP12 phenotype should be dominant over the other mutants in mixed culture, however it seems plausible that TNP12 produces some diffusible negatively-acting regulator of tetronasin biosynthesis.

Precisely why the remaining mutants produced tetronasin when grown in YEME is unknown. It is possible that the trait has a high reversion rate or that some mutations are leaky. To test this, each mutant was streaked out for single colonies of which ten each were picked and patched out individually on Emersons agar. After eight days growth the plates were bioassayed for tetronasin production. The results are presented in Table 3.4. These findings suggested the mutants to be reasonably stable on solid media. This implies that growth in YEME suppressed mutations in five of the strains. This may be possible, as indicated in Chapter 1.8.3,

TABLE 3.4; Stability Of Mutants Deficient In Tetronasin Production: Assay Of Ten Individual Colonies

strain	number of colonies with bioactivity	radius of bioactivity [from colony edge, mm]
4584	10	18
TNP12	1	1
TNP38	3	1
TNP44	5	1
TNP56	0	nd
TNP61	0	nd
TNP62	0	nd

nd = no bioactivity detected

if the mutations lie in regulatory circuits. Alternatively the new growth conditions may have induced hitherto silent genes for polyketide biosynthesis which complement the mutations in tetronasin biosynthesis. The mutants were therefore, still considered suitable for complementation tests on solid media, particularly as observed complementation/reversion can be tested in plasmid-loss experiments.

Only colonies with normal morphology were selected to avoid regulatory mutants which were impaired in development and which pleiotropically failed to synthesise antibiotics. Such selectivity may not have been wisely adopted after recent reports of the involvement of certain antibiotics in the differentiation cycle. Interestingly one of the two examples involves a polyketide anion-ionophore, pamamycin-607 (Kondo *et al.*, 1988), which at sub-lethal levels (0.1ug ml^{-1}), induced the formation of aerial mycelia in bald mutants of *S. alboniger* and stimulated differentiation in the parental strain. At higher concentrations (10ug ml^{-1}), pamamycin inhibited growth of the substrate mycelia and it has also been shown to have antibiotic activity against a wide range of eukaryotic and Gram-positive prokaryotic micro-organsims. This is strongly suggestive of a developmental regulatory role for pamamycin biosynthesis in *S. alboniger*. However the definitive experiment in which the effect of specifically disrupting pamamycin biosynthesis in the parental strain was not reported. With the omission of this information an alternative explanation cannot be discounted. Stimulation of differentiation through osmotic stress caused by the ionophoric properties of pamamycin could be viewed as analogous to the suppression of *bld* mutants of *S. coelicolor* by nutritional factors (see Chapter 1.8.3).

The involvement of a polyketide metabolite in differentiation of *S. coelicolor* is better established. Characterisation of *whiE* mutants of *S. coelicolor* (N. K. Davis, cited Chater, 1989), has shown that the physiological deficiency lay in the biosynthesis of a pigmented polyketide which was closely associated with the spores. The *whiE* locus was found to include the 6.7kb *Bam*HI fragment with homology to the *actI* fragment (D. A. Hopwood, pers. comm.), which directed the biosynthesis of a brown pigment when present in high copy numbers (Horinouchi and Beppu, 1985).

3.6 TRANSFORMATION OF S. longisporoflavus WITH PLASMID DNA

Streptomycete mycelia is not, in general, naturally competent for the uptake of DNA (Okanishi et al., 1966). The introduction of plasmid DNA into S. coelicolor and S. parvalus was first achieved only relatively recently (Bibb et al., 1978). The successful process, which is still the most widely used method today - although eletroporation (C. Binnie, pers. comm.) and conjugal transfer of shuttle plasmids from E. coli (Mazodier et al., 1989), have recently been shown to be possible - can be divided into four inter-related stages; growth of mycelia, formation of competent protoplasts, transformation of protoplasts and regeneration of mycelia. Each stage is characterised by a number of variables, whose influence is largely species dependent. Those which have been found to be generally important for optimal transformation efficiency include the following.

1. Growth of mycelia; the composition of the growth media, in particular, the levels of glycine and magnesium (Sagara et al., 1971; Thompson et al., 1982a), the growth phase of the culture when the mycelia is harvested (Baltz, 1978), and the incubation temperature (Baltz et al., 1981), have all been manipulated to increase the transformation efficiency of some strains.

2. Formation of protoplasts; the technique described by Hunter, (1985), for the preparation of protoplasts from S. coelicolor and S. lividans includes the cumulative findings of Okanishi et al., (1974), Bibb et al., (1978), and Thompson et al., (1982a), and is considered to be applicable to many species of Streptomyces. If difficulties are encountered, the concentration of degradative enzymes or the choice of osmotic buffer can be altered to good effect (Hunter, 1985; Czaplewski, 1989).

3. Transformation of protoplasts; polyethylene glycol-mediated transformation of protoplasts (Bibb et al., 1978) is commonly used. Positively charged liposomes have also been used to stimulate transformation (Rodicio and Chater, 1982), and the topology of the DNA has been shown to be important - highest efficiencies are obtained with ccc DNA (Bibb et al., 1980; Hopwood et al., 1986a).

4. Regeneration of mycelia; the regeneration of viable mycelia is a crucial stage in the process and the optimal

conditions for individual species can vary considerably. The constituents of the media, in particular the choice of osmotic buffer and divalent cation concentration is critical (Okanishi et al., 1974; Baltz et al., 1978; Czaplewski et al., 1989). The moisture content of the media must be low (Baltz and Matsushima, 1981; Thompson et al., 1982a), and sub-optimal growth temperature may also enhance regeneration efficiency (Baltz, 1978).

An initial investigation of protoplast transformation of S. longisporoflavus tested the importance of three variables: the effect of growth phase at the time of harvesting the mycelia; a comparison of regeneration of transformed protoplasts on the commonly used R2 and R9 media; and the effect of regeneration at sub-optimal growth temperature.

Of four liquid media tested (YEME, TSB, LB, and NB), YEME was chosen to support the growth of S. longisporoflavus 4584, because pellet formation in this media was less pronounced. A wire spring within the 250ml culture flask was used to ensure dispersed growth. Triplicate cultures were grown in 25ml YEME containing 5mM $MgCl_2$ and 0.5% glycine, from an inoculum of around $2x10^8$ spores. The mycelia was harvested from each flask after 2, 3, or 4 days by centrifugation at 5000g, 20°C, for 10 min.

Protoplasts were prepared according to the protocol of Hunter (1985), incubating the mycelia in lysozyme solution until most became osmotically sensitive. This was determined microscopically - protoplast spheres are phase dark in contrast to phase bright mycelia, and are lysed on the addition of water - and generally required around 30 minutes incubation.

PEG-mediated transformation was used to introduce 200ng of pIJ680, (prepared from S. lividans), into a 100ul aliquot of each batch of protoplasts. 100ul of each "transformed suspension" was spread, in quadruplicate, on either R2 or R9 regeneration plates (pre-dried as described in Chapter 2.10.1.3). After 24hrs incubation at 30°C or 28°C thiostrepton was added (see Chapter 2.10.1.3) to select for transformants. Surviving cells required a further 8 days incubation before the resultant colonies could be counted (Table 3.5).

The number of transformants recovered was not considered sufficient to allow the construction of a

TABLE 3.5; Transformation Frequency Of S. longisporoflavus 4584 With pIJ680.

mycelia harvested after (hrs)	Transformation efficiency (ug^{-1} ccc DNA) media R2		media R9	
	28°C	30°C	28°C	30°C
48	nd	nd	nd	nd
72	nd	nd	nd	nd
96	nd	nd	$1.3x10^{3*}$	$1.1x10^{3*}$

*this difference may not be significant

nd = not detected

representative gene library in that strain. The efficiency of transformation of blocked mutants is likely to be even less therefore complementation of mutants deficient in the production of tetronasin by self-cloning in S. longisporoflavus would be impractical without further work to increase the efficiency of transformation to manageable levels (at least around $1x10^5$ transformants ug^{-1} ccc DNA).

3.7 SUMMARY

S. longisporoflavus 4584 was shown to be sensitive to short wavelength UV light. The enhanced survival of UV-irradiated spores, when grown in visible light, indicated a photo-repair system to be present in this strain.

The development of a bio-assay, based on the sensitivity of B. subtilis to tetronasin and production of tetronasin on Emersons agar, allowed six UV-induced mutants of S. longisporoflavus, deficient in tetronasin production, to be identified. Two of these (TNP38 and TNP44), were bradiotrophic, whereas the other four (TNP12, TNP56, TNP61 and TNP62), were tight mutants, and all six were shown to have stable phenotypes on Emersons agar.

In co-synthesis tests on solid media, production of a bio-active molecule between TNP38 and TNP62 was detected. This suggested that the mutations in these two strains were different and that they were distinct from the other four mutants. After growth of the strains in YEME liquid media, five of the mutants "reverted" to production of a bio-active compound and confusing their phenotypic classification. Only TNP12 failed to synthesise a bio-active compound and intriguingly this phenotype predominated in pairwise growth of TNP12 with all other mutants in YEME.

Low efficiency transformation of S. longisporoflavus 4584, with plasmid DNA from S. lividans was achieved, using protoplasts prepared from 4 day old mycelia grown in YEME, and regeneration of transformants on R9 media. Efforts to improve the efficiency of transformation of S. longisporoflavus were not made because of the inordinate amount of time and effort that a careful study would require and the lessening importance of the successful application of this cloning strategy in view of progress in others.

The isolation of mutants, potentially blocked at different steps in the tetronasin biosynthetic pathway and the ability to introduce plasmid DNA into S. longisporoflavus may help confirm the function of DNA fragments, isolated by other means.

CHAPTER 4

CLONING AND PRELIMINARY ANALYSIS OF TETRONASIN-RESISTANCE GENE(S) FROM *Streptomyces longisporoflavus*

4.1 INTRODUCTION

As described in Chapter 1.6, the most pragmatic strategy for the isolation of genes for tetronasin biosynthesis reuires first the isolation of the tetronasin resistance gene(s). To adopt this approach, it is assumed that the antibiotic biosynthetic genes of Streptomyces are clustered on the genome - no exception has yet been found - and closely associated with the resistance gene(s), of which examples abound (see Chapter 1.6 Table 1.1). It should be remembered that not all resistance determinants need be closely associated with the gene cluster for antibiotic biosynthesis, in the producing strain. Examples, where "additional" resistance genes have been mapped outside biosynthetic gene clusters include otrC in S rimosus (Hunter and Baumberg, 1989), tlrA in S. fradiae (Seno and Baltz, 1989), aph in S. griseus (Distler et al., 1987), and srmA and srmC in S. ambofaciens (Nagaraja Roa, et al., 1988).

4.1.1 RESISTANCE MECHANISMS

Most micro-organisms which produce antibiotics are themselves intrinsically sensitive to its action. Survival therefore demands specific measures to avoid suicide (Thiara and Cundliffe, 1988). The streptomycetes which have the capacity to synthesise a huge diversity of antibiotics have co-evolved a variety of means, (Table 4.1), to resist the activity of the antibiotic that they produce.

4.1.1.1 Modification of the antibiotic:

This usually takes the form of phosphorylation, adenylation of hydroxyl groups, or acetylation of amino groups, but can include addition of larger residues to the antibiotic e.g. the addition of the tripeptide glutathione to the phosphoenolpyruvate analogue fosfomycin in S. fradiae (Suarez et al., 1988). Well-characterised examples of modifying activities from both actinomycetes and clinical isolates are common (Beneviste and Davies, 1973; Haas and Dowding, 1975). The biochemical modifications render the antibiotic less effective or ineffective in the producing organism. In some cases, but most notably in S. fradiae, two modifying enzymes (aminoglycoside phosphotransferase and acetyltransferase) act synergistically to give high level resistance to neomycin (Thompson et al., 1982b).

TABLE 4.1; Examples Of Cloned And Characterised Antibiotic Resistance Genes From Antibiotic-Producing Actinomycetes

gene	cloned from	mechanism	activity	reference
aphD	S. griseus	1	streptomycin-O-phosphotransferase	Tohyama et al., (1984)
kanR	S. kanamyceticus	1	aminoglycoside acetyltransferase	Murakami et al., (1983)
	S. vinaceus	1	viomycin-O-phosphotransferase	Skinner and Cundliffe, (1982)
aphI	S. fradiae	1	aminoglycoside 3'-O-phosphotransferase	Thompson et al., (1982)
aat	S. fradiae	1	aminoglycoside 3'-N-acetyltransferase	Davies et al., (1979)
ribR	S. ribosidificus	1	ribostamycin phosphotransferase	Murakami et al., (1983)
	S. fradiae	1	fosfomycin glutathione transferase	Suarez et al., (1988)
bar	S. hygroscopicus	1	phosphinothricin acetyltransferase	De Block et al., (1987)
	S. alboniger	1	puromycin acetyltransferase	Vara et al., (1985)
hyg	S. hygroscopicus	1	hygromycin B phosphotransferase	Zalacain et al., (1987)
pph	S. rimosus	1	paromomycin phophotransferse	Perez-Gonzalez and Jimenez, (1984)
pat	S. rimosus	1	paromomycin acetyltransferase	Zalacain et al, (1987)
tsr	S. azureus	2a	23S rRNA methylase	Cundliffe and Thompson, (1979)
ermE	Sac. erythraea	2a	23S rRNA methylase	Skinner and Cundliffe, (1982)
	S. tenjimariensis	2a	16S rRNA methylase	Skeggs et al., (1985)
gyrB'	S. sphaeroides	2b	novel DNA gyrase B	Thiara and Cundliffe, (1988)
otrA	S. rimosus	2b	novel Ef-Tu?	Doyle et al., (1988)
otrB	S. rimosus	3	efflux of oxytetracycline	Ohnuki et al., (1985a)

1 = modification of the antibiotic
2 = target site resistance
  a, modification of the target site
  b, synthesis of a novel antibiotic-resistant target site
3 = efficient export of the antibiotic

In contrast, enzymes catalysing the reverse reaction are not well documented. They may also be required, such that upon release from the cell the bioactivity of the antibiotic is regained. Majumdar and Majumdar, (1971), demonstrated a direct relationship between the levels of neomycin and an alkaline phosphatase with a high substrate specificity for neomycin phosphate in S. fradiae. Similarly a streptomycin-phosphate phosphohydrolase from S. griseus and an acetylkanamycin iminohydrolase from S. kanamyceticus have been described (Walker and Walker, 1971; Satoh et al., 1975).

Some antibiotic modifying enzymes may also be required for the catalysis of intermediate steps in antibiotic biosynthesis. This dual role was demonstrated for the bialaphos resistance gene (bar) product (phosphinthricin acetyltransferase: PAT), in S. hygroscopicus (Kumada et al., 1988). The bar gene can confer resistance to bialaphos in a heterologous host. Therefore PAT can presumably catalyse the acetylation of phosphinothricin post-synthetically. However, in S. hygroscopicus, PAT is also required to acetylate an intermediate in bialaphos biosynthesis, namely, demethylphosphinothricin (DMPT). bar$^-$ mutants accumulate DMPT, and fail to synthesise bialaphos. However they retain the ability to convert N-acetyl DMPT to bialaphos. This suggests that N-acetyl DMPT, but not DMPT, is a substrate for the next enzyme in the pathway, which catalyses the addition of alanylalanine to the carboxyl group of N-acetyl DMPT, forming N-acetyl demethylbialaphos. The bar gene therefore catalyses an essential step in the biosynthesis of bialaphos and ensures at the same time that S. hygroscopicus is protected from the antibiotic activity of the product. The de-acetylation of N-acetyl bialaphos is demonstrably catalysed in a later stage of bialaphos biosynthesis, removing the modification and restoring potential activity to the phosphinothricin moiety of the product.

Puromycin N-acetyltransferase may play a similar role in puromycin biosynthesis in S. alboniger (Vara et al., 1985). This enzyme can acetylate both puromycin and the precursor O-demethylpuromycin. However, N-acetyl-O-demethylpuromycin was shown to be a better substrate for the methylase enzyme than O-demethylpuromycin (Roa et al., 1969).

4.1.1.2 Target site resistance:

a. Enzymatic modification of the target site,

Several antibiotics bind to the prokaryotic ribosome to inhibit translation. One example is thiostrepton resistance (*tsr*) in *S. azureus*, which encodes a methylase which modifies the 23S rRNA. Thiostrepton binds almost irreversibly to the 50S subunit of prokaryotic ribosomes. It has been proposed (E. Cundliffe, personal communication), that thiostrepton recognises a particular conformation of the ribosome with resultant loss of function by effectively freezing the translational machinery in that state. Of course, it is equally plausible that the antibiotic simply binds to an active site of the RNA/protein complex preventing an essential reaction in protein synthesis. Perhaps favouring the latter explanation, is the observation that the methylation site for the thiostrepton-resistance gene product is located in a very highly conserved region of the 23S rRNA. This suggests a catalytic role for the stem-loop structure involved. However the importance of the domain with regard to ribosomal configuration cannot be discounted.

The ansamycin group of antibiotics including rifamycin, streptovaricin and tolpomycin, inhibit DNA dependent RNA polymerase. Self resistance in the producing organisms, manifested by RNA polymerase insensitivity, is widespread (Roza *et al.*, 1986). However the molecular mechanism of resistance is unknown, and therefore it is not clear whether the ansamycin producers should occupy this category or the next.

b. Synthesis of a novel resistant component,

This is epitomised by novobiocin resistance in *S. sphaeroides*. Novobiocin is a powerful inhibitor of DNA gyrase subunit B. DNA gyrase is a heterodimer topoisomerase, comprising subunits A_2B_2, which introduces negative supercoils into covalently-closed circular DNA. The different subunits have defined activities - the A protein is a nuclease, whereas the B protein binds and hydrolyses ATP energising the reaction. In concert with topoisomerase I (which relaxes negatively-supercoiled DNA), DNA gyrase controls the superhelical density of DNA within the cell.

Thiara and Cundliffe (1988) cloned and identified from *S. sphaeroides* a novel DNA gyrase B subunit (*gyrB'*) whose function was intrinsically resistant to novobiocin and

was induced by novobiocin. Curiously, S. sphaeroides possesses an alternative, novobiocin-sensitive, DNA gyrase B subunit (gyrB). Why gyrB has not been rendered obsolete by the resistant subunit is not clear. It may infer a role in antibiotic biosynthetic gene regulation for gyrB' or possibly the recent acquisition, in evolutionary terms, of novobiocin synthesis by streptomycetes. Regulation of the expression of gyr B' is most interesting, being demonstrably under the control of a promoter sequence with sensitivity to the superhelicity of the DNA. In a manner reminiscent of E. coli gyrB, ompF and tonB regulation (Higgins et al., 1988), gyrB' was induced when the superhelicity of the genomic locus was reduced - a consequence of inhibiting the normal DNA gyrase by novobiocin - and repressed by an increase in superhelicity which can result from DNA gyrase overactivity or high osmotic stress of the culture. It is unknown whether the indigenous gyrB is sensitive to superhelicity and its isolation is eagerly awaited, to permit the investigation not only of the regulation of gene expression but also to facilitate a comparison of the resistant and sensitive forms of the two proteins. This should achieve a better understanding of how this species has solved the problem of auto-immunity.

c. Absence of target site,

For those antibiotics with biological activity against target sites of higher organisms a resistance mechanism within the producing streptomycete may be obsolete. Examples include the avermectins, glycosylated macrolide structures produced by S. avermitilis ATCC 31267 (patent number NL 7703810), and a similar series of unglycosylated compounds, the milbemycins which are the products of Streptomyces sp. NRRL 5379 (patent number DT 2329486). Both groups are commercially-important antihelmintics (the avermectins have more potency), and also have insecticidal and acaricidal properties. The debatable issue here, concerns the need for a specific avermectin and/or milbemycin resistance gene in the producing organism. Avermectin is thought to act via the gamma-aminobutyric acid (GABA) receptors of neuromuscular synapses (Kass et al., 1984). GABA is an inhibitory neuromuscular transmitter in nematodes. Its action results in hyperpolarisation of muscle cells by increasing the transport of Cl^- ions (del Castillo et al., 1964). Over exposure to GABA causes a flaccid

paralysis in Caenorhabditis elegans (Lewis et al., 1980). By comparison, avermectin paralyses both Ascaris and C. elegans, but does not produce a flaccid response (Kass et al., 1980). The absence of any known sensitive micro-organism suggests that the target site is restricted to these higher organisms. Such speculation makes it difficult to propose an attractive teleological justification for the biosynthesis of these compounds, unless one imagines free living nematode worms are predators of streptomycetes or at least in competition for nutrients. The genes determining the biosynthesis of avermectin and related antibiotics may therefore represent positive evidence for the selfish gene hypothesis which was discussed in Chapter 1.2. It is possible that a group of sensitive organisms exist which constitute more reasonable grounds for an evolutionary selective advantage. However these organisms still remain unidentified, despite the endeavours of industry to find a more practical bioassay than activity against C. elegans.

4.1.1.3. Export of the antibiotic from the cell:

Active efflux of the antibiotic from the cell avoids intracellular accumulation to inhibitory levels. OtcII (now renamed otrB), cloned from S. rimosus 15883 by Rhodes et al (1984), provides an example of this resistance mechanism. OtrB is one of three resistance genes of the oxytetracycline producer S. rimosus 15883. In contrast to the knowledge accumulated on the subject of antibiotic transport into sensitive organisms (Chopra, 1988) very little is known about the mechanism of antibiotic transport from the producing cell. OtrB mediates oxytetracycline resistance by preventing accumulation of the antibiotic in the cell. In this respect it is reminiscent of tetracycline resistance found in Gram-negative enteric bacteria. DNA sequence analysis of otrB (I. S. Hunter, pers. comm.), the methylenomycin resistance determinant - mmr (Neal and Chater, 1987) - and a region of the act gene cluster (D. A. Hopwood, pers. comm.) has indicated that resistance to methylenomycin and actinorhodin as well as oxytetracycline might be conferred by integral membrane proteins. The predicted product of mmr also exhibits some homology at the amino acid level to the N-terminal 200aa of TetA from Tn10. Furthermore the arrangement of ORF's around tetA, mmr, otrB and the act region were similar. Interestingly the divergent transcript

from the act region exhibits homology to tetR from Tn10 and is therefore postulated to function as a repressor of the putative actinorhodin resistance gene.

Resistance to certain macrolide antibiotics might also occur by similar means. Fierro et al., (1987 & 1988) reported that resistance to the macrolide antibiotics, spiramycin, carbomycin, tylosin and oleandomycin, in the respective producers - S. ambofaciens, S. halstedii (and S. tendae), S. fradiae and S. antibioticus - was not mediated by modification of the ribosome or antibiotic. Mycelia of the producing-strains was shown not to absorb exogenous antibiotic from the culture media, which led the authors to propose that the cell walls of the producing-organisms were impermeable to the active-extracellular antibiotic.

4.1.2 SYNERGISM BETWEEN RESISTANCE DETERMINANTS: A CAUTIONARY NOTE FOR GENE CLONING

It is important to remember that a number of resistance mechanisms acting in concert may be used by S. longisporoflavus. Antecedent examples of possible synergistic resistance genes include, oxytetracycline resistance in S. rimosus and neomycin resistance in S. fradiae (see below), and tylosin resistance in S. fradiae (Seno and Baltz, 1989; A. Orekhov, pers. comm.). There also exists a growing number of cases where multiple resistance genes within the one antibiotic-producing strain have been identified, but whose interaction has not been investigated; these include carbomycin resistance in S. thermotolerans (Epp et al., 1987; Schoner et al., 1988), and spiramycin resistance in S. ambofaciens (Nagaraja Rao et al., 1988).

As mentioned in section 4.1.1.1 resistance to neomycin in S. fradiae is mediated by two resistance genes (aphI and aat) encoding neomycin modifying enzymes (aminoglycoside phosphotransferase and acetyltransferase respectively), which function synergistically to give high level resistance to neomycin (Thompson et al., 1982b). Oxytetracycline resistance in S. rimosus may also depend on synergism between resistance mechanisms. Two resistance determinants, OtrA and otrB (which flank the oxytetracycline biosynthetic gene cluster - Butler et al., 1989) are inducible two fold with pre-exposure to sublethal levels of oxytetracycline (Ohnuki et al., 1985). In isolated studies in the heterologous host S. griseus, otrA

and otrB were shown to determine maximal inducible levels of resistance to 250ug ml^{-1} and 300ug ml^{-1} tetracycline respectively. The parental strain - in these studies S. rimosus ATCC 10970 - would tolerate a maximum of 1200ug ml^{-1} tetracycline. A third gene OtrC was found to confer only low levels of resistance in heterologous hosts (I. S. Hunter, pers. comm.). The discrepancy in these resistance levels, suggests that the level of resistance found in the parental strain to be the result of synergism between the three genes. However, the ability of each gene to confer tetracycline resistance in a heterologous host may not reflect its activity in the parental strain. This is an important point, because an isolated piece of DNA, outwith the regulatory control circuit of the genomic locus (see Chapter 1.8.7), and now contained on a high copy number plasmid, may confer a very different phenotype on the heterologous host. These are exactly the conditions which pertain in many gene libraries. Therefore, selection or screening for specific gene products should not be too stringent and should allow clones with "imperfect" phenotypes to be examined further. This should prevent genes which normally function synergistically or have an altered activity, from being rejected in the initial stages of selection.

4.1.3 TETRONASIN RESISTANCE

The mode of action of polyether ionophore antibiotics (see Chapter 1.4.1) makes it difficult to conceive a simple effective means of auto-immunity. Faced with active extracellular tetronasin (spent culture broth has bioactivity against several organisms, see Chapter 3.4), how does S. longisporoflavus prevent depolarisation of its cell membrane? Modification of the antibiotic although practicable does not seem pragmatic. Even if S. longisporoflavus modifies the antibiotic intracellularly, the modification is removed upon export from the cell. Clearly with the cell membrane as the target site problems are created for a resistance mechanism based on modification of the antibiotic. Even if the modifying activity was an integral part of the cell membrane, its affinity for tetronasin would require to be high to protect against depolarisation.

Ionophore antibiotics have been shown to have a depolarising effect on many different substrates, including

microorganisms, animal cells, mitochondria, and simple synthetic lipid bilayers (Pressman, 1976). It is therefore difficult to imagine that resistance to tetronasin is mediated via modification of the cell membrane or synthesis of a novel form of cell membrane which is intrinsically resistant to tetronasin activity. If genetic modification of the cell membrane is a common strategy, then it does not confer cross resistance to all other ionophore antibiotics because as described in this chapter, S. albus G153, a known polyether-producer, is highly sensitive to tetronasin.

A variation of this theme which may be worth consideration, is to propose the existence of a barrier between the cell membrane and the extracellular environment which restricts the access of extracellular ionophore to the cell membrane. This barrier could be a facet of the cell wall or an extracellular matrix with selective permeability. Whether the coding genes for such a resistance mechanism could be cloned on a small fragment of DNA is an open question. However to be effective it may require the existence of a tetronasin efflux system which may be possible to isolate in such a manner. An efflux system located in the cell membrane may not necessarily be sufficient to confer resistance to a high titre of antibiotic, so cloned DNA conferring low level tetronasin-resistance properties should be characterised. A similar role could be performed by cation "pumps" or tetronasin modifying enzymes located in the cell membrane, provided that the antibiotic was activated upon efflux.

Other potential mechanisms could include sequestration or trapping of the antibiotic. A protein associated with the outer surface of the cell could be imagined to bind tetronasin thus preventing the ionophore from crossing the cell membrane.

Hopefully analysis of resistance determinants isolated from S. longisporoflavus will further the understanding of how self-resistance to ionophore antibiotics is mediated.

4.2 CONSTRUCTION OF A GENOMIC LIBRARY OF Streptomyces longisporflavus 83E6 DNA

4.2.1 CHOICE OF CLONING RECIPIENT

It was hoped to isolate the resistance gene(s)

for tetronasin by screening a genomic library of S. longisporoflavus DNA constructed in a cloning host which was sensitive to the drug. This was not the sole criterion of a suitable cloning host. Efficient protoplast transformation and a restriction minus phenotype for DNA from S. longisporoflavus were required. Four species: S. albus G153, S. coelicolor G94, S. lividans TK24, and S. rimosus G7 were investigated as putative cloning recipients for this experiment.

a. Sensitivity To Tetronasin

The sensitivity of each of the four putative cloning recipients to tetronasin was studied. A number of important variables were tested;

(i) Concentration of tetronasin;

To determine the minimum inhibitory concentration for each species, and indicate a suitable level to select for resistance gene(s), a series of agar plates were poured with increasing levels of tetronasin (0-100ug ml^{-1}). Each of the four test species were inoculated onto the agar plates. Growth of the inocula under selection was compared by visual inspection (a reasonable assay considering tetronasin-resistance clones would need to be identified by eye) with growth in the absence of the drug.

(ii) Inoculum material;

Spores and mycelia represent two very different growth stages of Streptomyces. Metabolically, spores are relatively inactive, exhibiting only a low level, turnover respiratory rate or are absolutely dormant (Hirsch and Ensign, 1976), waiting to germinate in response to environmental signals. By comparison, mycelial metabolism is actively converting extracellular nutrients into cellular material, and at the same time maintaining a homoeostatic intracellular environment in the face of changing ambient conditions. They also differ in their respective cell wall structures. Although there is little data on the subject, the spore comprises an outer layer of loosely associated, unidentified hydrophobic material surrounding a spore sheath of chitin (Smucker, 1984). Streptomycete spores are not particularly resistant structures although there is some protection from desiccation, light and enzymatic attack (Ensign, 1978). Spore structure is better defined in other genera. For example, studies on Bacillus sp. have indicated

the complex cell wall structure of the endospore and the presemce of dipicolinic acid to be fundamental to resistance to desiccation and radiation damage and protection from attack by either enzymatic or chemical agents (Gould and Hurst, 1969).

Spore suspensions were prepared as described in Chapter 2.8.2.1 mycelial fragment suspensions were isolated from 5 day old liquid cultures; spinning out the mycelia at 10 000g for 10 minutes, washing once in distilled water before resuspending in distilled water. 10ul of both preparations were pipetted onto the agar plate and spread using a sterile tooth pick. Substrate mycelium may vary in sensitivity to tetronasin with age, although no attempt was made to assess the effect at this stage.

(iii) Growth medium;

The level of growth inhibition by tetronasin was determined on several substrates. Each species was tested on TSB agar, Emersons agar, and MMT agar. S. lividans and S. coelicolor were also tested on protoplast regeneration medium R2, while S. albus and S. rimosus were tested on the protoplast regeneration medium R9.

The results of these experiments (Tables 4.2, 4.3, and 4.4) indicated the suitability of S. albus G153 as a tetronasin-sensitive cloning recipient. The spores and mycelia of this species were highly sensitive to 10ug ml^{-1} tetronasin on all substrates tested.

S. rimosus G7 was sensitive to 10ug ml^{-1} tetronasin in R9 agar, but a low level of growth with spore inocula on Emersons and TSB agars indicated spore germination and outgrowth to occur in the presence of tetronasin on these substrates. No growth of S. rimosus spores on MMT in the absence of tetronasin, precluded an estimation of the sensitivity of the strain to the drug on this substrate.

In general the growth of all four Streptomyces sp. on MMT was poor.

S. lividans and S. coelicolor spores both exhibited some resistance to tetronasin which was enhanced on the regeneration medium R2. S. lividans mycelia was sensitive throughout the experiment however working with spore inocula is more practicable, particularly for replica plating transformants from protoplast regeneration agar to selective media. TSB agar did not allow sporulation of S. lividans.

TABLE 4.2; Growth Of *S. coelicolor* G94 In The Presence Of Tetronasin

Media	Inoculum	Concentration of tetronasin (ug ml^{-1}) 0	10	20	40	70	100
TSB	spores	***	*	*	*	*	*
	mycelium	***	*	*	*	*	*
Emersons	spores	***	*	-	-	-	-
	mycelium	***	*	-	-	-	-
MMT	spores	***	*	*	*	*	*
	mycelium	***	*	*	*	*	*
R2	spores	***	***	***	***	***	***
	mycelium	***	**	**	**	**	**

Growth was estimated on a scale of * to *** where *** represents growth in the absence of antibiotic. The same key pertains for Tables 4.3, 4.4 and 4.5.

TABLE 4.3; Growth Of *S. lividans* TK24 In The Presence Of Tetronasin

Media	Inoculum	Concentration of tetronasin (ug ml^{-1}) 0	10	20	40	70	100
TSB	spores	***	*	-	*	-	-
	mycelium	***	-	-	-	-	-
Emersons	spores	***	*	*	*	*	*
	mycelium	***	-	-	-	-	-
MMT	spores	***	*	*	*	*	*
	mycelium	***	-	-	-	-	-
R2	spores	***	**	**	**	**	**
	mycelium	***	-	-	-	-	-

TABLE 4.4; Growth Of *S. rimosus* G7 And *S. albus* G153 In The Presence Of Tetronasin

Strain	Media	Inoculum	Concentration of tetronasin (ug ml^{-1}) 0	10	20	40	70	100
G7	TSB	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	MMT	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	Emers.	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	R9	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
G153	TSB	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	MMT	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	Emers.	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-
	R9	spores	***	-	-	-	-	-
		mycelium	***	-	-	-	-	-

Selection of tetronasin resistance determinant(s) in S. coelicolor with spore inocula might have been possible on Emersons agar, but background growth was observed with all other substrates.

As a practical test to assess selection after replica plating and to check that the thiostrepton selectable marker of streptomycete plasmids did not confer tetronasin resistance pleiotropically, sporulated colonies of each putative cloning recipient, containing pIJ702, were transferred from protoplast regeneration agar plates to media with both thiostrepton and tetronasin by replica plating via a velvet impression. The results of this experiment (Table 4.5) indicated that there was no background growth of S. albus G153/pIJ702 when replica plated to Emersons or R9 agar containing 10ug ml^{-1} thiostrepton after 7 days, even at 10ug ml^{-1} tetronasin. S. rimosus could also be used to screen for tetronasin resistance, particularly on R9 based selection media. S. lividans TK24, however, still exhibited unacceptable levels of background growth - despite the retarded growth rate it was considered unlikely that a tetronasin resistance clone could be identified in a nearly confluent lawn of sensitive cells.

b. Transformation Of The Putative Cloning Recipients With Plasmid DNA

The preferred method (see section 3.7) for the introduction of plasmid DNA or naked phage DNA into streptomycetes requires first the removal of much of the cell wall with lysozyme. Competence of these osmotically-labile protoplasts can be very high, provided that optimal conditions for protoplast formation, transformation and regeneration are discovered. The technique adopted here (Chapter 2.10.1) was essentially the same as used by Hunter (1985) which is based on previous work by Okanishi et al., (1974), Bibb et al., (1978) and Thompson et al., (1982a). Minor modifications, in particular to the growth phase of the culture when harvested are detailed in Chapter 2 for high efficiensy transformation of both S. albus and S. lividans.

Previous work (Thompson et al., 1982; Okanishi et al., 1984; Baltz and Matsushima, 1981) implicated the importance of the age of a streptomycete culture when harvested, for the preparation of protoplasts. A preliminary experiment indicated that the most competent S. lividans protoplasts

TABLE 4.5; Transfer And Growth Of The Putative Cloning Hosts Transformed With pIJ702, To Media Containing Thiostrepton (10ug ml^{-1}) And Tetronasin

Strain	Media	Conc^{n} tetronasin (ug ml^{-1})	Growth (days) 1	2	3
S. lividans TK24/pIJ702	MMT	0	***	***	***
		10	-	*	**
		40	-	*	**
S. albus G153/pIJ702	Emers.	0	***	***	***
		10	-	-	-
		40	-	-	-
	R9	0	***	***	***
		10	-	-	-
		40	-	-	-
S. rimosus G7/pIJ702	Emers.	0	***	***	***
		10	-	*	*
		40	-	-	-
	R9	0	***	***	***
		10	-	-	-
		40	-	-	-

were prepared from a 65hrs culture (Table 4.6). In a similar test the best S. albus protoplasts were found to be obtained from a 24hr culture (Table 4.7), verifying the work of Hunter and Friend, (1984). Unfortunately plasmid DNA, from S. lividans, could not be introduced into S. coelicolor or S. rimosus with high efficiency. The reason for the importance of the growth phase for the preparation and transformation of S. lividans and S. albus, was not investigated. It may well reflect the condition of the cell wall and its susceptibility to lysozyme degradation. It is equally plausible to suggest that the number of protoplasts in a transformation reaction may effect the observed frequency of plasmid uptake and establishment in the cell. For example the amplified number of cells in a three day culture of S. lividans may simply supply an increased number of competent protoplasts for interaction with the DNA, resulting in nearly a 100 fold increase in transformation efficiency over protoplasts prepared from younger cultures. With S. albus, a reduction in the number of cells may decrease the concentration of extracellular DNase, or as likely, reduce the number of non-protoplasts which might interact with the DNA, but not result in uptake and stable maintenance of the plasmid. The latter explanation is more credible given that S. albus G153, although deficient in the restriction enzyme SalG1, harbours another restriction system which, as described later in this chapter, is active against plasmid and genomic DNA from S. longisporoflavus. Using a low titre of S. albus protoplasts in a transformation reaction may saturate the DNA uptake sites on the surface of the protoplasts, resulting in optimal internalisation of the DNA and increased chance of any molecule escaping degradation to become established in the cell. The presence of mycelial fragments in protoplast preparations has also been implacated to inhibit the regeneration in some species (Baltz and Matsushima, 1981).

Another parameter investigated with the aim of improving the transformation efficiency of S. albus, was the incubation temperature of the regenerating protoplasts. The literature suggests that a sub-optimal growth temperature for regenerating protoplasts gives improved results, (Baltz, 1978). To test this S. albus protoplasts transformed with pIJ702 were plated in duplicate on R9 agar plates and allowed to regenerate at 30, or 34^{o}C. The results (Table 4.7)

TABLE 4.6; The Relationship Between The Age Of A *S. lividans* TK54 Culture At The Time Of Harvesting And The Competence Of The Protoplasts Obtained

mycelia harvested after (hrs)	average No. of transformants	dilution factor	transformants (ug^{-1} pIJ702)
30	312	10	7.8×10^5
46	128	10^{-2}	3.2×10^6
65	392	10^{-3}	9.8×10^7

TABLE 4.7; The Importance Of The Age Of The Culture At The Time Of Harvesting The Mycelia, And The Regeneration Temperature Of The Protoplasts, For Transformation Of *S. albus* G153

mycelia harvested after (hrs)	transformants per 100ul aliquot (ug^{-1} pIJ702) regeneration temp. (^{o}C)	
	30	34
24	$2.2x10^{6}$	nt
48	$2.1x10^{5}$	$7.8x10^{4}$
72	$1.0x10^{5}$	nt

nt = not tested

indicated that regeneration at 30°C yielded more transformants.

The experiments conducted here define conditions for very high transformation efficiency of S. lividans and adequately high transformation efficiency of S. albus for the construction of a genomic library in that strain.

c. Restriction Status Of The Putative Cloning Hosts;

The restriction status of the putative hosts was the final criterion for the selection of a suitable cloning recipient. This may be simply assessed by measurement of infection efficiencies by actinophage between hosts.

The importance of divalent cations in phage infection of Streptomyces is well established (Dowding, 1973). The concentration of Mg^{2+} and Ca^{2+} giving best efficiency of infection varies with the host strain. The conditions for the infection of S. albus, S. lividans and S. rimosus by the actinophage used are given in Chapter 2.10.3. The infection of S. longisporoflavus 4584 by four actinophage was tested at both high and low salt concentrations. S. longisporoflavus was found to be a host, at both high and low salt concentrations, for three of the four actinophage tested:

R4; isolated from S. albus, widely reported to be most sensitve to restriction (Chater and Carter, 1979).

R4G2; a mutant of R4 with a wider host range, less sensitive to restriction (Chater and Carter, 1979).

øC31; well characterised and developed into cloning vectors by Chater and Bruton (1983). One such attachment site deleted vector, øKC515, gave lytic plaques as expected, on S. longisporoflavus.

S. longisporoflavus was not a host for the actinophage TP1, characterised by Czaplewski, (1989).

Infection efficiencies of actinophage on Streptomyces can be measured by the standard double layer plate assay (Chapter 2.10.2). Comparison of the efficiency of infection of R4 on a restrictive host with a non-restrictive

host, after propagation in a non-modifying host, should be manifested as a substantial decrease in infection of the restrictive host. To check whether R4 could reflect a plasmid restriction barrier, a known plasmid restriction barrier between S. lividans TK24 (r-) and S. coelicolor G94 (r+) was tested. The result of this experiment (Table 4.8) was inconclusive. The literature suggests that phage restriction is not a feature of strain G94 (Lomovskaya et al., 1980), however the two log difference in plaque formation efficiency between S. coelicolor and S. lividans, may be explained by restriction, if modification of R4 DNA in S. coelicolor is incomplete. Alternatively the lower efficiency of plaquing observed in S. coelicolor may simply reflect a reduced efficiency of adsorption to the host cell, as the consistency of the result, irrespective of the previous host, would seem to testify.

A clear result showing the absence of a restriction barrier for actinophage R4, between S. longisporoflavus, and the potential cloning hosts - S. albus G153, S. lividans TK24 and S. rimosus G7 - in either direction was obtained (Table 4.9). This was not a surprising result with S. albus G153, because R4 was isolated from soil by infection of a SalGI restriction mutant of S. albus G. Its interaction with a second restriction system of S. albus may therefore be minimal. Whether this data would reflect the restriction status of S. albus G153 for plasmid constructs containing DNA from S. longisporoflavus was open to speculation.

A summary of the findings (Table 4.10) identified S. albus G153 as a suitable cloning host for the construction of a genomic library of S. longisporoflavus DNA. Subsequently (section 4.2.4) failure to construct a representative library in the preferred host was shown to be due to a plasmid restriction barrier between S. longisporoflavus and S. albus G153. S. lividans was then chosen as an alternative host. The extremely high transformation efficiency obtained with S. lividans was very attractive for the purpose of library construction. Transfer of the library to S. albus would be required to identify tetronasin resistance clones unless a refined screen in the primary host could be developed.

TABLE 4.8; Efficiency Of Propagation Of Actinophage R4 On *S. coelicolor* G94 In Comparison With *S. lividans* TK24.

previous hosts of R4*	e.o.p.
TK24,	5.0×10^{-3}
TK24,G94,	1.3×10^{-1}
TK24,G94,TK24,	7.5×10^{-2}
TK24,G94,TK24,G94,	2.5×10^{-2}
TK24,G94,TK24,G94,TK24,	3.2×10^{-2}

*Hosts are ordered, so that the last host is given last. The number of plaques produced on strain TK24 was always greater and this value was thus denoted unity.

TABLE 4.9; Efficiency Of Actinophage R4 Propagation On S. albus G153, S. lividans TK24, And S. rimosus G7 In Comparison With S. longisporoflavus 4584

previous hosts of R4	e.o.p.
4584,	1.12
4584,G153,	1.02
4584,	1.36
4584,TK24,	0.82
4584,	0.26
4584,G7,	0.38

Host strains are ordered as for Table 4.8. In each independent experiment the number of plaques produced on strain 4584 was denoted unity.

TABLE 4.10; A Comparison Of The Four Putative Cloning Recipients

species	growth on tetronasin at 10ug ml^{-1}	restriction of phage R4 from strain 4584	protoplast trans. effic. (ug^{-1} pIJ702)
S. albus G153	sensitive	absent	$2x10^6$
S. rimosus G7	sensitive	absent	poor
S. lividans TK24	variable	absent	$1x10^8$
S. coelicolor G94	variable	nt	poor

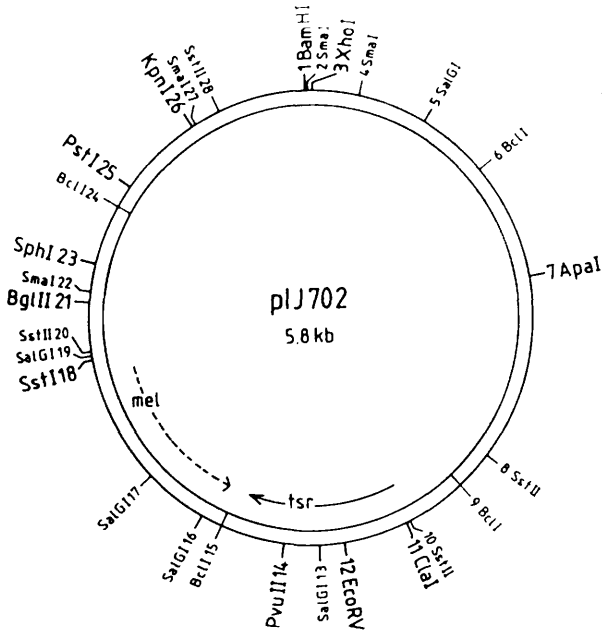
nt = not tested

4.2.2 CHOICE AND PREPARATION OF CLONING VECTOR

Four plasmid vectors were used at various points in this study. All four are non-conjugative derivatives of the natural pIJ101 replicon, and as such are high copy number vectors (40-300 copies per chromosome - Kieser *et al.*, 1982). Each carries the *tsr* gene (from *S. azureus*), which confers resistance to thiostrepton by methylation of the rRNA (Thompson *et al.*, 1982b), providing a selectable marker for propagation in *Streptomyces*.

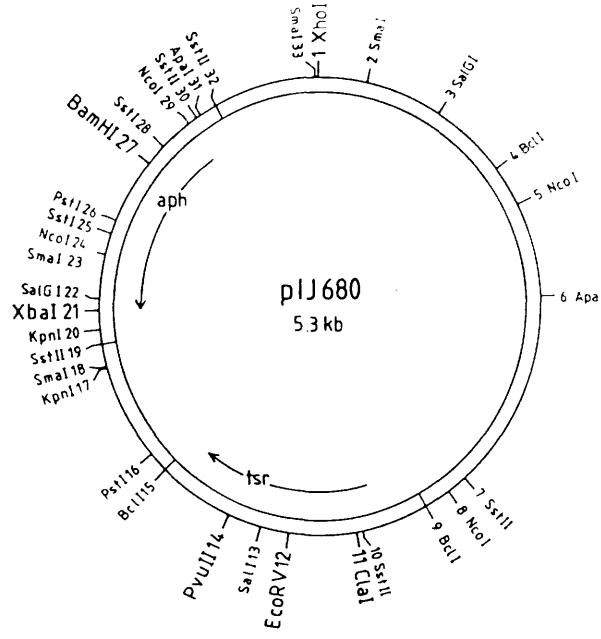
pIJ702; A 5.65kb plasmid (Figure 4.1a), which carries the *mel* gene from *S. antibioticus* (Katz *et al.*, 1983). Cloning *Sau*3A fragments into the *Bgl*11 site, introduces DNA 5' to the *mel*C gene. This plasmid was used in initial experiments to construct a genomic library in *S. albus* G153. Its use was discontinued, in favour of pIJ680, when it became apparent that the *mel* fragment phenotype was unstable in cloning experiments. The *mel*C gene encodes tyrosinase which catalyses the formation of melanin. Transformed colonies should be dark brown on suitably supplemented medium. However a number of *mel*$^-$ transformants were isolated, which contained non recombinant pIJ702, indicating that screening for cloned DNA by following the expression of the chromogenic marker was unreliable. The number of *mel*$^-$, thiostrepton resistant transformants increased in cloning experiments, but a significant proportion of these were found to be non-recombinant. This appeared to be the result of both the removal of the *Bgl*II sticky ends during preparation of the vector DNA, and the intrinsic instability of the phenotypic expression of the marker (Table 4.14). Conceivably, brown transformants with recombinant plasmids could also occur because DNA fragments with promoter activity, cloned into the *Bgl*11 site, may direct transcription of the *mel*C gene.

pIJ680; A 5.3kb plasmid (see Figure 4.1b), which carries the *aph*I gene, i.e. the neomycin phosphotransferase from *S. fradiae* (Thompson *et al.*, 1982b). The removal of the *Bam*HI site of pIJ101 (Eckhardt, T. cited Hopwood *et al.*, 1985a) allowed the unique *Bam*HI site in the *aph*I gene to be utilised for insertional inactivation. Here again, neomycin sensitivity of the cells was not a totally-reliable indicator of recombinant plasmids because *aph*I, as noted in section



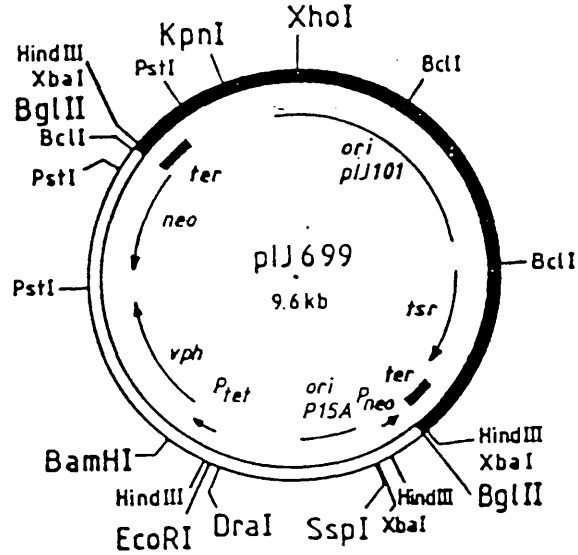
a. pIJ702

(after Hopwood et al., 1985a)



b. pIJ680

(after Hopwood et al., 1985)



c. pIJ699

(after Keiser and Melton, 1988)

FIGURE 4.1; Restriction Maps Of The Cloning Vectors Used In This Chapter

4.1.2, does not confer a high level of resistance, even in high-copy numbers (see 4.2.5). This rendered phenotypic classification of transformants somewhat ambiguous. Although a representative genomic library was constructed using pIJ680, it contained a high background of non-recombinant cells. This problem was circumvented by using pIJ699.

pIJ699; A 9.6kb vector (Figure 4.1c) developed by Kieser and Melton, (1988). The vector comprises both an *E. coli* (P15A) and a streptomycete (pIJ101) origin of replication, separated by transcriptional terminators from phage *fd*. It could therefore be used as a shuttle vector with unique *Bam*HI, *Dra*I, and *Eco*RI restriction sites. In this study however it was employed, by virtue of its twin phage *fd* terminators, as a positive-selection cloning vector to maximise the frequency of recombinant plasmids in a cloning experiment. Digestion of pIJ699 with *Bgl*II released the 5kb fragment containing the pIJ101 origin, *tsr* and two copies of the 331bp phage *fd* terminator sequences, found in indirect repeat at either end of the linear molecule. Circularisation of this molecule generates a perfect palindrome which will not be maintained in *S. lividans*. Plasmids containing an insert between the two terminators are therefore favoured upon transformation of *S. lividans*.

High copy-number vectors were chosen for ease of manipulation, and because it was hoped that low level tetronasin-resistance determinants might have an enhanced phenotype at high copy number. If a tetronasin resistance gene was not isolated from a representative library it would then be prudent to attempt the cloning in a low copy-number vector, because high copy-number expression of the gene or linked genes might be deleterious to the cell.

All vectors were prepared for use in cloning experiments in a similar manner. To accept genomic DNA partially digested with *Sau*3A digestion of the plasmid DNA with a restriction enzyme which generates complimentary 5' overhangs (sticky ends) was required. For pIJ702 and pIJ680, *Bam*HI was used, and for pIJ699 *Bgl*II was used. pIJ699 was also digested simultaneously with *Eco*RI. This double digest was carried out, with high efficiency, in 1xKGB buffer (Chapter 2.14), and cleaved the 4.6kb "stuffer" fragment into 2.9kb and 1.7kb fragments, to ease purification of the 5kb linear molecule.

After digestion the DNA was treated with phenol to remove the restriction enzyme. The aqueous phase was extracted twice with chloroform and once with ether, prior to precipitation with ethanol. The DNA pellet was resuspended in 1 x Calf Intestinal Alkaline Phosphatase (CIAP) buffer and treated with CIAP to remove the 5' phosphate group from the linear DNA. In principal phosphatasing the vector, increases the frequency of clones containing an insert, by inhibiting the rejoining of the complimentary ends of the vector molecule. This should not be necessary with the positive-selection cloning vector pIJ699; however the treatment was completed to ensure maximal intermolecular ligation by preventing intramolecular "self" ligation. After phosphatasing, the solution was again treated with phenol and the aqueous phase was recovered and loaded directly onto a 0.8% (w/v) agarose gel with TAE running buffer. The desired fragment (linearised pIJ702 and pIJ680 or the 5kb pIJ699 BglII fragment) was physically purified from the gel using a "Gene Clean" kit (Chapter 2.18.1). The gel purification step not only facilitated the complete removal of CIAP from the DNA but also purified the desired fragment from any contaminating DNA (this will include uncut or partially digested plasmid DNA, or smaller fragments generated by the digestion of non-ccc DNA).

The vector DNA was tested to verify the efficiency of phosphatasing and to ensure that the ability to ligate with "insert" DNA was retained. This was conveniently assayed by visualising the ligation products after gel electrophoresis, - a worthwhile precaution which could identify preparations of vector DNA which were not usable, avoiding time-consuming transformation and regeneration of Streptomyces protoplasts. Figure 4.2 illustrates the performance of a good preparation of phosphatased pIJ699 in the aforementioned assay, using pUC18 linearised at the BamHI site as "dummy insert".

Lanes 10 and 11 indicate that phosphatased vector would not self ligate. However when a suitable dummy insert (pUC18 cut with BamHI) was available the band representing the 5kb vector DNA disappeared (lanes 8 and 9). Vector DNA ligated to one or more copies of pUC18 is manifested in lane 9 as a complex pattern of ligation products. This "ladder" differs markedly from those generated by self ligation of pUC18 (lane 7) and unphosphatased vector

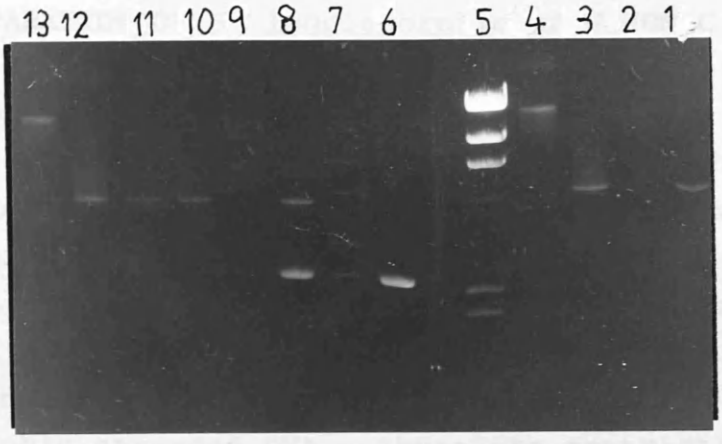


FIGURE 4.2; Assay Of Efficiently-Phosphatased Vector

Lane
1. vector (5kb BglII fragment of pIJ699) before ligation
2. vector after ligation
3. vector + insert (5-15kb Sau3A fragments of *S. longisporoflavus* 83E6 genomic DNA) before ligation
4. vector + insert after ligation
5. bacteriophage lambda DNA cut with HindIII
6. dummy insert (pUC18 cut with BamHI) before ligation
7. dummy insert after ligation
8. phosphatased vector + dummy insert before ligation
9. phosphatased vector + dummy insert after ligation
10. phosphatased vector before ligation
11. phosphatased vector after ligation
12. phosphatased vector + insert before ligation
13. phosphatased vector + insert after ligation

The concentration of reactants in each ligation was constant:
concentration of vector DNA, 3ng ul^{-1}
concentration of insert DNA;
5-15kb Sau3A fragments, 7ng ul^{-1}
linearised pUC18, 5ng ul^{-1}
Less pUC18 "dummy insert" was added because its small size (2.9kb) provided a higher molar ratio of "sticky ends" to DNA concentration than found with the size selected Sau3A fragment preparation.

(lane 2), confirming the formation of heterologous products.

A comparison of lanes 3 and 4 with lanes 12 and 13 shows little difference. However it cannot be assumed that ligation conditions did not favour intramolecular self ligation therefore the two well defined bands of DNA in lane 3 could represent mainly vector circularisation and concatenation as displayed in lane 2.

4.2.3 PREPARATION OF S. longisporoflavus GENOMIC DNA

High molecular weight genomic DNA was prepared from S. longisporoflavus strain 83E6 (Chapter 2.11.2). The length of the DNA fragments recovered was roughly estimated as 50kb to >100kb (Figure 4.3; lane 2). The construction of the genomic library required the generation of a large collection of random genomic DNA fragments in the size range of 5-15kb. This was achieved by partial digestion with the restriction endonuclease Sau3A. Sau3A recognises the tetramer GATC in double stranded DNA - therefore restriction sites are common. Cleavage of the DNA occurs between the cytosine residue and the next base, generating a four base overhang (sticky end) which is complementary to those generated by BamHI and BglII. DNA fragments generated by Sau3A digestion can therefore be cloned into vectors with BamHI (pIJ702, pIJ680), or BglII (pIJ699) sites.

Conditions for the cleavage of the genomic DNA which yielded most fragments in the 5-15kb size range had to be determined. This can be conveniently ascertained by either titrating the enzyme and continuing the digestion for a set time or use of a constant amount of enzyme and altering the duration of the digest. The temporal approach was adopted because it avoids dilution of the restriction endonuclease, which can alter the activity of the enzyme. The reaction products of the test were separated by agarose gel electrophoresis, and a suitable level of digestion chosen after examining the intensity of the ethidium bromide staining of each track. In the pilot experiment 3ug of genomic DNA was dissolved in 25ul of 1 x Sau3A restriction buffer, and equilibrated to 37^{o}C for 15 minutes before the addition of the enzyme. Prior to the addition of 1U Sau3A, 4ul of the DNA sample was removed as the zero time control. During the digestion, 4ul aliquots were removed after 15 and 30 minutes, then every 30 minutes, and the reaction was

FIGURE 4.3 Partial Digestion Of Genomic DNA From *S. longisporoflavus* 83E6 By Restriction Endonuclease *Sau*3A

Lane

1. bacteriophage *lambda* DNA cut with *Hin*dIII
2-7. 500ng genomic DNA from *S. longisporoflavus* 83E6 cut with *Sau*3A for 0, 15, 30, 60, 90, & 120 minutes

terminated by adding the sample directly into gel loading buffer held at 70°C. After 10 minutes the samples were placed on ice, until all six samples were recovered, when the reaction products were separated on a 0.8% (w/v) agarose gel (Figure 4.3).

Seed et al., (1982) determined theoretically that a partial digestion of genomic DNA giving a maximum of fluorescence, by ethidium bromide staining, at 20kb comprises an average fragment size of 10kb. Fifteen minutes of digestion by Sau3A was therefore chosen as the time point generating most fragments in the desired size range. The reaction volume and quantity of enzyme added, were scaled up accordingly to accommodate 1mg of genomic DNA. The DNA concentration and temperature of the reaction were held constant. Fragments between 5kb and 15kb in length were isolated from a 0.8% (w/v) agarose gel by gel electro-elution (Chapter 2.18.2). The size-selected DNA was shown to efficiently concatenate by comparison, in a 0.8% (w/v) agarose gel, of a 100ng aliquot before and after ligation. This was confirmed by the ligation of these DNA fragments to unphosphatased and phosphatased pIJ699 vector shown Figure 4.2 (lanes 3 & 4, and lanes 12 & 13 respectively).

4.2.4 INABILITY TO CONSTRUCT A REPRESENTATIVE LIBRARY OF *S. longisporoflavus* DNA IN *S. albus*: EVIDENCE FOR A SECOND RESTRICTION SYSTEM IN *S. albus*

Digestion of DNA from S. coelicolor protoplasts lysed, in situ then treated with restriction enzymes with recognition sites which occur infrequently in streptomycete DNA, followed by and pulse field gel electrophoresis of the products, enabled Kieser et al., (1988) to determine the genomic complexity of the species. Assuming the size of the S. longisporoflavus genome to be similar (5848kb), the number of recombinants required to constitute a genomic library can be calculated. The binomial theorem (see section 4.2.5) predicts that 5848 clones with an average insert size of 10kb would contain ten genomic equivalents, and give greater than 99.99% probability of complete representation in the gene bank. According to Bibb et al (1980), and Hunter (1985), the transformation efficiency of a primary gene bank can be 100 fold less than when supercoiled, covalently closed, circular substrate is used. S. albus G153 protoplasts, with

transformation efficiency of $6x10^5$ transformants ug^{-1} uncut vector, were used in the first attempt to construct a gene bank. With ligation products these were expected to transform at a level giving $6x10^3$ transformants ug^{-1} vector DNA. Realistically, even after CIAP treatment maybe only half of the transformants would represent recombinant molecules. Using these figures, calculation of the required amount of vector DNA to generate a genomic library was possible.

$$\text{No. of clones required} \Big/ \text{fraction of recombinants expected} \Big/ \text{expected transformation efficiency}$$

$$= 5848 \Big/ 1/2 \Big/ 6x10^3 = 1.96ug$$

2ug of pIJ702 vector, cut with BamHI and phosphatased was ligated to 8ug of 5-15kb size selected Sau3A fragments from S. longisporoflavus 83E6. Ligation was performed at a concentration of 20ug ml^{-1} DNA, with 1U of T4 DNA ligase per microgram of DNA. The reaction was allowed to continue for 2hrs at room temperature, prior to precipitation with ethanol and resuspension of the pellet in 50ul distilled water. Ten aliquots of S. albus G153 protoplasts were each transformed with 5ul of the ligation mix.

Only 75 transformants were regenerated of which 49 were white, ostensibly representing an insert frequency of 65%. This indicated a protoplast transformation efficiency of 37 transformants ug^{-1} vector DNA - much less than expected. Transformation of a further ligation experiment into S. albus G153, incorporated the control transformation of phosphatased vector, before and after ligation. (Table 4.11).

The data indicated digestion with BglII and phosphatase treatment of the vector to have been reasonably successful, with a low number of transformants recovered after self-ligation of vector treated with CIAP. The transformation efficiency of phosphatased vector and DNA Sau3A fragments after ligation however remained low, with very few, if any recombinant molecules expected. The background level of transformants represents a mixture of both, molecules which still have a 5' phosphate group and

TABLE 4.11; Comparison Of The Efficiency Of Transformation Of *S. albus* G153 With Various DNA Substrates

substrate	transformation efficiency (transformants ug^{-1} vector)
supercoiled pIJ702	1.8×10^5
phosphatased vector	2.5×10^2
self ligated phosphatased vector	7.5×10^2
phosphatased vector ligated to insert (*Sau*3A fragments)	7.1×10^2

retain the ability to self ligate (about 2/3 of the transformants), and either contamination of the prepared vector with uncut molecules (only 2.5ng would be required to give 10^2 transformants) or transformation of linear molecules, kinased and ligated intracellularly. If this were to represent a major problem, the extent of transformation by linear molecules can be tested by treatment with S1 nuclease. Linear DNA, after denaturation at high temperature, will be effectively digested by S1 nuclease. The transformation efficiency with DNA treated in this manner would therefore be due to covalently closed, circular molecules.

The problem was not the inability of phosphatased vector to ligate to the insert DNA, but specifically transformation of S. albus G153, because ligation products which transformed this strain giving only 90 transformants ug^{-1} vector DNA, yielded $1.3x10^5$ transformants of S. lividans ug^{-1} vector DNA (only a 50 fold decrease from efficiency of transformation, measured with a supercoiled substrate).

The potential reasons for the enigmatic decrease in transformation of S. albus by these types of ligation mixtures were tested rigorously.

a. Competence of S. albus protoplasts for the uptake of relaxed DNA and sensitivity to ligation buffer.

These are not held to be general problems in Streptomyces, although similar difficulties are recognised in transformation of other genera. For example, competent Bacillus subtilis cells exhibited a preference for the uptake of supercoiled multimers (Canosi et al., 1978) - although this could be overcome in PEG-mediated transformation of protoplasts (Chang and Cohen, 1979). The transformation efficiency of E. coli decreased markedly in the presence of ligation buffer but the problem was circumvented by a 5 fold dilution. No individual component of the ligation buffer was responsible, implicating a synergistic combination of factors (Hanahan, 1983). To test these hypotheses, a preparation of pIJ702 linearised with BamHI was self ligated and transformed into S. albus G153. As a control, S. lividans was transformed with half the ligated DNA. The results (Table 4.12), indicated that neither DNA in a relaxed state nor the presence of ligation buffer adversely affected transformation efficiency - a small drop in efficiency could be explained by multimerisation of the plasmid DNA in vitro.

TABLE 4.12; Comparison Of The Transformation Efficiency Of *S. albus* G153 And *S. lividans* TK24 Protoplasts With Supercoiled pIJ702 And pIJ702 Linearised With *Bam*HI, Which Has Been Self Ligated

strain	transformation effic. with supercoiled pIJ702 (ug^{-1} DNA)	transformation effic. with relaxed pIJ702 (ug^{-1} DNA)
S. lividans	7.3×10^6, 10% whi	2.6×10^6, 46% whi
S. albus	6.0×10^5, 18% whi	1.5×10^5, 33% whi

TABLE 4.13; Efficiency Of Transformation Of *S. albus* G153 With Plasmids Of Various Sizes

plasmid	pIJ702	pPZ43	pPZ46	pPZ30	pPZ67
size (kb)	5.8	10.9	12.5	13.0	18.7
transformation efficiency ($x10^6$ per molecule)	5.4	1.9	3.4	6.0	2.0

An unforeseen problem was however highlighted by this experiment. The occurrence of *mel*$^-$, thiostrepton resistant transformants with non recombinant pIJ702 was unacceptably high to permit the use of *mel* insertional inactivation as a measure of cloning efficiency. The loss of tyrosinase activity, more frequent after cutting with *Bgl*II, suggested exonuclease activity was removing part or all of the 5' overhang generating a frameshift mutation. Phenotypic expression of *mel*C was also unreliable with untreated supercoiled pIJ702. This was not alleviated by retransformation of *S. lividans* protoplasts with plasmid DNA observed previously to direct production of large amounts of melanin. The expression of *mel* in *S. lividans* and *S. albus* was therefore concluded to be unstable.

Exonuclease activity during preparation of linear phosphatased vector could in theory be responsible for the poor yield of transformants. The inability of blunt ended DNA to ligate with *Sau*3A overhangs, combined with the inhibition of recircularisation after treatment with CIAP, could render the vector useless. This however was in disaccord with the result reported in section 4.2.4. The efficiency of phosphatase treatment was tested, to specifically eliminate those preparations which had lost the ability to ligate with compatible sticky ends (represented by both pUC18 cut with *Bam*HI, and genomic DNA cut with *Sau*3A).

b. Transformation efficiency of *S. albus* protoplasts with respect to plasmid size.

The efficiency of transformation of *S. albus* protoplasts might have been limited to small DNA molecules, and selection against uptake and maintenance of larger recombinant plasmids might operate. To test this, *S. albus* G153 protoplasts were transformed with a series of plasmids (sizes ranging from 5.8kb to 18.7kb). The efficiency of transformation was measured for each, and the results expressed in Table 4.13 as the number of transformants per molecule of DNA. These figures, calculated assuming the weight of 1kb of DNA to be 1.19×10^{-12} ug, permitted the comparison of transformation efficiencies for different plasmids. Clearly plasmid size was not an important factor in transformation of *S. albus*, at least up to 18kb.

c. Plasmid restriction in *S. albus* G153.

In section 4.2.1 the restriction status of *S.*

albus G153 was discussed. To reiterate, S. albus G153 is restriction-minus for Sal GI and no restriction barrier was apparent between the host strain and S. longisporoflavus when actinophage R4 had been used. The possibility that R4 avoids restriction by a second enzyme, thus concealing a plasmid restriction barrier was not investigated at that time.

To test for plasmid restriction between the donor (S. longisporoflavus) and the recipient (S. albus), plasmid DNA was prepared from S. longisporoflavus. A 200 fold drop in transformation efficiency was observed (Table 4.14) when pIJ385 from S. longisporoflavus was introduced into S. albus G153 protoplasts. This reduction was not seen with S. lividans protoplasts suggesting a specific plasmid restriction barrier between S. longisporoflavus and S. albus.

The nature of this second restriction system in S. albus was not investigated. The specificity for restriction of S. longisporoflavus DNA implied that DNA from S. lividans was not recognised as foreign in S. albus, i.e. modified in the same way, or the restriction enzyme involved was specific for the modified DNA from S. longisporoflavus. In the former instance S. lividans TK24 would have to represent a restriction-deficient, modification-proficient phenotype, given that it does not restrict DNA from S. longisporoflavus. In the latter case restriction of modified DNA may be similar to the now well characterised examples in E. coli. The mar (Blumenthal et al., 1985), mcr A (Raleigh and Wilson, 1986), and mcr B (J. Heitman, and P. Model, sited in Blumenthal, 1986), loci are known to be involved in restriction by E. coli of methylated DNA, and are implicated - a convenient precedent - to bias genomic libraries (Blumenthal, 1986). Methyl-specific restriction is not confined to the genus Escherichia, systems have been reported in Diplococcus (Lacks and Greenberg, 1977) and Mycoplasma (Sladek et al., 1986). Restriction of DNA containing N^6-methyladenine or 5-methylcytosine has also been reported in S. avermitilis (MacNeil, 1988), and the author purports the widespread occurrence of methyl-specific restriction in the genus, with 7 out of 9 species testing positive.

4.2.5 CONSTRUCTION OF A REPRESENTATIVE LIBRARY OF S. longisporoflavus DNA IN S. lividans

From the previous section it was concluded

TABLE 4.14; A Comparison Of The Efficiency Of Transformation Of *S. albus* G153 and *S. lividans* TK24 With DNA Prepared From *S. longisporoflavus* (pIJ385) And *S. lividans* (pIJ702)

	trans. effic. with DNA ex *S. lividans*		trans. effic. with DNA ex *S. longisporoflavus*	
strain	No. ug^{-1}	No. $molecule^{-1}$	No. ug^{-1}	No. $molecule^{-1}$
S. lividans	$7.3x10^{6}$	$5.0x10^{-5}$	$2.9x10^{7}$	$2.0x10^{-4}$
S. albus	$7.9x10^{5}$	$5.4x10^{-6}$	$4.2x10^{3}$	$3.0x10^{-8}$

that, although it was the strain which was most sensitive to tetronasin, S. albus G153 was an inadequate host for a primary gene library from S. longisporflavus. The absence of restriction of S. longisporoflavus DNA in S. lividans and the high transformation efficiency obtained with this strain, indicated its suitability for the construction of the primary library. The availability of mutants of S. lividans - the genotypes of strains TK54 and TK64 are his/leu, and pro respectively - would allow the genetic complementation of the auxotrophic markers to confirm representation of S. longisporoflavus genome.

Furthermore the empiric inability to use tyrosinase inactivation in pIJ702 as an accurate measure of cloning efficiency, rendered assessment of genomic representation a laborious task. Two alternative vectors pIJ680 and pIJ699 were employed instead.

a. Using pIJ680

100ng of pIJ680 (Figure 4.1b) cut with BamHI and treated with CIAP was ligated to 200ng of size-selected Sau3A DNA fragments from S. longisporoflavus 83E6. The total DNA concentration of the reaction was 16ng ul^{-1} and 1U of T4 DNA ligase was added.

The efficiency of transformation of S. lividans TK54 with these ligation products and control DNA was tested (Table 4.15).

The results indicate the advantages of using efficiently dephosphorylated vector. The illuminating differentials, between the transformation efficiency of cut and phosphatased vector, self-ligated and ligated to insert (f/d = 0.11) and cut vector, self-ligated and ligated to insert (e/g = 1.17), imply strong selection for recombinant molecules with phosphatased pIJ680. Comparison of the transformation efficiency achieved with self-ligated, phosphatased and unphosphatased, vector clearly shows that dephosphorylation inhibited recircularisation. The results also show that S. lividans can uptake and establish linear DNA but cannot efficiently kinase and re-ligate a dephosphorylated molecule (dephosphorylation reduces transformation with linears by two thirds). The data implies that in a gene bank constructed with phosphatased vector, 90% of the transformants should be recombinant molecules. This statistic was not corroborated by the proportion of neomycin-

TABLE 4.15; Construction Of A Gene Bank Of *S. longisporoflavus* DNA, Based On Cloning Vector pIJ680 And Contained In *S. lividans* TK54

cloning vector			No. transformants	
BamHI cut	CIAP treated	ligated to	of strain TK24[a] (ug^{-1} vector)	efficiency a/b
+	+	insert	6.0×10^6	0.06[d]
-	-	-	9.8×10^{7b}	1
+	-	-	9.8×10^5	0.01
+	-	self	2.8×10^7	0.29[e]
+	+	-	2.9×10^5	0.003
+	+	self	6.5×10^5	0.007[f]
+	-	insert[c]	2.4×10^7	0.24[g]

[c]ligation conditions used were 15ng ul^{-1} total DNA concentration, 1:5 vector to insert molar ratio.

sensitive transformants; of 1358 colonies tested 922 proved sensitive to 10ug ml^{-1} neomycin, implying a 68% insert frequency. The test was performed by replica plating thiostrepton-resistant transformants from regeneration plates to Emersons agar plates containing thiostrepton and 10ug ml^{-1} neomycin. A second replicate to Emersons agar with thiostrepton was made from the same velvet to confirm that spores were transferred from all transformants. Despite a reasonable sample number in each of six tests, the variability was high, ranging from 34% to 76% colonies which were sensitive to neomycin. The number of colonies tested argued against random variation. The scepticism was confirmed, when plasmid DNA prepared from sixteen neomycin sensitive, ostensibly recombinant clones, revealed insert DNA present in only five. The sizes of the inserts were measured to be 5.4, 5.8, 6.5, 5.7 and 4.3kb, giving an average of 5.5kb. Each of the eleven non-recombinant plasmids still retained the unique BamHI restriction site; thus a frameshift mutation resulting from exonuclease digestion of the BamHI cut vector was not the cause of the observed sensitivity. The sixteen isolates were patched out on R2 agar plus thiostrepton and replica plated to a series of plates containing a range of neomycin concentrations (0.1ug ml^{-1} to 20ug ml^{-1}). Replicates were taken from the same velvet to the highest concentration of neomycin first, and lowest concentration last. All sixteen were resistant to 1ug ml^{-1} neomycin, six were sensitive to 5ug ml^{-1} neomycin (only one did not contain an insert), the remaining ten were sensitive to 10ug ml^{-1} neomycin. The synergistic nature of neomycin resistance in S. fradiae, was discussed in the introduction to this Chapter. The inability of aphI to confer high-level resistance independently has been observed previously (Thompson et al., 1982b; Bibb and Jansen, 1987), despite high levels of expression and translation of aphI which may constitute up to 10% of soluble protein in the cell (Thompson and Gray, 1983). The usefulness of the gene as a marker for insertional inactivation in this type of experiment was clearly limited here.

Assuming insert DNA to be absent in the neomycin resistant colonies an estimate of the representation of the S. longisporoflavus genome in the library could be calculated.

proportion of neomycin-sensitive colonies found to contain recombinant plasmids / proportion of colonies found to be sensitive to neomycin

= 5/16 of 34% = 10.6% = an estimate of the proportion of recombinants in the library

therefore 10.6% of 70 740 (total number of transformants) contain an insert
= 7 500 regenerants

the average insert size = 5.5kb

$$N = \frac{\ln(1-p)}{\ln(1-x/y)}$$

where, N = number of recombinants
p = probability
x = insert size (kb)
y = genome size (5848kb for S. coelicolor)

therefore,

$$N = \frac{\ln(1-0.99)}{\ln(1-5.5/5848)} = 4894 \text{ clones}$$

thus the binomial theorem predicts the gene bank to represent the S. longisporoflavus genome at the 99% level.

A tetronasin resistance gene, if present, would have to be screened from a large background of colonies which were partially tolerant of the antibiotic. A differential screen was developed which would hopefully identify any resistance-determinant that conferred increased resistance to the host cell. When replica plating Streptomycetes, firm pressure was required to ensure all colonies transferred spores from the master plate to the velvet. This procedure transferred a substantial amount of spores and aerial mycelia from most colonies, resulting in dense background growth on the selective plate. To circumvent the problem a replicate of the master plate was first made to minimal media (supplemented with leucine, which allows selection of the His gene from S. longisporoflavus). Material was then transferred

from this plate to a second velvet from which replicate plates were taken, first to selective (TSB agar + 50ug ml^{-1} tetronasin) then non-selective media (R2 agar + thiostrepton). This manipulation reduced the amount of material transferred to the tetronasin-resistance selection plates but growth on the R2 agar confirmed transfer of spores from each colony.

Unfortunately no resistance gene reproducibly conferring resistance to tetronasin could be identified. Five putative clones complementing the lesion in histidine metabolism and three clones which interfered with the metabolism of endogenous antibiotic production in S. lividans were found. The plasmids in the latter class, denoted pAAP2, 6 and 8, contained non-identical inserts of 5.2, 4.5 and 4.3kb respectively, and reproducibly directed the early synthesis of red pigments of differing shades by S. lividans transformants grown on R2 agar. These plasmids were not characterised further but they indicated that recombinant clones could be selected with a simple screen.

With such large numbers required to constitute a library, and the high background of spores containing parental, non-recombinant vector, transfer of plasmid DNA to S. albus G153 for screening seemed impractical. Indeed in plasmid DNA prepared from a culture inoculated from 9000 colonies (enough to constitute one genomic equivalent), the basal vector pIJ680 was by far the most predominant form.

b. Using pIJ699

1ug of pIJ699 (Figure 4.1c) prepared from S. lividans TK64 was digested with both BglII and EcoRI, the mixture was treated with calf intestinal alkaline phosphatase to remove the 5' phosphate group from the linear DNA and maximise intermolecular ligation by preventing intramolecular 'self' ligation. The 5kb BglII fragment was then separated from the mixture of products by agarose gel electrophoresis and recovered using a "Gene Clean" kit.

100ng of vector DNA was ligated to 400ng of size selected Sau3A fragments of chromosomal DNA from the high producing strain 83E6.

Ligation conditions used were 10ng/ul total DNA concentration, 1:2 vector to insert molar ratio, with 1 unit T4 DNA ligase for 2 hrs at 20°C. Control ligations of vector to itself and to dummy insert were performed and the products

TABLE 4.16; Construction Of A Gene Bank Of *S. longisporoflavus* DNA, Based On The Cloning Vector pIJ699 And Contained In *S. lividans* TK64

5kb *Bgl*II fragment CIAP treated	ligated to	No. transformants of strain TK64 (ug^{-1} vector)
+	insert	5.6×10^6
+	self	2.5×10^4
-	insert	6.2×10^6
-	self	3.9×10^5

analysed by gel electrophoresis (see Figure 4.2, section 4.2.2). Transformation of S. lividans TK64 with small amounts of the ligated DNA generated the data contained in Table 4.16. The products of the ligation between dephosphorylated vector and DNA Sau3A fragments transformed S. lividans TK64 protoplasts with an efficiency generating $5.6x10^6$ transformants ug^{-1} DNA. Phosphatased-vector ligated to itself under similar conditions transformed these protoplasts at 1/200 of the efficiency of the ligation mix, indicating that selection of recombinants was very efficient.

4.3 COMPLEMENTATION OF THE pro LESION IN TK64

To confirm that the library was representative of the S. longisporoflavus genome, 40 000 colonies were replica plated to agar based minimal media in order to identify clones complementing the lesion in proline metabolism in strain TK64. Sixteen colonies were selected for secondary screening. Plasmid DNA was prepared from eight isolates which complemented the mutation well. These eight plasmids contained inserts of average size 6kb which were shown to be homologous by restriction analysis and Southern blotting (Figures 4.3 and 4.4 respectively). All the constructs overlapped by at least 2kb, so the complementing sequences must be largely located within the 1.9kb SalI fragment common to all plasmids. Complementation of the pro lesion was confirmed by showing that TK64 protoplasts retransformed with each of the plasmids grew well on Ludox based minimal media (supplemented with histidine) while the parental strain did not (Figure 4.5).

4.4 INCREASED SENSITIVITY OF S. lividans TO TETRONASIN; A REFINED SCREEN

The biological activity of ionophore antibiotics is attributed to the ability to complex with monovalent and/or divalent cations and transport them across lipid bilayers to equilibrium thus depolarising the membrane.

Different ionophores have differing affinities for the cations with which they will complex. The effect of elevated concentrations of various cations on enhancement of the sensitivity of S. lividans to Tetronasin was tested. Of the four cations, (Na^+, K^+, Mg^{2+}, Ca^{2+}) tested, surprisingly the monovalent Na^+ and divalent Ca^{2+} proved most effective

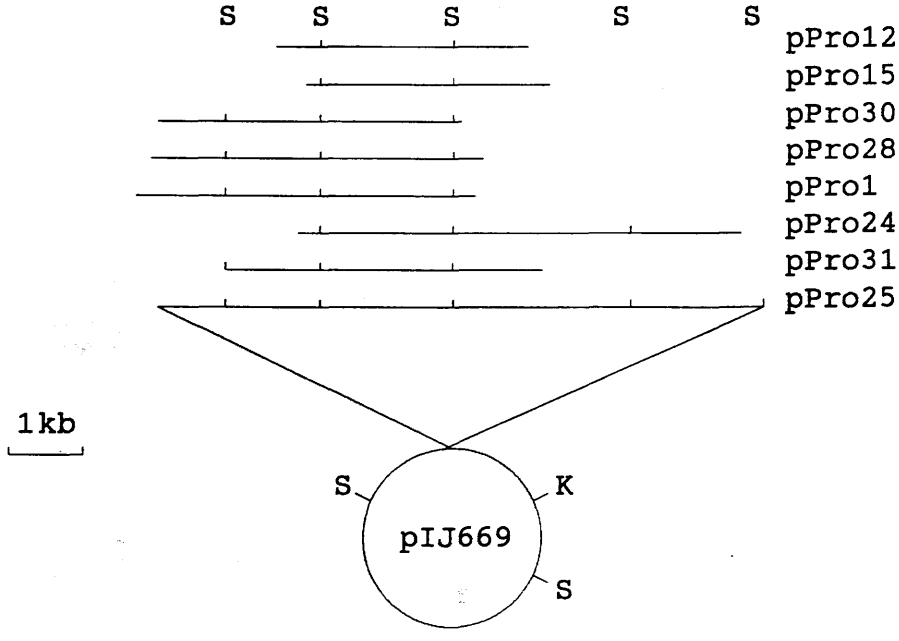


FIGURE 4.3; SalI Restriction Map Of The Eight Recombinant Plasmids Which Were Shown To Complement The Lesion In Proline Biosynthesis In S. lividans TK64

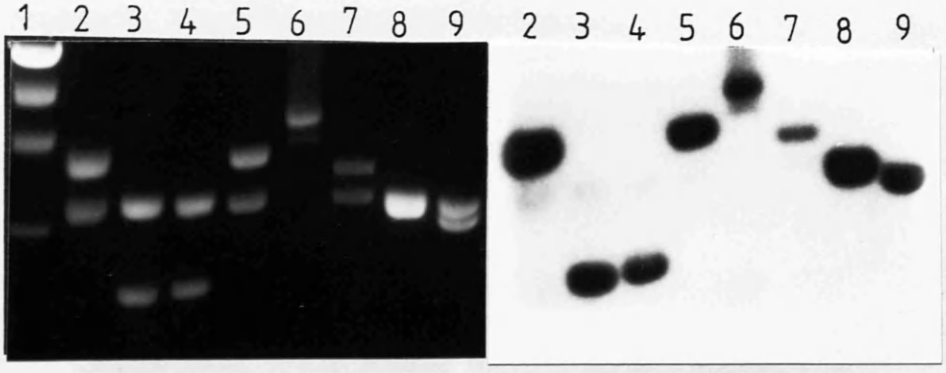


FIGURE 4.4; Southern Analysis Of Recombinant Plasmids Complementing The pro Lesion Of S. lividans TK64

Lane

1. phage lambda cut with HindIII
2-5. pPro1, Ppro12, pPro15 and pPro24 cut with HindIII
6. gel purified HindIII insert from pPro25
7-9. pPro28, pPro30 and pPro31 cut with HindIII

The 5kb DNA fragment common to all lanes (except 1 & 6), is the streptomycete replicon from pIJ699. Random primed DNA synthesis (see Chapter 5.1) from the gel purified insert of pPro25 provided the radiolabelled probe for this analysis.

FIGURE 4.5; Complementation Of The pro Lesion Of S. lividans TK64.

Plate gelling agent was Ludox. Proline, (40ug ml^{-1}), was included only in the left hand plate. Strain order; top row, working left to right; S. lividans TK24 (prototroph) containing pIJ699, S. lividans TK64 (pro) containing plasmids pPro1, pPro12, pPro15, pPro24, bottom row; S. lividans TK64 containing plasmids pIJ699, pPro25, pPro28, pPro30, pPro31.

(Figures 4.6a and b).

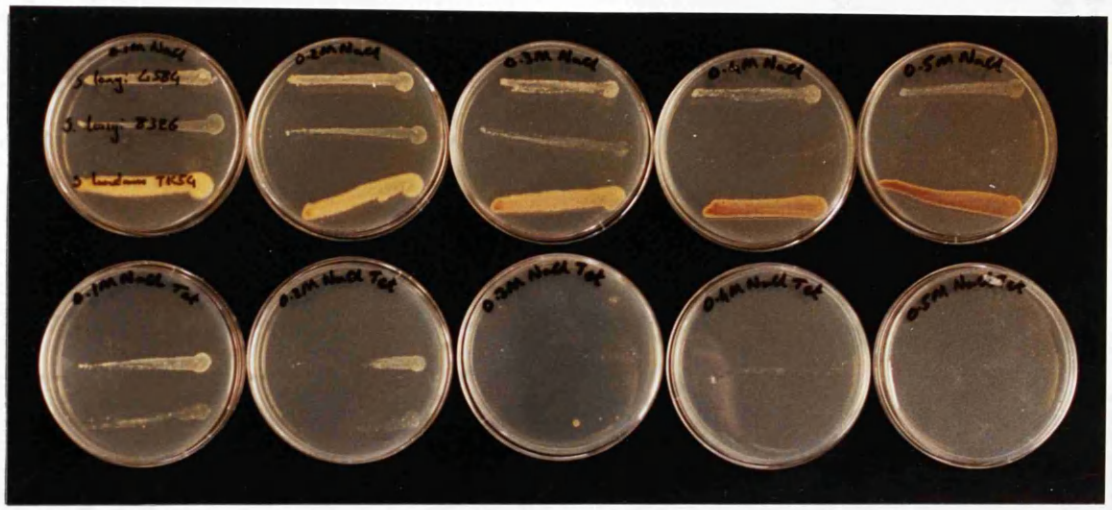
The greater sensitivity of S. longisporoflavus strain 83E6 compared to S. longisporoflavus 4584, at high sodium concentration in the absence of added tetronasin, may be due to the high production of tetronasin by strain 83E6. This will generate high local concentrations of the antibiotic under culture conditions which may make it more sensitive to its own metabolite. This conclusion is supported by the greatly increased sensitivity of strain 4584, a low tetronasin producer, in the presence of added exogenous tetronasin.

4.5 SCREENING FOR TETRONASIN RESISTANCE

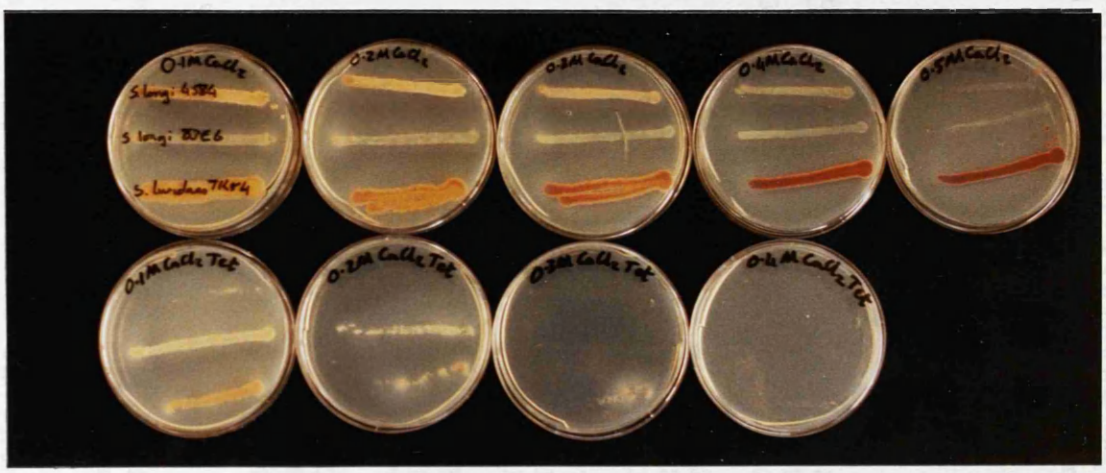
Thiostrepton resistant colonies from regenerated protoplasts which had been recovered from transformation with the DNA described in section 4.2.5b were screened, as before, first replica plating to minimal media then taking a second replicate from the minimal media plate to a selective plate. Five putative tetronasin resistant clones were selected for further analysis from the 40,000 colonies tested for growth on nutrient agar containing 0.2 M NaCl, 10 ug ml^{-1} Tetronasin. Two of these colonies contained plasmids pTetR1 and pTetR5 (with insert sizes 5kb and 5.5kb respectively) which when re-introduced into S. lividans TK64 increased resistance to tetronasin substantially. Table 4.17, and Figures 4.7a and b show the survival of S. lividans TK64 containing pTetR1, pTetR5 and pIJ699 on increasing levels of Tetronasin and NaCl respectively.

Cells containing either pTet1 or pTet5 appeared sooner on selection plates and had a larger final colony size, than cells containing pIJ699 alone. (Figure 4.8).

The high sensitivity of a proportion of the spores thought to contain ptetR1 or ptetR5 could most likely be explained by the precise deletion of the sequences separating the indirectly repeated phage fd terminators in these cells. The presence of a small plasmid species which was observed in DNA preparations of pTetR1 and pTetR5 was considered to be supporting evidence for this hypothesis (Figure 4.9a). pIJ699 sequences were also unstable; Figure 4.9b shows a preparation of the 5kb streptomycete replicon from pIJ699 with two species of DNA present. Neither was digested with either



A



B

FIGURES 4.6 (A) AND (B); Effect Of Increasing Na^{+} (A) Or Ca^{2+} (B) Concentration On The Sensitivity Of S. lividans TK64, And S. longisporoflavus Strains 83E6 (high producer) And 4584 (Low Producer) To Tetronasin.

Plates are ordered with increasing cation concentration (0.1M to 0.5M) from left to right, in the absence of tetronasin (top row of plates) and presence of 10 ug/ml^{-1} tetronasin (bottom row of plates).

TABLE 4.17; Numbers Of Surviving Colonies Of S. lividans TK64 Containing pIJ699 (Top Of Each Triplet*), ptetR1 (Middle+) And ptetR5 (Bottom#) On Difco Nutrient Agar With Varying Concentrations Of Tetronasin And NaCl

tetronasin (ug/ml)	0	2.5	5	10	25	50
NaCl (M)						
0	2140±46*			609±25		
	974±31+			639±25		
	1066±33#			781±28		
0.01				518±23		
				514±23		
				672±26		
0.1	421±21	140±12	127±11	45±7	8±3	0±0
	832±29	360±19	299±17	241±16	142±12	136±12
	603±25	485±21	351±19	175±13	30±5	41±6
0.2				1±1		
				222±15		
				50±7		
0.4				0±0		
				21±6		
				15±4		

The spore samples were suspended in cooled agar (45°C), therefore the resulting colonies were of discreet size and embedded in the agar which enabled large sample numbers (where possible) to be counted with confidence in the accuracy of the result. The error factor was calculated as the square route of the number of colonies counted, and although it does not represent a valid statistical test it does give an indication of the inherent error involved when the sample number was necessarily small.

FIGURE 4.7a; Survival Of S. lividans TK64 Spores Containing PIJ699, ptetR1 Or ptetR5 On Media Containing 5ug ml^{-1} Thiostrepton, 10ug ml^{-1} Tetronasin And Increasing Concentrations Of NaCl.

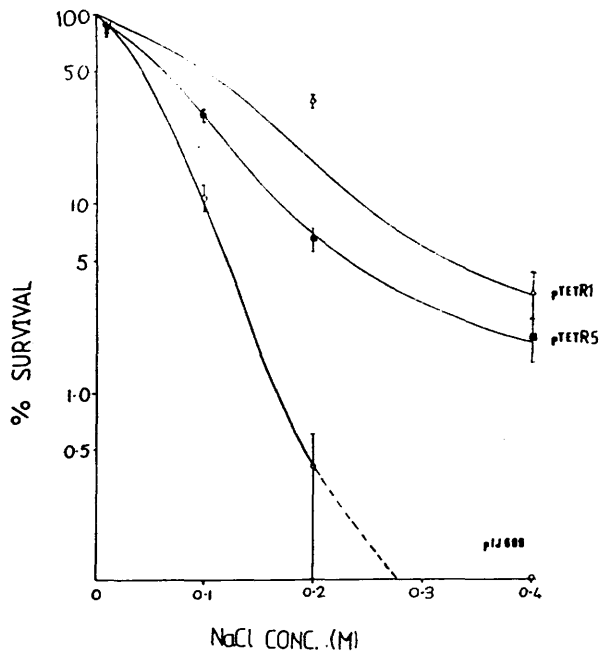
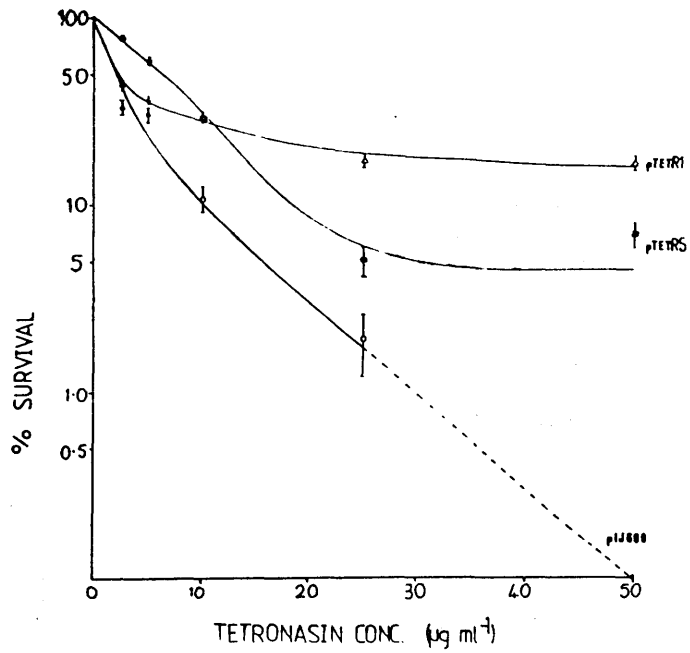


FIGURE 4.7b; Survival Of S. lividans TK64 Spores Containing pIJ699, ptetR1 Or ptetR5 On Media Containing 5ug ml^{-1} Thiostrepton, 0.2M NaCl And Increasing Concentrations Of Tetronasin.



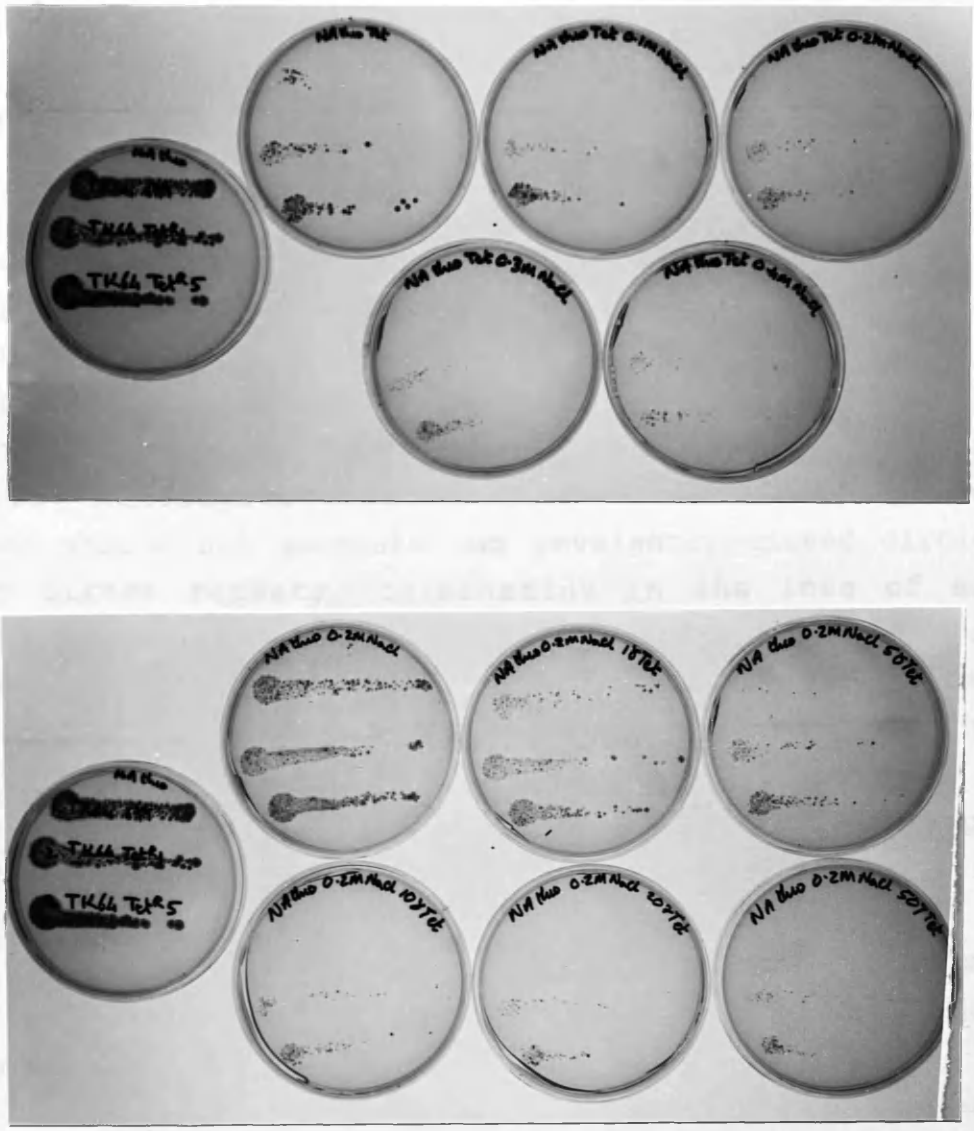


FIGURE 4.8; Growth Of S. lividans TK64 Containing pIJ699, ptetR1 Or ptetR5 On Media Containing 5ug ml^{-1} Thiostrepton And 10ug ml^{-1} Tetronasin With Increasing Concentrations Of NaCl (Top), Or 0.2M NaCl With Increasing Concentrations Of Tetronasin (Bottom).

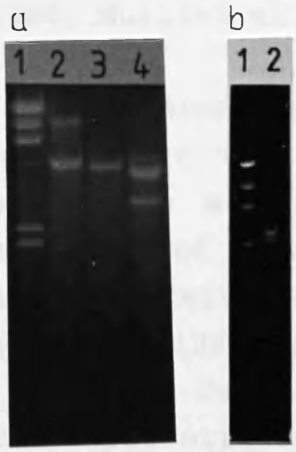


FIGURE 4.9; Plasmid DNA Preparations Of ptetR1, ptetR5 And pIJ699 Contain Deletion Derivatives

Lane

a, 1. phage lambda cut with HindIII
2. ptetR1 cut with KpnI
3. ptetR4 cut with KpnI
4. ptetR5 cut with KpnI

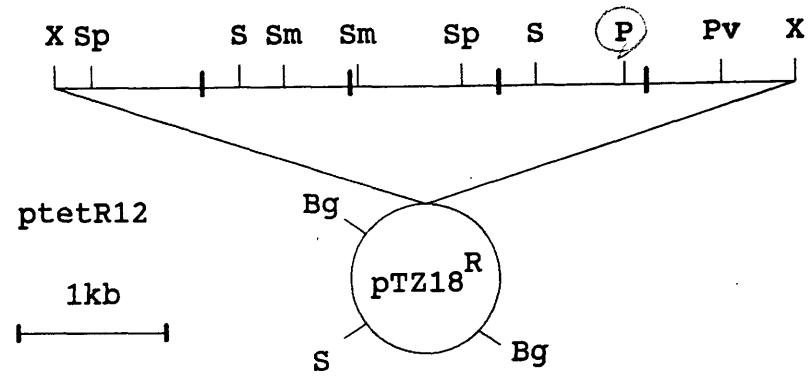
b, 1. phage lambda cut with HindIII
2. gel purified preparation of the 5kb BglII streptomycete replicon of pIJ699 with contaminating fragment

EcoRI or DraI, and both contained a single ClaI site inferring that both recovered fragments had originated from an overlapping region of pIJ699. The smaller of the two is most likely derived from pIJ699 by precise deletion of the stuffer fragment along with both copies of the phage fd terminator (this probably contributed to the $3.9x10^5$ transformants of S. lividans recovered after self-ligation of the gel-purified 5kb BglII fragment. With long stretches of DNA separating the indirect repeats they can be maintained in S. lividans but the stability of such a construct is questionable. In E. coli similar substrates are recognised by recombination enzymes and the topology of potential secondary structures have been implicated in the loss of intervening sequences. Homologous recombination between the repeated sequences should not generate two covalently closed circles as with direct repeats, culminating in the loss of any molecule without an origin of replication. Instead recombination of indirectly repeated sequences causes inversion of the intervening DNA - reported by Kieser and Melton (1988), as a rare occurrence in pIJ699. If this was a serious problem, propagation of the plasmid in S. lividans JT46 (Tsai and Chen, 1987; Chen et al., 1987), deficient in intraplasmid recombination may be the solution.

Two models to interpret the precise deletion of DNA between and including indirect repeats have been proposed. Both require the formation of secondary structure:

1. Formation of a cruciform structure with the insert DNA looped out. Recognition by endonucleases and cleavage at the base of the structure would precisely excise the insert and the repeated sequences. Evidence for recognition of Holliday junctions or artificial cruciform structures by nucleases in other organisms has been reported (Kleff et al., 1988; Mueller et al., 1988; Kemper et al., 1988; West et al., 1988).

2. Formation of a stem and loop structure in ssDNA with the insert DNA looped out. Replication slippage by DNA polymerase at the base of the stem would result in the synthesis of a daughter strand without the repeated sequences or the intervening DNA. This model was described by Brunier et al., (1988) and is supported by data of Leach et al., (1988) who described experiments which show that cleavage of the input strands is not necessary to cause the inviability



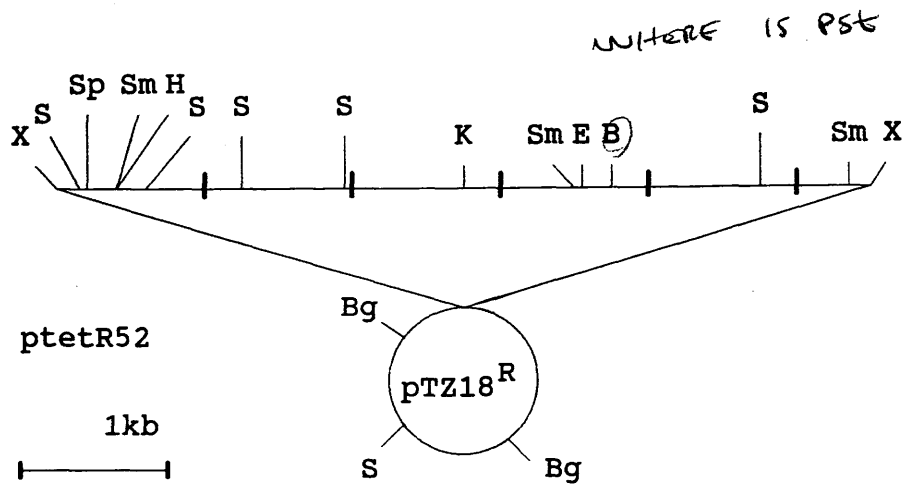


FIGURE 4.10; Restriction Endonuclease Map Of ptetR12 And ptetR52

Key, B = BamHI
Bg = BglI
Bgl = BglII
E = EcoRI
EV = EcoRV
H = HindIII
K = KpnI
M = MluI
P = PstI
Pv = PvuII
S = SalI
Sp = SphI
Sm = SmaI
X = XbaI
Xh = XhoI

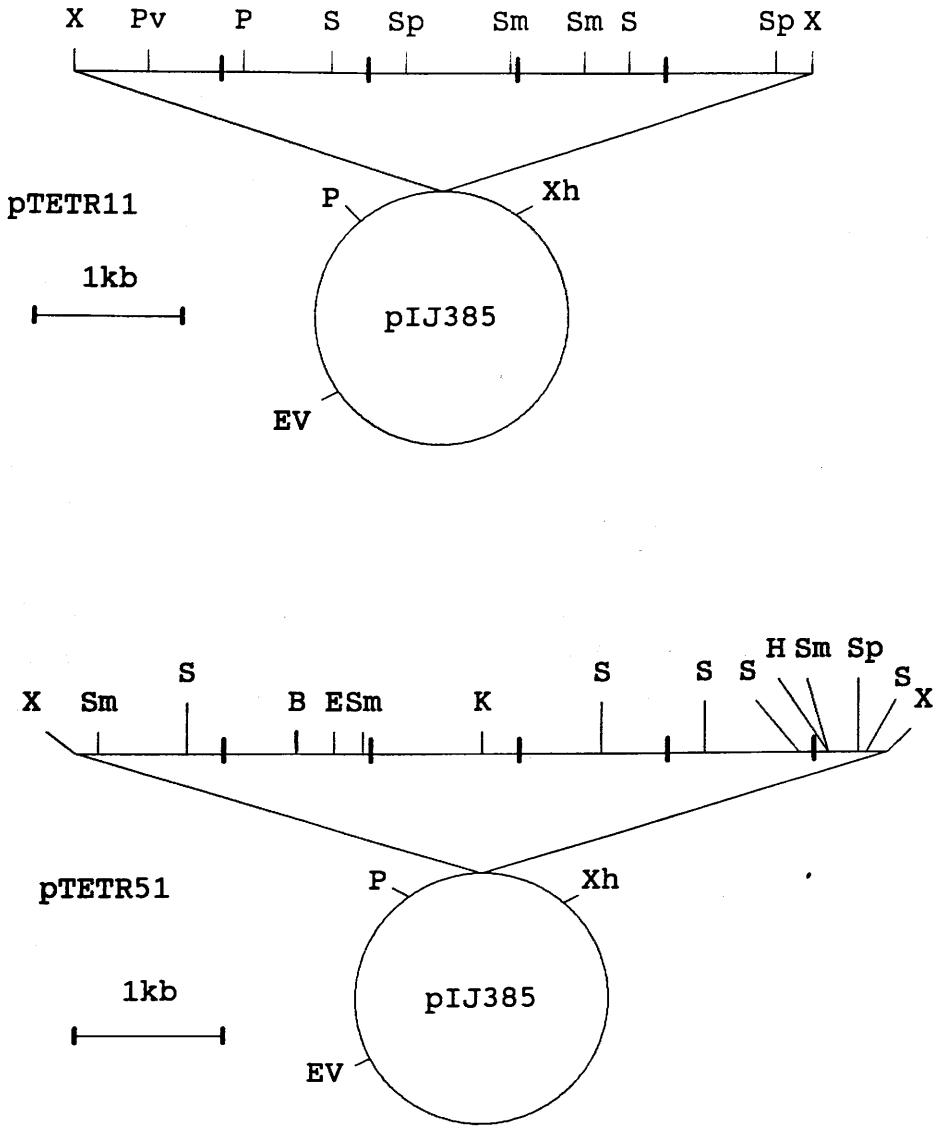


FIGURE 4.11; Restriction Endonuclease Maps Of ptetR11 And ptetR51

Key, as for Figure 4.10

of palindromic DNA. Plasmid DNA in Streptomyces may be particularly prone to rearrangement by this mechanism. It is replicated via a single strand intermediate (Schrempf and Pigac, 1986), and the omission of the sti region - probably an efficient site of initiation of lagging strand synthesis (Deng et al., 1988) - during construction of many cloning vectors (including pIJ699), means a high proportion of the DNA is present in single strand form.

The proportion of deletion derivatives in preparation of ptetR1 and ptetR5 hindered phenotypic analysis and restriction mapping of the insert DNA in this form. The insert DNA was therefore excised by digestion with XbaI and subcloned into pTZ18^{R} (giving plasmids ptetR12 and ptetR52 respectively) and propagated in E. coli JM109. Restriction maps were derived from analysis of this DNA (Figure 4.10).

The loss of insert DNA from ptetR1 and ptetR5 by similar means could explain the non-uniform survival of spores under selective conditions and biased the data presented in Table 4.17 and Figures 4.7a and b. The insert DNA of both constructs were therefore also subcloned into pIJ385 in the orientations shown in Figure 4.11 giving plasmids ptetR11 and ptetR51 respectively. The absence of smaller deletion derivatives in DNA preparations of either of these plasmid suggested that the stability of the constructs had indeed been improved.

ptetR11 and ptetR51 were introduced into S. albus G153 and the level of resistance conferred by each was determined (Figure 4.12). Clearly ptetR51 conferred resistance at least up to 1ug ml^{-1} tetronasin on S. albus (in the presence of 0.2M NaCl) whilst the host strain containing pIJ385 was sensitive to 0.1ug ml^{-1} of the drug. In contrast ptetR11 did not appreciably elevate the resistance of S. albus G153 to tetronasin in this test. A comparison of the phenotypes of ptetR11 and ptetR51 in S. lividans TK24 is also provided in Figure 4.12. ptetR11 conferred resistance up to 20ug ml^{-1} and mycelia containing ptetR5 continued to grow even in the presence of 100ug ml^{-1} tetronasin.

4.6 TRANSCRIPTIONAL ANALYSIS OF TetR1 AND TetR5

Low resolution S1 mapping was performed as described in Chapter 5.8 for isolated restriction fragments of both clones. A 1.1kb PstI fragment from TetR12 and a 1.5kb

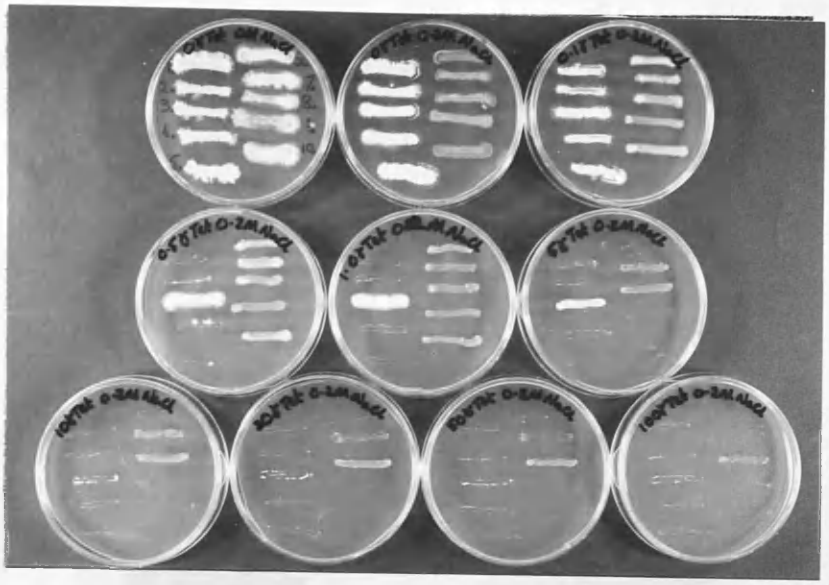


FIGURE 4.12; Resistance Levels Of S. albus G153 And S. lividans TK24, Harbouring Various Pasmid Constructs, To Tetronasin In The Presence Of 0.2M NaCl

Strain order,

1. S. albus\pIJ702
2. S. albus\ptetR11
3. S. albus\ptetR51
4. S. albus\pKJL1
5. S. albus\pKJL2
6. S. lividans\pIJ385
7. S. lividans\ptetR11
8. S. lividans\ptetR51
9. S. lividans\pKJL1
10. S. lividans\pKJL2

Constructs pKJL1 and pKJL2 are described in Chapter 5.

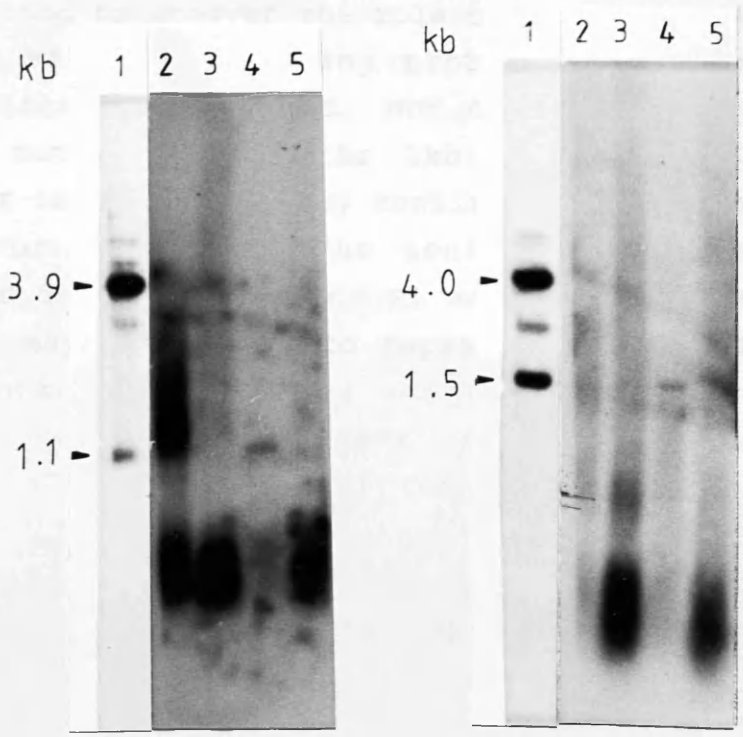


FIGURE 4.13; Low Resolution S1 Analysis Of tetR1 And tetR5

Lane

a. 1. ptetR12 digested with BamHI and PstI
   2. 3.9kb BamHI\PstI fragment from tetR12+ total RNA
   3. 3.9kb BamHI\PstI fragment from tetR12+ tRNA
   4. 1.1kb PstI fragment from tetR12+ total RNA
   5. 1.1kb PstI fragment from tetR12+ tRNA

b. 1. ptetR52 digested with BamHI and PstI
   2. 4kb BamHI fragment from tetR52+ total RNA
   3. 4kb BamHI fragment from tetR52+ tRNA
   4. 1.5kb BamHI\PstI fragment from tetR52+ total RNA
   5. 1.5kb BamHI\PstI fragment from tetR52+ tRNA

total RNA was prepared from a culture of S. longisporoflavus 4584 grown for 7 days under conditions which favour tetronasin production.
tRNA = tRNA from S. cerevisiae.

The nucleic acid in lanes 2-5 was denatured by raising the temperature to 80°C for 10 minutes before renaturation in conditions which favour the formation of DNA:RNA heteroduplexes and treatment with S1 nuclease (see Chapter 2.19).

BamHI/PstI fragment from TetR52 were shown to be specifically and completely protected by hybridization with RNA prepared from a 6 day culture of S. longisporoflavus 4584 (Figure 4.13). These fragments should prove useful for gene disruption to uncover the role of the respective genes in the parent strain. Convincing protection of the 4kb fragments from either clone was not observed, although an intense smear centred around the 1kb mark was found with TetR1. Further investigation may confirm this to be a transciptional start or end point. The tentative conclusions drawn in Chapter 5 still holds for S1 mapping of large DNA fragments and it may be prudent to repeat this work with smaller DNA fragments.

4.7 SUMMARY

S. albus G153 was identified as a potentially suitable cloning recipient in which to construct a library of S. longisporoflavus 83E6 DNA for the isolation of tetronasin resistance determinants. Protoplasts could be prepared with reasonable transformation efficiency (around 10^6 ug^{-1} DNA) and the strain was very sensitive to tetronasin. However, the inability to obtain a gene library in this strain was shown to be due to a plasmid restriction barrier between S. longisporoflavus and S. albus, which had not been detected by the passage of actinophage R4 between the two strains.

S. lividans TK64 was chosen as an alternative host and two gene libraries were constructed using the cloning vectors pIJ680 and pIJ699. Complete represention of the S. longisporoflavus genome in the library contained in pIJ699 was confirmed by the independent isolation of eight recombinants, with homologous inserts, which complemented the lesion in proline biosynthesis in strain TK64.

Elevated Na^+ concentrations in the growth medium was shown to enhance the sensitivity of S lividans to tetronasin and a screen was developed which allowed the direct selection of tetronasin resistance determinants in the primary cloning host. Two recombinants, ptetR1 and ptetR5, were isolated which contained non-homologous, unlinked inserts, which when retransformed into S. lividans increased resistance to tetronasin. Subcloning the respective 5.0kb and 5.5kb inserts into $pTZ18^R$ allowed the restriction endonuclease maps of the two clones to be determined. The inserts were also subcloned into pIJ385 giving ptetR11 and ptetR51 respectively, to alleviate the instability of the constructs in pIJ699. ptetR51 elevated the resistance of S. albus to 1ug ml^{-1} tetronasin in the presence of 0.2M NaCl.

Transcriptional analysis was also carried out and low resolution S1 mapping showed that the 1.1kb PstI fragment from TetR1 and the 1.5kb BamHI/PstI fragment from TetR5 were completely internal to transcription units.

CHAPTER 5

ISOLATION OF S. longisporoflavus DNA WITH HOMOLOGY TO ACTINORHODIN AND MONENSIN-A BIOSYNTHETIC GENES

5.1 INTRODUCTION

The structural similitude of polyketide antibiotics and similarity of their biosynthetic origins - discussed in Chapter 1.4 - can be exploited through nucleic acid hybridization. Application of this potentially powerful technique allows researchers to profit from the cloning of polyketide synthases from more amenable systems. For example, characterisation of sequences which rescued mutations in the earliest steps of biosynthesis of the pigmented antibiotic actinorhodin revealed the actI and actIII genes to encode components of the polyketide synthase (PKS); actI is believed to represent the condensing enzyme (B-keto-acyl synthase) with actIII being a Beta-ketoacyly-ACP-reductase (Malpartida et al., 1987; Sherman et al., 1989; Hallam et al., 1988). The availability of these genes from the John Innes Institute, has simplified the isolation of PKS genes and accelerated progress in the study of antibiotic biosynthesis. The actI gene was used by Malpartida to screen a genomic library of S. cinnamonensis ATCC 15413. A 4.3kb BamHI fragment with strong homology to both actI and actIII was isolated and is believed, by virtue of DNA sequence homology with genes involved in the biosynthesis of polyketides from other strains (Bibb et al., 1989; Hallam et al., 1988; Sherman et al., 1989), to encode the condensing enzyme and keto-reductase components of the monensin synthase (Robinson, J. A., pers. comm.) The actI fragment contained on a 2.2kb BamHI fragment, and the putative monensin synthase fragment (monI) were both used to detect sequences with homology in total DNA prepared from S. longisporoflavus 83E6. With considerable structural similarity between tetronasin and monensin-A (both are branched chain, polyoxygenated polyketides) the sanguine expectation was for a tetronasin PKS sequence with better homology to monI than actI. The improved rate of hybridization and hybrid stability in Southern hybridization would be manifested as stronger signal, simplifying the isolation of the complementary sequence.

The detection of heterologous sequences by Southern analysis is dependent on a number of factors. These can be divided into broad categories; DNA condition and transfer to solid support, properties of the labelled probe, hybridization conditions, and methods of detection.

5.1.1 DNA CONDITION AND TRANSFER TO SOLID PHASE

High molecular weight total DNA was prepared from a S. longisporoflavus culture, in which eight out of eight isolates were shown, by bioassay (see Chapter 3.4), to be producing tetronasin. Spectrophotometric absorbance of 2 abs. units at 260nm for a 1/10 dilution of the DNA indicated the concentration to be 1mg ml^{-1}. The products of digestion with various restriction endonucleases were fractionated by gel electrophoresis. Depurination by 0.5M HCl for 10min was used to fragment the DNA to facilitate transfer of large molecules (Wahl et al, 1979). Small fragments of DNA do not bind well to solid supports (Meinkoth and Wahl, 1984) therefore longer treatment times were avoided.

Nylon membrane was preferred to nitrocellulose because it is resilient and allows multiple hybridization cycles to be performed. Transfer of the DNA to Amersham Hybond N membrane was performed by passive diffusion (Southern, 1975), in alkaline conditions (Chapter 2.22.1). Alkali blotting transfers DNA in denatured form under conditions which promote covalent binding to the surface without UV cross linking or baking at high temperature. This improves the availability of filter bound sequences for hybridization to probe.

5.1.2 PREPARATION OF LABELLED PROBE

A number of methods for radiolabelling nucleic acids have been developed. These provide probes which, when used in Southern analysis, give different levels of sensitivity, which are, in general, inversely proportional to the resolution obtained. For detection of heterologous sequences in filter hybridization, high sensitivity is a prerequisite. The level of sensitivity is dependent on both the specific activity of the probe and ability to detect the signal. ^{32}P-radionucleotides offer high efficiency of detection (see section 5.1.4) and labelled dCTP was used throughout this work. Uniform labelling methods; nick translation (Rigby et al, 1977), and random prime synthesis (Feinberg and Vogelstein, 1983 and 1984) are in common use and provide probes with high label density.

Nick translation

In a nick translation reaction, dsDNA could be labelled to a specific activity, (s.a.), of around $5x10^7$ dpm

ug^{-1}. Pancreatic DNaseI is used to introduce single strand nicks into the DNA. DNA polymerase I from E. coli, sequentially adds nucleotide residues, of which dCTP is usually radiolabelled, to the 3' hydroxyl terminus of the nick, whilst removing nucleotides from the adjacent 5' phosphoryl terminus. Nick translation provides labelled fragments of average length 500nt (Cunningham and Mundy, 1987) although this can be manipulated by altering the concentration of DNaseI (Meinkoth and Wahl, 1984).

Random primed DNA synthesis

Using Boehringer Mannheim Random Primer DNA Labelling kit (Cat. No. 1004760) probes with specific activity of $5x10^9$ dpm ug^{-1} were routinely obtained. A mixture of all possible hexanucleotides is supplied for hybridization to the template DNA. The Klenow fragment of DNA polymerase, using the hexanucleotides as primers, incorporates radiolabelled dCTP into the growing complementary strand. The average length of probe generated is around 200nt but individual reactions have been reported to be highly variable in this respect (Cunningham and Mundy, 1987).

Despite the smaller length and lower concentration of probe obtained, random priming was the preferred method, because the high specific activity generated was very reproducible, and only a small amount of template DNA was required - 20ng, compared with 500ng for nick translation.

Labelling reactions were always performed with gel-purified DNA fragments, which had contaminating agarose removed using a "Gene Clean" kit. This ensured that most of the radiolabel was incorporated into sequences of interest and not into vector DNA.

5.1.3 HYBRIDIZATION PARAMETERS

Nucleic acid hybridization is a reversible process. Therefore conditions which affect stability of the duplex must be considered. This is particularly true if detection of imperfect hybrids is desired, and a dsDNA probe is used. For the factors mentioned below, not only is the effect on the rate of hybridization discussed but also, (where applicable) the stability of the heteroduplex is considered.

a. Prehybridization

The first step in mixed phase hybridization is to precoat the filter with substances which mask sites to which probe DNA will bind non-specifically. This reduces the level of background "noise". The method of Denhardt, (1966), using ficoll, polyvinylpyrrolidone and bovine serum albumen, each at 0.02% (w/v), and denatured heterologous DNA, was employed. Denhardt's solution was also a component of the hybridization fluid. Stimulation of the hybridization rate by neutral polymers such as ficoll cannot be ruled out. Experiments by Chang et al., (1974) demonstrated a 50% increase in solution hybridization rate in the presence of 5.7% (w/v) ficoll. This was purported to be due to molecular exclusion of the DNA from the fraction of solution occupied by the polymer, effectively increasing the nucleic acid concentration. A similar theory has been proposed for the action of dextran sulphate (see section f); however with such low concentrations of ficoll, perturbation of the rate of hybridization is likely to be minimal.

b. DNA; molecular weight, concentration, and mismatches

The rate of hybridization of probe DNA to immobilized, filter-bound target DNA is affected by the concentration of both molecular species. Mixed phase hybridization is a two-stage process, requiring first the diffusion of the probe to the filter then hybridization to the target DNA. At high concentrations of filter-bound DNA, the diffusion of the probe to the filter becomes rate limiting. At low concentrations, nucleation between probe and target is the important parameter (Anderson and Young, 1985). High concentrations of both reactants would therefore promote the forward rate. When using a dsDNA probe, reassociation of probe DNA is also accelerated by high concentration. However this phenomenon can be exploited to the advantage of reaction sensitivity if the probe has been produced in a manner which generates a multiplicity of small overlapping fragments (see section f, effect of dextran sulphate).

The molecular weight of the probe DNA also affects the rate of filter hybridization. Observations by Flavell et al., (1974), and Birnsteil et al., (1972), indicated that in a nucleation-limited reaction, the rate was independent of molecular weight. In a diffusion-limited reaction, the rate was inversely proportional to the molecular weight. Therefore with increasing probe molecular weight, reduction in the rate

of hybridization followed. The complexity of the process is compounded with a dsDNA probe because increased probe length promotes solution hybridization; and this reassociation of the probe results in a reduction of the availability of sequences for the formation of hybrids.

The stability of hybrids formed is a function of the length of the shortest strand in the DNA duplex. Generally, duplex stability of the duplex is improved by increased length, but the situation is complicated by the number and distribution of mismatches. For a duplex of 150bp or more, the melting temperature (Tm) decreases 1°C for every 1% mismatched base (Bonner et al., 1973). With shorter hybrids the Tm is more sensitive and decreases by 5°C for every mismatch in a 20bp duplex. If the mismatches are clustered, leaving long stretches of perfect, or near perfect, homology the stability of the double helix will be greater i.e. evenly-dispersed errors destabilise the duplex.

Nick translation and random priming both produce probes of short length. The former generates probes of favourably high concentration; however the effective concentration of random-primed probes can be manipulated to be effectively higher using anionic dextran polymers (see section f).

c. Temperature

Perfectly-matched sequences hybridize most efficiently at 20°C below the Tm (Bonner et al., 1973). For cross hybridization of imperfectly-matched sequences, the bell-shaped temperature dependence curve is centred around lower temperatures. As noted above, the Tm itself is also decreased, to a degree dependent on the frequency and distribution of mismatches. The temperature can thus be manipulated to achieve the highest rate of formation of heteroduplexes without optimising the rate of probe reassociation. This will never favour cross hybridization over reannealing of probe but a temperature which will give the best chance of finding a poorly matched sequence can be discovered empirically.

With unknown levels of homology between the probe sequences and S. longisporoflavus DNA, earliest hybridizations were carried out at low stringency - determined in aqueous hybridization by temperature and ionic strength (see next section) - followed by iterative washes at

increasing stringency, checking the signal level at each step. This procedure will indicate empirically a suitable level of stringency for hybridization. It does not define the exact conditions at which hybridization should be carried out, because hybridization and washing are dependent on different parameters - nucleation rate and hybrid stability respectively (Anderson and Young, 1985). However a stringency range will be identified, within which small modifications of hybridization conditions may result in improved signal to noise ratio.

d. Ionic strength

At high temperatures nucleic acid is unstable, particularly in solution. Therefore prolonged incubation at elevated temperatures gradually degrades the probe DNA. For this reason it is preferable to use a combination of temperature and ionic strength to define stringency.

Wetmur and Davidson, (1968), were the first to investigate the effect of ionic strength on DNA reassociation. They reported that the rate of hybridization increased with increasing Na^+ concentration. The relationship also held for mixed phase hybridization. The dependence on salt concentration was most evident at low ionic strengths. Above 0.1M Na^+ the influence of further increase was diminished, but still measurable even in 3M solution. Elevated salt concentrations also affected duplex stability. Therefore washing was performed in solutions of sodium chloride and trisodium citrate to stabilise mismatched hybrids.

The influence of ionic strength upon the Tm of the duplex is defined by the following equation (Dove and Davidson, 1962);

$$Tm_{(u^2-u^1)} = 18.5\log_{10}(u_2/u_1)$$

where u_1 and u_2 are the ionic strengths of the two solutions.

Using this equation the stringency of washing can be manipulated in a rational manner by a combination of temperature and ionic strength.

Duplex destabilising agents, such as formamide, can also be used to reduce the working temperature of hybridization without relaxing the stringency (Casey and

Davidson, 1977). However this can cause a reduced rate of hybridization. With formamide, the rate in a 50% (v/v) solution has been estimated as 2x slower than found aqueous conditions (Hutton, 1977). These agents were omitted from the hybridization fluids.

e. Duration of hybridization

Mixed phase hybridization of a dsDNA probe to non-homologous target sequences does not benefit from prolonged incubation, because the reaction may never reach an equilibrium. The monitored product - formation of a mismatched hybrid - is in competition with probe renaturation. Mismatches in the base pairing lower the Tm of a duplex. Therefore the stability of the hybrid is unlikely to be high. Given the reversible nature of the reaction, prolonged incubation under hybridization conditions (approaching the Tm of the hybrid, but much lower than the Tm of the renatured probe), will favour probe reassociation and the formation of stable duplexes. Furthermore at high temperatures, nucleic acids are unstable - particularly in solution - so the probe DNA will gradually degrade.

If $C_0t_{1/2}$ is the time taken for one half of the initial reactants to be consumed, Maniatis *et al.*, (1982), advise that after 3 x $C_0t_{1/2}$ the amount of probe available for hybridization to filter bound sequences is negligible. $C_0t_{1/2}$ can be calculated (in hours), by the equation;

$$C_0t_{1/2} = 1/X \times Y/5 \times Z/10 \times 2$$

where, X is the total weight of probe DNA added (ug)
Y is the complexity of the probe i.e. the number of bases in non-repetative DNA sequence (kb)
Z is the volume of hybridization fluid (ml)

For 100ng of random primed probe, prepared from a DNA fragment of length 2.2kb (size of *actI* fragment), and a reaction volume of 5ml, the $C_0t_{1/2}$ will be approximately 4hrs. According to Maniatis after 12hrs the reaction is effectively complete. Further incubation is only likely to decrease the strength of the signal, so a reaction time of 12hrs was closely adhered to.

f. Dextran sulphate

Stimulation of the rate (10-fold) of reassociation of nucleic acids by 10% (w/v) dextran sulphate was first observed by Wetmur (1975). The effect was repeated in mixed phase hybridization. Wahl et al., (1979), reported a 4-fold increase in hybridization rate with a ssDNA probe and a 100-fold increase using a dsDNA probe with a concommitant increase in the amount of hybrid formed. The exclusion of the nucleic acid from the volume occupied by the anionic polymer, resulting in an effective increase in the probe concentration is thought to be responsible for the accelerated rate of association with ss probes. The elevated rate and yield observed with dsDNA probes is attributed to the formation of networks through the hybridization of overlapping complementary strands. Single strand regions of the network are available for hybridization to target sequences on the filter. The formation of networks is dependent on length, with polynucleotides of more than 250 bases being most effective (G. Wahl, cited in Meinkoth and Wahl, 1984). Small probes are less likely to initiate or maintain the formation of stable networks.

Random-primed labelling, generating fragments of average length 200 nucleotides may not be the most appopriate probes. However an increase in sensitivity was recorded when using dextran sulphate (section 5.2). It should be noted that where quantitation is important, the formation of networks is undesirable and dextran sulphate should be avoided with dsDNA probes.

5.1.4 DETECTION OF HYBRIDS

Detection of cross hybridizing sequences was the primary aim of this experiment. The conditions and materials used had therefore to be optimised for high sensitivity. Uniformly labelled, high specific activity, ^{32}P probes fulfil this requirement. The high energy B particles emitted from ^{32}P have a long mean path length and can produce silver grains on the emulsion layers on both sides of X-ray film (Kodak X-omat). B emissions can also pass through the film to excite an intensifying screen, and the light generated will contribute to the amount of silver grain production. At $-70^{o}C$ the flourescence of the screen is prolonged so the strength of signal is enhanced 5-fold (Anderson and Young, 1985). This

arrangement reduces the resolution of the experiment, but fortunately this was not a high priority.

5.2 SOUTHERN ANALYSIS, CROSS HYBRIDIZATION OF actI AND monI TO S. longisporoflavus 83E6 TOTAL DNA

In an initial experiment 2ug aliquots of S. longisporoflavus 83E6 DNA were digested with restriction endonucleases chosen because they have a unique site in the pTZ18^{R} polylinker - any fragment with homology to actI or monI would therefore be a candidate for cloning into this vector using E. coli as a host. The products were separated in a 0.8% (w/v) agarose gel, and transferred to nylon membrane. Probe DNA was prepared by nick translation of 500ng pIJ2330 (pBR325 containing a 9kb PstI fragment of the actinorhodin biosynthetic gene cluster, spanning both the class I and class III genes (Malpartida and Hopwood, 1984). Hybridization of the probe (s.a. 2.8×10^{7} dpm ug^{-1}), to the filter was performed in Denhardts solution with 5xSSC at 60^{o}C. Washes, carried out at this relaxed stringency, should have allowed sequences of both weak and strong homology to bind. Figure 5.1a shows that this was indeed observed, with probe bound in several distinct bands in most tracks and also to high molecular weight molecules. To determine the stability of the hybrids and identify any specific fragments with strong homology to the probe (good candidates for tetronasin biosynthetic genes), the stringency of subsequent washes was increased. Washing at 65^{o}C in 5xSSC (Figure 5.1b) identified three hybrids, 9.6kb SstI, 2.9kb KpnI, and 1.5kb SalI, which were stable under these conditions. The signal to noise level was improved by a further increase in stringency, washing at 70^{o}C, in 1xSSC (Figure 5.1c). The probe DNA remained bound to these sequences even at 70^{o}C, 0.1xSSC, 0.1% SDS (w/v), although improvement in the signal to noise ratio could not be discerned (Figure 5.1d).

This experiment showed the presence of sequences in the S. longisporoflavus genome with homology to genes involved in actinorhodin biosynthesis. Furthermore the hybrids formed, were stable in a high stringency wash. To simplify the identification of clones containing the cross hybridizing sequences the hybridization conditions were manipulated to increase the signal to noise ratio. The following experiment served to illustrate several points. Its

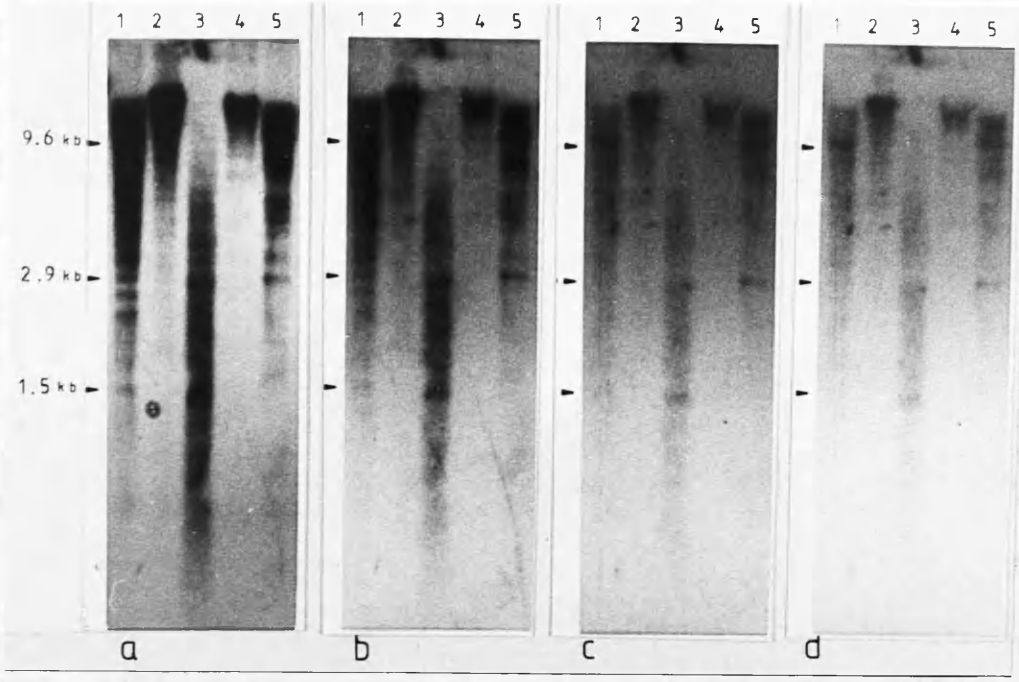


FIGURE 5.1; Hybridization Of pIJ2330 To Total DNA (2ug) Prepared From S. longisporoflavus 83E6

Lane

1. S. longisporoflavus total DNA digested with Sst1
2. S. longisporoflavus total DNA digested with SphI
3. S. longisporoflavus total DNA digested with SalI
4. S. longisporoflavus total DNA digested with PstI
5. S. longisporoflavus total DNA digested with KpnI

The s.a. of the probe was 2.8×10^7 dpm ug^{-1}.
Hybridization was carried out in Denhardts solution with 5xSSC at 60°C. Washing conditions; a, 5xSSC, 60°C b, 5xSSC, 65°C c, 1xSSC, 65°C d, 0.1xSSC, 70°C, 0.1% SDS (w/v).

main objective was to determine hybridization conditions which permitted formation of a duplex between the actinorhodin class I fragment and the putative tetronasin synthase sequence, whilst minimising the amount of non-specific hybridization of probe with S. longisporoflavus DNA. Four identical agarose gels were blotted under alkaline conditions to nylon membrane. Genomic DNA from S. hygroscopicus and S. rimosus was included to provide both weak and strong, positive controls for hybridization. S. hygroscopicus produces the polyketide milbemycin. Strong cross hybridization with actI was instrumental in the isolation of DNA fragments from this strain, which are thought to be involved in milbemycin biosynthesis (Malpartida et al., 1987). Genes for oxytetracycline biosynthesis have much less homology with actI and indeed Malpartida et al., were unable to demonstrate cross hybridization of actI to total DNA from S. rimosus under the washing conditions; 0.2xSSC, 70°C, 0.1% (w/v) SDS, (calculated by the authors to destabilise duplexes with less than 80% homology - although this must be dependent on the distribution of the mismatches).

Random primed probe DNA was prepared using, as a template, 25ng of the 2.2kb BamHI fragment isolated from pIJ2330 and known to contain the coding sequence of the actinorhodin condensing enzyme. The s.a. of the probe was calculated at $1x10^8$ dpm ug^{-1} DNA. The low level of activity obtained was due to the use of 800 Ci $mmol^{-1}$ dCTP in the reaction, and only 30% incorporation. Hybridization was performed in Denhardts solution plus 6xSSC varying the temperature as detailed in Table 5.1. Washing was carried out in 0.1% (w/v) SDS using the temperature and salt conditions also given in Table 5.1.

The reduction in the salt concentration for washing of blots 5.2b and d was standardised so the stringency of the individual experiments could be compared. From the equation formulated by Dove and Davidson (section 5.2d), a 3 fold drop in salt concentration should result in a decrease in the Tm of about 9°C. This would be equivalent to washing blots 5.2b and d in 6xSSC at 69°C and 74°C respectively, so ostensibly blot 5.2b was washed in more stringent conditions than blot 5.2c.

In spite of a lack of hybridization between the probe and

TABLE 5.1; Temperature And Salt Conditions Of Hybridization And Washing For Figures 5.2a To 5.2d Using The act Class I Probe

Figures	hydridization		washing	
	temp. (^{o}C)	salt conc. (xSSC)	temp. (^{o}C)	salt conc. (xSSC)
5.2a	60	6	60	6
5.2b	60	6	60	2
5.2c	65	6	65	6
5.2d	65	6	65	2

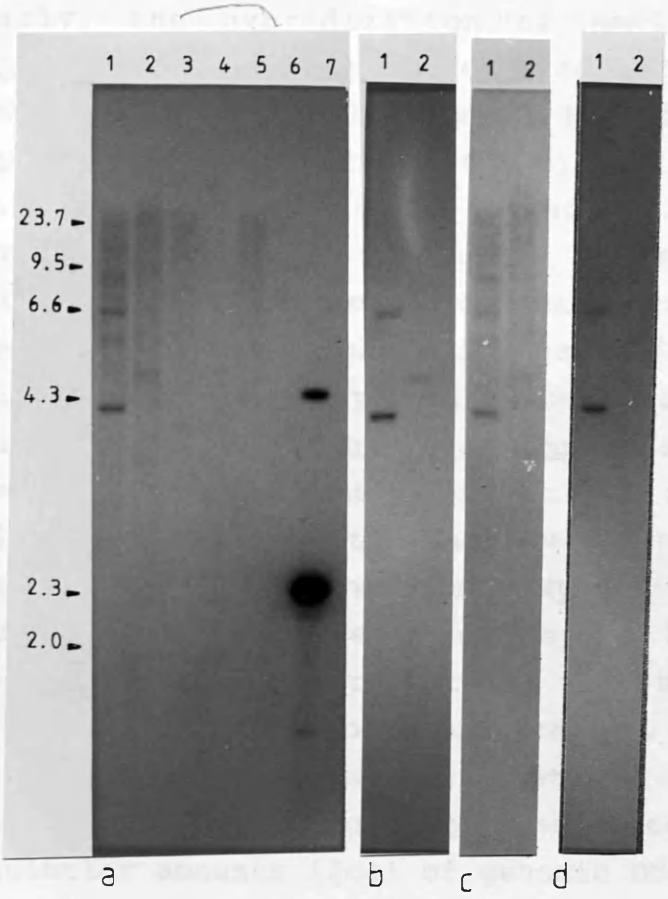


FIGURE 5.2; Hybridization Of actI To Total DNA From S. hygroscopicus And S. rimosus; A Comparison Of The Effects Of Small Manipulations Of The Stringency Of Hybridization And Washing

Lane
1. S. hygroscopicus total DNA digested with BamHI
2. S. rimosus total DNA digested with BamHI and BglII
3. S. longisporoflavus total DNA digested with SstI
4. S. longisporoflavus total DNA digested with SalI
5. S. longisporoflavus total DNA digested with KpnI
6. phage lambda digested with HindIII
7. gel purified fragments of PKS genes (monI, actI and otcY 5ng of each)

Lanes 1 and 2 only, are shown for 5.2b-d.
The s.a. of the probe (actI, 2.2kb BamHI fragment) was $1x10^8$ dpm ug^{-1}. The hybridization fluid contained Denhardts solution. The hybridization and washing conditions for each experiment are given in Table 5.1.

S. longisporoflavus sequences, several conclusions regarding the methodology could be drawn from examination of the hybridization of actI to DNA from S. hygroscopicus and S. rimosus (Figure 5.2a, b, c, d).

Firstly, the hybridization of actI to DNA from S. hygroscopicus and S. rimosus endorsed the validity of the approach. The act class I fragment hybridized strongly with two BamHI fragments (6.6kb and 4.0kb) of S. hygroscopicus and somewhat less strongly to the expected 5.0kb BamHI/Bgl II fragment of S. rimosus. Comparison of the signal to noise ratio of these bands under the different conditions indicated that hybridization at higher stringency (blots 5.2c and d), ensured less background hybridization while maintaining - at least in the case of S hygroscopicus - a similar level of positive signal. This must reflect the sensitivity of the rate of hybridization, determined by the nucleation rate, to the stringency of the conditions. Hybrid stability was less sensitive to the stringency employed, because blot 5.2b washed in more stringent conditions than blot 5.2c, retained higher background levels of hybridization.

The level of homology between the act class I fragment and S. longisporoflavus sequences appeared to be low. Equimolar amounts (2ug) of genomic DNA from each of the species were used. The level of homology with DNA from S. hygroscopicus proved sufficient for Malpartida et al., (1987), to isolate recombinants containing milbemycin biosynthetic genes. The 4.0kb BamHI homology observed in this study was consistent with the published restriction map of the milbemycin gene cluster. The 6.6kb fragment, with less homology, is of unknown function. Malpartida and his co-workers were unable to show cross-hybridization with S. rimosus genomic DNA, and required the use of cloned DNA from S. rimosus to identify regions of weak homology. To achieve hybridization with S. rimosus genomic DNA therefore indicates that this experiment was relatively sensitive. Lane 7 of Figure 5.2 contained 20ng of the 4.3kb monI fragment and 10ng of the 2.1kb actI fragment. Also included were 10ng of a 2.2kb fragment and 5ng of a 1.5kb fragment from the otcY region of the oxytetracycline biosynthetic gene cluster. Thus a mixture of DNA fragments were provided to which the level of probe hybridization could be compared. Annealing of the probe to the actI fragment represented the level of

hybridization obtained when the sequences were perfectly matched. In comparison the level of hybridization to monI and in particular to the 1.5kb otcY fragment was greatly reduced and is likely to directly reflect the number of mismatched base pairs in the heteroduplexes formed (unfortunately the hybridization signal from the 2.2kb otcY fragment was obscured by the level of hybridization to the 2.1kb actI fragment).

For cross hybridization to be an efficacious strategy required a refined technique to generate a strong signal, clearly discriminated from the background noise. This was achieved by altering three parameters in the next experiment. Again three identical agarose gels were used which allowed the stringency of hybridization to be modulated. However, the amount of DNA digested and fractionated in the agarose gel was increased to 4ug per lane, decreasing the dependence of the rate of hybridization on the nucleation rate and providing more fragments, hopefully to increase the number of hybrids formed. The specific activity of the probe DNA (act class I fragment), was increased to 2.4×10^9 dpm ug^{-1} by using 3000 Ci $mmol^{-1}$ dCTP (89% incorporation). Finally the inclusion of 10% (w/v) of dextran sulphate was anticipated to promote the rate of hybridization and formation of networks. Figures 5.3a, b, and c show the results of hybridization at 65°C, with 6x, 4x, and 2xSSC respectively. The welcome improvement in signal was accompanied by an increase in non-specific background hybridization - deemed to be a result of hybridization at relaxed stringency. The probe DNA was stripped from the membrane (see Chapter 2), and hybridization was repeated with freshly prepared probe at 65°C in 1x (Figure 5.3d), 0.5x (Figure 5.3e), and 0.2xSSC. No hybridization of actI to S. longisporoflavus DNA was observed at 65°C in 0.2xSSC although the expected hybrids were formed with S. hygroscopicus and S. rimosus DNA (not presented). Hybridization of actI to DNA from S. longisporoflavus at 65°C in 0.5xSSC approached the discrimination and visual acuity achieved with hybridization of actI to S. hygroscopicus and S. rimosus DNA (Figure 5.3e). This was viewed optimistically for the applicability of the technique to the isolation of tetronasin biosynthetic genes.

One surprise finding was the change in hybridization profile from the first Southern (Figure 5.1).

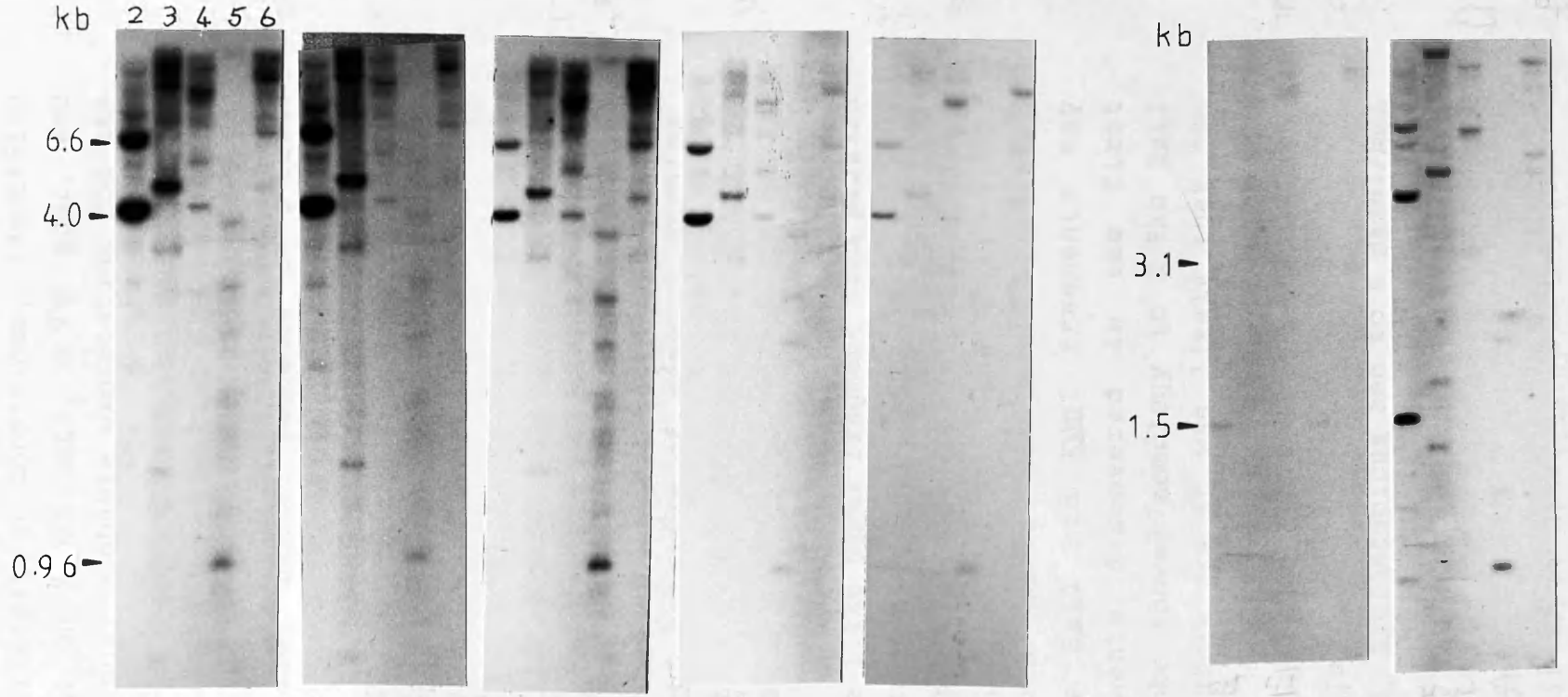


FIGURE 5.3; Cross Hybridization Of actI (a-e), actIII (f) And monI (g) To Total DNA From S. longisporoflavus 83E6, S hygroscopicus And S. rimosus

Lane
1. phage lambda digested with HindIII
2. S. hygroscopicus total DNA digested with BamHI
3. S. rimosus total DNA digested with BamHI and BglII
4. S. longisporoflavus total DNA digested with SstI
5. S. longisporoflavus total DNA digested with SalI
6. S. longisporoflavus total DNA digested with KpnI

The s.a. of the probes were, 1.5×10^{9}dpm ug^{-1} for actI (a, b and c), 2.4×10^{9}dpm ug^{-1} for actI (d and e), 9.6×10^{6}dpm ug^{-1} for actIII (f), and 1.0×10^{9}dpm ug^{-1} for monI (g).
Hybridization was carried out in Denhardts solution and 10% (w/v) dextran sulphate at 65°C with, a, 6xSSC b, 4xSSC c, 2xSSC d, 1xSSC e,f, and g, 0.5xSSC.
Washing was carried out at the same stringency as hybridization.

The strongest homology to actI was found on a 9.8kb SstI fragment, a 14kb KpnI fragment, and a 0.96kb SalI fragment. The previous finding, using a probe synthesized from total pIJ2330 and different hybridization conditions, identified the homology to be present on 9.6kb SstI, 2.9kb KpnI, and 1.5kb SalI fragments. The most probable explanation for the inconsistency was that the hybrids formed in the first experiment did not represent cross homology between the condensing enzymes of the actinorhodin and tetronasin synthases, but rather between another sequence on pIJ2330 (most likely involved in actinorhodin biosynthesis but one cannot dismiss pBR325 sequences) and sequences in the S. longisporoflavus genome. The 8.8kb PstI fragment contained in pIJ2330 complements several classes of actinorhodin mutants (Malpartida and Hopwood, 1986); therefore it carries the genes for biosynthetic steps I, III, IV, VB, and VII and part of the positive-regulatory gene II. Homology to the actIII gene has been demonstrated (Malpartida et al., 1987), to be closely linked to the actI homology of several strains, namely S. rimosus (oxytetracycline), S. hygroscopicus (milbemycin), and S. violaceoruber (granaticin), although a similar arrangement was not found with S. glaucescens (tetracenomycin). Using the 1.1kb BamHI fragment from pIJ2330 as a probe the hybridization pattern of actIII to S. longisporoflavus DNA was investigated (Figure 5.3f). Despite the low s.a. of the probe, 1.5kb SalI, 3.1kb and 14kb KpnI and 9.8kb SstI fragments could be detected in S. longsporoflavus DNA. The SalI and KpnI fragments may correspond to the fragments discovered in the first experiment. The actIII probe showed homology to 9.8kb SstI and 14kb KpnI fragments. Therefore it was likely that the actI and III homologues were closely linked in the S. longisporoflavus genome, spanned by the two SstI sites but on separate SalI fragments. The actIII gene hybridized to a 1.5kb BamHI fragment from S. hygroscopicus and to a BamHI/BglII fragment from S. rimosus of approximate size 18kb, as expected from the data of Malpartida et al, (1987). Hybridization of the monI gene - a 4.3kb DNA fragment cloned from S. cinnamonensis by virtue of homology with actI/III - to DNA from S. longisporoflavus was also investigated. Hybridization and subsequent washing at 65^{o}C, 0.5xSSC, using a probe with s.a. 1x10^{9} dpm ug^{-1}, provided a curious result

(Figure 5.3g). The monI probe hybridized strongly with the expected 4.0kb BamHI fragment from S. hygroscopicus and had homology with a 6.6kb fragment producing a similar banding pattern to that generated by actI. But the monI probe also hybridized strongly with an additional 1.5kb fragment. Superimposition of the autoradiograph of actIII hybridized to the same Southern blot, indicated the smallest fragment with homology to monI to be exactly the same size as the actIII homologue. This result confirmed that monI shares homology with both the Beta-ketoacyl synthase and Beta-ketoacyl-ACP reductase of actinorhodin biosynthesis, and further implicates it in having a role in monensin biosynthesis in S. cinnamonensis. This deduction correlates well with hybridization of monI to 5.0kb and approximately 18kb fragments of a BamHI/BglII digest of S. rimosus DNA (predicted sizes of fragments encoding the condensing enzyme and ketoreductase for oxytetracycline biosynthesis).

In view of this homology between actI and monI and the shared homologues in S. hygroscopicus and S. rimosus DNA, the hybridization of monI and actI/III to DNA from S. longisporoflavus was expected to be similar. The observed result however, was not consistent with the expectations. Fragments with homology to monI were measured at 14, 6.0 and 3.6kb for SstI (6.0kb gave the strongest signal), 2.1, 1.1, and 0.74kb for SalI (0.74kb gave the strongest signal), and 15, 5.2 and 4.6kb for KpnI (15kb gave the strongest signal). Only the weak 4.6kb KpnI homology correlated with a weak homology to actI, and this may have been coincidental in the absence of other corresponding bands.

At this stage the only evidence for the involvement of the 4.3kb BamHI fragment from S. cinnamonensis in monensin biosynthesis was circumstantial cross-homology with actI, whereas actI itself had been shown, by complementation of mutants and gene disruption, to be involved in actinorhodin biosynthesis in S. coelicolor. It seemed sensible therefore to clone the actI homologue as the main objective but also to isolate the monI homologue in case sequence divergences of the tetronasin synthase was such that it was only distantly related to actI. The prudence of this approach was confirmed by comparison of the DNA sequence of monI (J. A. Robinson, pers. comm.) with the polyketide synthases for granaticin and tetracenomycin biosynthesis (Sherman et al, 1989; Bibb et

al, 1989). In Southern analysis all three are homologous with actI, and sequence analysis has shown conserved homology, at the amino acid level, with B-ketoacyl synthase of the yeast multifunctional fatty acid synthase. Furthermore, not only are the primary nucleotide sequences of monI, tcm1a and the granaticin polyketide synthase closely related, the arrangement of open reading frames is similar - providing more convincing evidence for the role of monI in polyketide biosynthesis. If actI and monI both encode bona fide components for a polyketide synthase and assuming tetronasin biosynthesis to require only one - whichever - what is the function of the other homologue? It is possible that the B-ketoacyl synthase and B-ketoacyl-ACP reductase sequences of monI are too distantly diverged from the equivalent genes of S. longisporoflavus for cross hybridization and other seqences on the 4.3kb BamHI (perhaps the ACP or another more esoteric component of polyether synthases) provide the homology with S. longisporoflavus DNA. It is equally plausible that one of the probes has hybridized with DNA encoding the fatty acid synthase or even another polyketide synthase for an unidentified antibiotic. The perceived solution to the dilema was to clone the strongest cross hybridizing sequences (described in section 5.5). Complementation of blocked mutants or gene disruption of wild type sequences, could then be tested to determine the function of the isolated DNA.

5.3 RESTRICTION MAP OF THE actI HOMOLOGUE, LOCUS IN S. longisporoflavus

Use, only of purified DNA of the appropriate size for constructing a genomic sub-library, reduces the number of recombinants required to be screened by cross hybridization. Further enrichment for the desired DNA fragment can be achieved, if the restriction sites surrounding the genomic locus of the homologue are known. This enables a DNA fragment of known length with non self-compatible sticky ends to be targeted. With vector DNA cut with the same restriction endonucleases, recircularisation is prevented, and only ligation to insert DNA with compatible sticky ends will generate a viable plasmid. Cleavage of the DNA with restriction endonucleases which have no site within the targeted fragment also reduces the number of competing DNA

fragments which could give rise to a covalently-closed circular molecule.

In the following three experiments DNA from S. longisporoflavus was digested with three restriction enzymes, individually, in all pairwise combinations and as a triple digest. The enzymes were chosen because they each have a unique restriction site within the polylinker region of the pTZ18^{R} vector. The reaction products were separated in 0.8% (w/v) agarose gels and transferred, in alkali, to Hybond-N. Hybridization of radiolabelled actI was performed at 65^{o}C, 0.5xSSC giving the results shown in Figure 5.4.

The size of each hybridizing fragment was calculated and tabulated in Table 5.2.

The absence of smaller fragments (Figure 5.4) after digestion with two enzymes in any of the combinations tested, indicated (within the limits of resolution of the gel) that in each experiment the single enzyme restriction fragments did not overlap partially. The 0.96kb SalI fragment was completely internal to all the other sized fragments. The 2.5kb SmaI fragment was completely internal to the 14kb KpnI fragment. The 5.0kb BamHI fragment was completely internal to an EcoRI fragment (of undetermined size), and to a 10.05kb SstI fragment which was internal to the large PstI fragment.

A further experiment was undertaken to test for HindIII, XbaI, KpnI or SphI fragments overlapping with the 5.0kb BamHI fragment. From Figure 5.5a it can be seen that a 13kb SphI fragment was identified, but a partial overlap of the BamHI fragment was not found.

In the absence of an obvious partial overlap of the restriction fragments identified, a forced cloning strategy was not adopted. The 5.0kb BamHI homologue, a convenient size for cloning into a plasmid vector, was therefore targeted for direct cloning into pTZ18^{R}. In order to minimise ligation of the vector to itself, it was treated with CIAP to remove the 5' phosphate groups from the linear molecules.

5.4 BamHI FRAGMENTS FROM S. longisporoflavus WITH HOMOLOGY TO monI

The actI probe was stripped from Southern blot 5.5. It was re-probed with the monI fragment and the strongest S. longisporoflavus homology was calculated to be present on a 7.5kb BamHI fragment (Figure 5.5b). Despite candidate

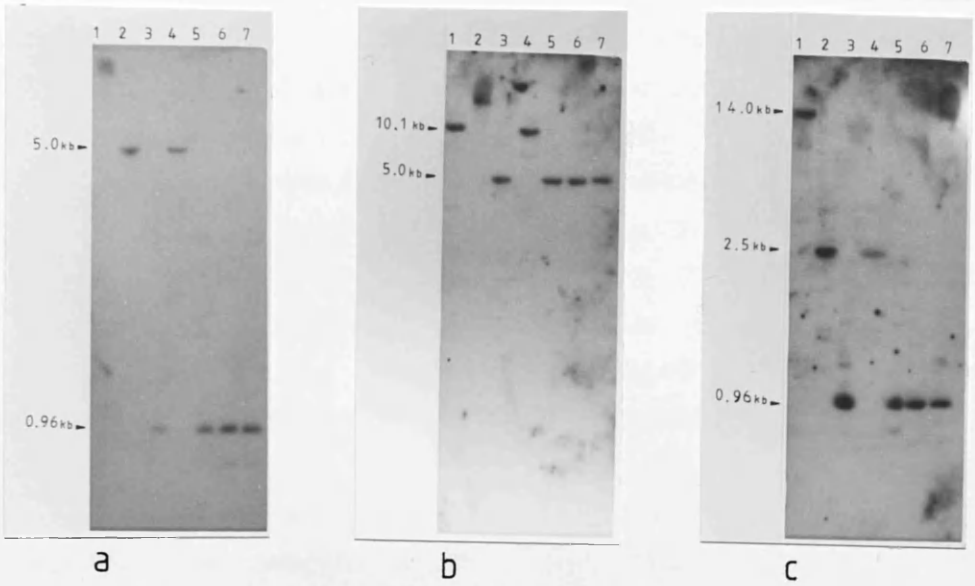


FIGURE 5.4; Cross Hybrdization of actI To Total DNA From S. longisporoflavus 83E6 Digested With Various Combinations Of Restriction Endonucleases

S. longisporoflavus total DNA digested with

Figure 5.4a	Figure 5.4b	Figure 5.4c
1. E	1. Ss	1. K
2. B	2. P	2. Sm
3. S	3. B	3. H
4. E+B	4. Ss+P	4. K+Sm
5. E+S	5. Ss+B	5. K+H
6. B+S	6. P+B	6. Sm+H
7. E+B+S	7. Ss+P+B	7. K+Sm+H

Key, B = BamHI P = PstI
E = EcoRI S = SalI
H = HincII Sm = SmaI
K = KpnI Ss = SstI

The s.a. of the probe was 9.8×10^8dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.5xSSC at 65^oC. Washing was performed at the same stringency.

TABLE 5.2; Length Of DNA Fragments With Homology To *actI*

Restriction Enzyme	Length (kb)
SalI (*HincII*)*	0.96
SmaI	2.5
BamHI	5.0
SstI	10.05
KpnI	14.0
PstI	>23

**HincII* an isoschizomer of *SalI* was substituted for *SalI* in expt. 3 because it was more active in the buffer required for efficient *KpnI* and *SmaI* digestion.

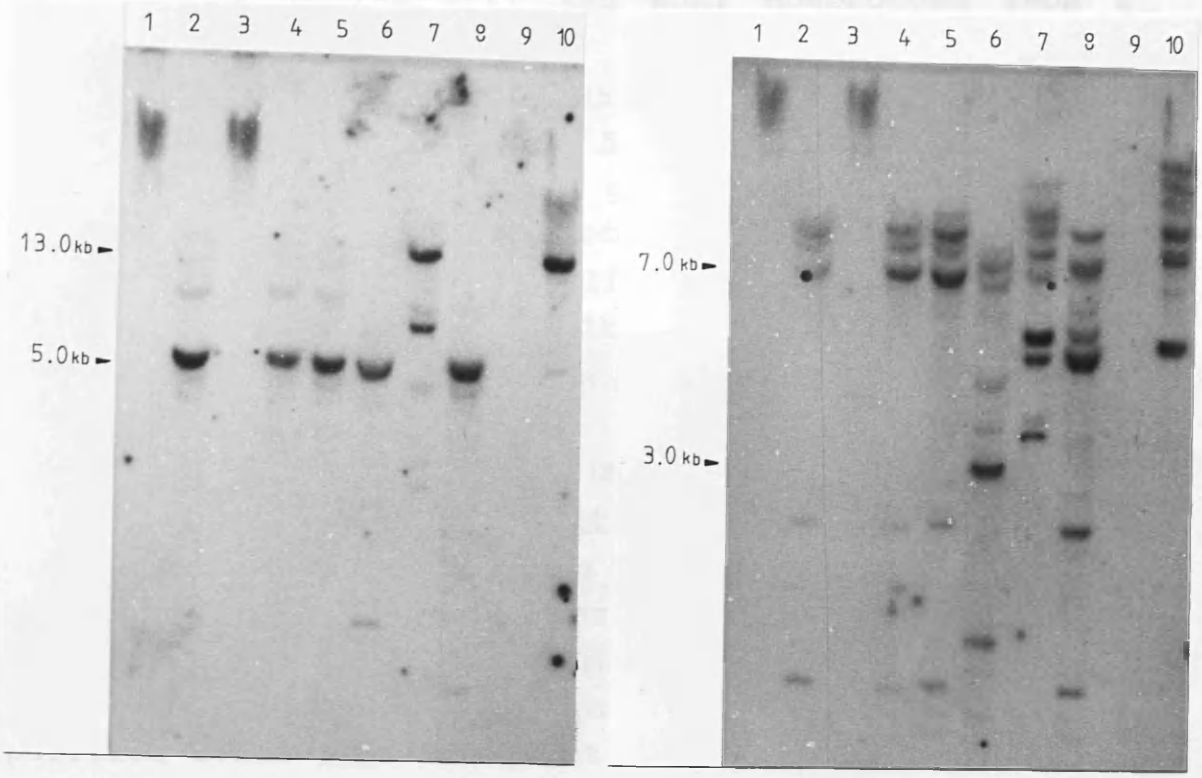


FIGURE 5.5; Cross Hybrdization Of actI (a) And monI (b) To Total DNA From S. longisporoflavus 83E6 Digested With Various Combinations Of Restriction Endonucleases

Lane

1. S. longisporoflavus total DNA digested with HindIII
2. S. longisporoflavus total DNA digested with HindIII + BamHI
3. S. longisporoflavus total DNA digested with XbaI
4. S. longisporoflavus total DNA digested with XbaI + BamHI
5. S. longisporoflavus total DNA digested with BamHI
6. S. longisporoflavus total DNA digested with BamHI + KpnI
7. S. longisporoflavus total DNA digested with KpnI
8. S. longisporoflavus total DNA digested with BamHI + SphI
9. phage lambda DNA digested with HindIII
10. S. longisporoflavus total DNA digested with SphI

The s.a. of the probes were 9.8×10^8dpm ug^{-1} for actI (a), and 1.5×10^9dpm ug^{-1} for monI (b). Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.5xSSC at 65°C. Washing was performed at the same stringency.

BamHI/Sph I and BamHI/KpnI fragments suitable for forced cloning, the 7.5kb BamHI fragment was targeted for cloning into pTZ18^{R}.

5.5 CLONING OF THE actI AND monI HOMOLOGUES FROM S. longisporoflavus

1mg of S. longisporoflavus 83E6 total DNA was digested with restriction endonuclease BamHI. The reaction products were separated by TAE agarose gel electrophoresis using an extended well width to accommodate the large volume. Using phage lambda cut with HindIII as a guide, the gel was fractionated between the 4.3kb and 9.5kb size markers. The DNA migrating between these markers was purified using a "Gene Clean" kit.

50ng of the recovered DNA, migrating between 4.3 and 6.6kb (likely to contain the actI homologue), was loaded onto a 0.8% (w/v) agarose gel. 30ng of pTZ18^{R} linearised at the unique BamHI site was also included to provide a negative control for hybridization stringency. Hybridization of the actI probe (made by random primed DNA synthesis, using gel purified 2.1kb BamHI fragment as a template), yielded a strong signal from pTZ18^{R} compared with only weak levels from S. longisporoflavus DNA (Figure 5.6b).

This posed a serious problem. Under the conditions used it would be impossible to identify a recombinant carrying the actI homologue from a gene bank. It was hoped that conditions could be found which prevented formation of these hybrids. However the hybrids were stable (increased stringency of washing did not melt the duplex), nor could nucleation be prevented by increasing the stringency of hybridization (Figure 5.7a). The problem was common to hybridization with monI (shown in Figure 5.7b), and the level obtained at 70^{o}C, 0.25xSSC, - at which neither actI nor monI hybridize with S. longisporoflavus DNA - was indicative of association of homologous sequences. pBR325 and pBR327, the parental vectors of pIJ2330 and pMon respectively, and pTZ18^{R} are related derivatives of the natural E. coli plasmid colE1. The presence of vector sequences within the probe DNA would explain the high levels of hybridization observed. Contamination of the gel-purified fragment was attributed to the presence of linear molecules, generated during purification of the plasmid DNA. These molecules will have

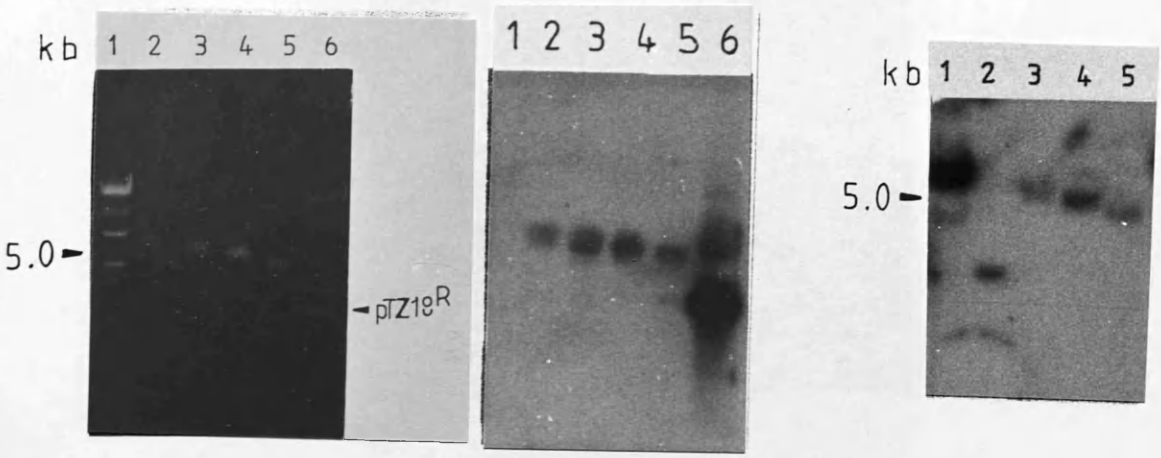


FIGURE 5.6; Cross Hybrdization Of actI Prepared From pIJ2330 (b) And pLoractI (c) With Gel-Purified DNA Fractions Of BamHI Digested Total DNA From S. longisporoflavus 83E6

Figure 5.6a (agarose gel stained with ethidium bromide), Figures 5.6b and c (autoradiograph)
Lane
1. phage lambda DNA digested with HindIII
2. fraction A
3. fraction B
4. fraction C
5. fraction D
6. $pTZ18^R$ digested With BamHI

Figure 5.6c,
1. phage lambda DNA digested with HindIII
2. $pTZ18^R$ digested With BamHI
3. fraction B
4. fraction C
5. fraction D

The s.a. of the probes were 8.6×10^8 dpm ug^{-1} for actI prepared from pIJ2330 (b), and 1.5×10^9 dpm ug^{-1} for actI prepared from pLoractI (c). Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.5xSSC at 65^oC. Washing was performed at the same stringency.

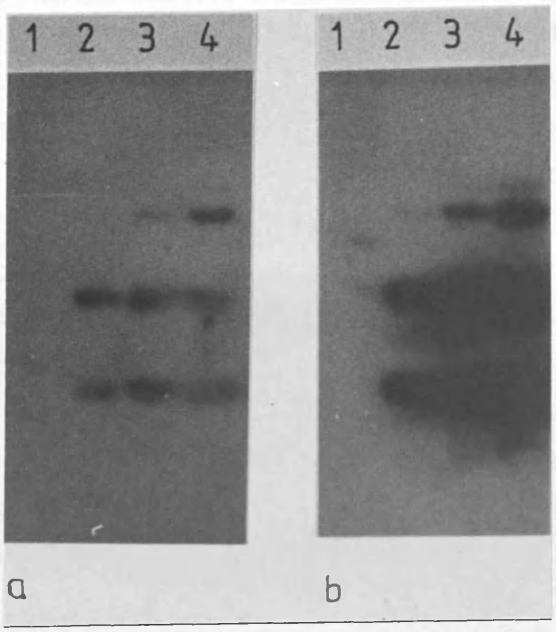


FIGURE 5.7; Hybrdization Of The "actI" Probe Prepared From pIJ2330 (a) And The "monI" Probe Prepared From pMonI (b) To $pTZ18^R$

Lane
1. phage lambda DNA digested with HindIII
2. 5ng of $pTZ18^R$ digested with BglI*
3. 10ng of $pTZ18^R$ digested with BglI*
4. 50ng of $pTZ18^R$ digested with BglI*

The s.a. of the probes were 5.0×10^8dpm ug^{-1} for actI (a) and 2.0×10^9dpm ug^{-1} for monI (b). Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.25xSSC at 70°C. Washing was performed at the same stringency.

*The largest fragment in each lane is linearised $pTZ18^R$ due to partial digestion of the plasmid with BglI.

been linearised at random positions in the circle. Digestion with restriction endonucleases therefore yields a population of DNA fragments of all sizes smaller than the longest expected restriction fragment. For most purposes this would not be considered a problem - the majority of the DNA would be in ccc form, and the small amount of linear DNA would give a background smear after digestion, which would not be detected by ethidium bromide staining. However only a very small amount of template DNA is required for random primed DNA synthesis and the use of dextran sulphate in hybridization promotes the formation of networks of overlapping sequences which allows the actI or monI labelled DNA to contribute to the signal from pTZ18^{R}. Accepting that the presence of labelled vector DNA in the probe was inevitable, and given the low level of homology between actI or monI and their S. longisporoflavus counterparts (requiring long exposure times for the autoradiographs), it was not surprising that the problem arose.

To confirm the nature of the problem and also hopefully alleviate it, the 2.1kb BamHI, actI and 4.3kb BamHI, monI fragments were subcloned into the lambda based vector, pLorist6 (Gibson et al., 1987).

pLorist 6 was digested with BamHI, phosphatased, and gel purified. The desired BamHI fragments from pIJ2330 and pMon were also gel-purified using a "Gene Clean" kit. Ligation was performed at 20ng ul^{-1} with 0.1U of T4 DNA ligase ug^{-1}. Plasmid DNA was prepared from single colonies (see Chapter 2.11.1.3) containing putative recombinants, pLoractI and pLormonI respectively. Separation of the DNA by agarose gel electrophoresis, (Figure 5.8), identified recombinants by their retarded mobility through agarose gels.

Labelled actI DNA - purified from pLorist6 - whilst hybridizing with S. longisporoflavus DNA (albeit only weakly - probably due to the low specific activity of the probe) gave only a faint signal with pTZ18^{R} (Figure 5.9). Note that despite gel purification of the actI template away from the pLorist6, there was high level hybridization to pLorist6 and to the lambda 6.6kb HindIII fragments on the filter, which confirmed that the previous problem in hybridization to pTZ18^{R} with the actI probe was due to random priming of vector fragments in the fraction containing the purified actI band.

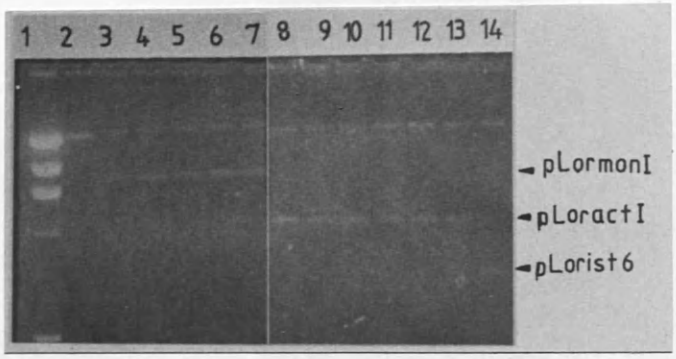


FIGURE 5.8; Plasmid DNA Preparations From Single Colonies Of *E. coli* Which Contain Putative pLoractI And pLormonI Recombinants

Lane

1. phage lambda DNA digested with *Hin*dIII
2-7. DNA prepared from single colonies containing putative pLormonI recombinants
8-13. DNA prepared from single colonies containing putative pLoractI recombinants
14. DNA prepared from a single colonies containing pLorist6

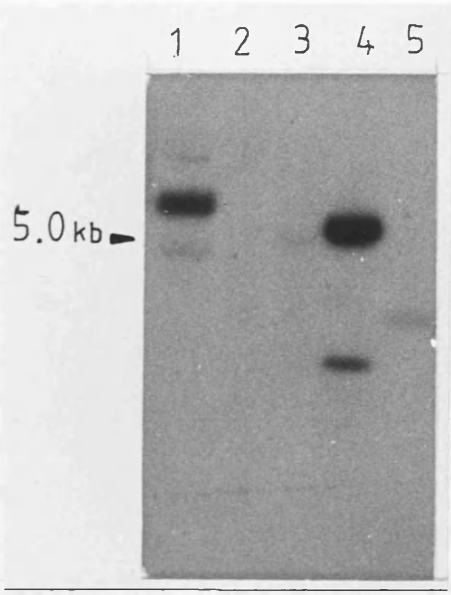


FIGURE 5.9; Cross Hybrdization Of actI Prepared From pLoractI With Total DNA From S. longisporoflavus 83E6, Phage Lambda DNA And pTZ18^{R}

Lane
1. phage lambda DNA digested with HindIII
2. 4ug Of S. longisporoflavus total DNA digested with SalI
3. 4ug Of S. longisporoflavus total DNA digested with BamHI
4. 50ng pLoractI digested with BamHI
5. 50ng pTZ18^{R} digested with BamHI

The s.a. of the probes was 2.5×10^{8}dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.5xSSC at 65^{o}C. Washing was performed at the same stringency.

Hybridization of the actI probe prepared from pLorist 6, to the purified BamHI-cut DNA fractions from S. longisporoflavus, indicated fraction 3 to contain the desired homologue (Figure 5.6c). Low level hybridization of the probe to $pTZ18^R$ was not viewed as an insurmountable problem, as equimolar amounts of the homologue would be expected to intensify the signal from a positive clone.

Highly competent E. coli JM109 cells were prepared to facilitate construction of the enriched gene library. JM109 allowed identification of recombinants through gene disruption of the $pTZ18^R$ encoded B galactosidase subunit 2. The Hanahan, (1983), protocol II for making competent cells suitable for frozen storage was followed strictly, and cells with a transformation efficiency of $3.2x10^7$ transformants ug^{-1} of $pTZ18^R$ were recovered.

Ligations were performed in a volume of 25ul, with phosphatased vector DNA concentration of 1ng ul^{-1} and a 2:1 molar ratio of S. longisporoflavus sequences to $pTZ18^R$. 0.1U of T4 DNA ligase was added and the reaction was allowed to proceed for 2hrs at 22^oC before diluting 5-fold with 0.5xTE. After transformation of E. coli JM109 with 15ul of ligation mix (equivalent to 3ng vector DNA), the number of blue and white transformants were scored (Table 5.3).

From subsequent transformations almost 1200 and 800 recombinants from the enriched gene banks for the respective actI and monI homologues were recovered. These were patched in duplicate onto L-agar containing ampicillin. Several positive (oxytetracycline synthase gene in both high and low copy), and negative (non-recombinant pLorist6), control colonies were included on the master plates. The colonies were transferred to Hybond-N and allowed to continue growth for a further 48hrs before lysis and binding of the DNA to the membrane. Prehybridization of all eighteen 9.5x9.5cm filters was performed in a large volume (100ml), at 65^oC, 0.5xSSC. Radiolabelled probes were made by random primed DNA synthesis from the actI and monI, BamHI fragments purified from pLoractI and pLormonI respectively. The s.a. of both probes was calculated to be $2x10^9$ dpm ug^{-1}. Hybridization was performed in a 10ml volume for each duplicate gene bank (twelve filters for actI and eight filters for monI), at the same stringency as prehybridization. Figure 5.10 shows the result from filter AC (A=actI, filter C). The filter

TABLE 5.3; Cloning Of *actI* And *monI* Homologues From *S. longisporoflavus* Into *E. coli* JM109: Statistics Of The Enriched Gene Bank Contained In $pTZ18^R$

Ligation	Number of colonies counted	Trf. effic. (ug^{-1})	Number of whites	percentage recombinant
$pTZ18^R$ to *BamHI* fraction 3	93	$3x10^5$	77	83
$pTZ18^R$ to *BamHI* fraction 5	128	$4x10^5$	95	74

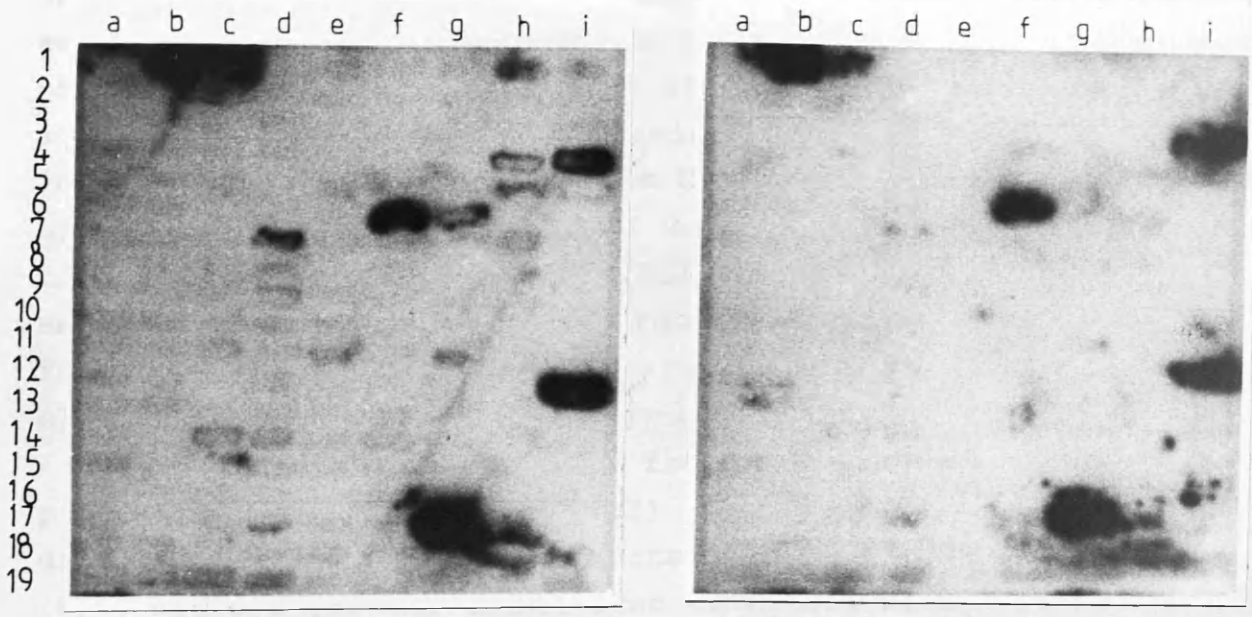


FIGURE 5.10; Hybridization Of actI Prepared From pLoractI To Duplicate Filters Of DNA From 171 E. coli JM109 Colonies (a1 - i19) Which Contained Part Of A Gene Library From S. longisporoflavus 83E6 Biased For BamHI Fragments Of Around 5kb In Length

Control colonies,
-ve, a1, e6, f17 = JM109 containing pLorist6
+ve, b1, f6, g17 = C1400 containing pPZ508
c1, g6, h17 = C1400 containing pPZ510
d1, h1, f17 = ED8767 containing pPZ518
putative tetronasin synthase clones;
i 4 = AC36 and i12 = AC108

The s.a. of the probe was $2x10^9$dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate with 0.5xSSC at 65°C. Washing was performed at the same stringency.

contained DNA from 171 individual colonies, patched out in a regular array. Colonies a1, e6 and f17 were negative controls (JM109 containing pLorist6). Colonies b1, f6 and g17 were positive controls (C1400 containing containing pPZ508, - part of the oxytetracycline synthase gene cluster in high copy number). Colonies c1, g6 and h17 were also positive controls (C1400 containing pPZ510, - part of the oxytetracycline synthase gene cluster, contiguous with the pPZ508 insert, in high copy number). Colonies d1, h1 and f17 were sensitive positive controls, (ED8767 containing pPZ518 - the oxytetracycline synthase gene in single copy). Clearly most of the sequences homologous to actI was contained on pPZ508, and at high copy numbers, probe association was obvious. With pPZ510 and pPZ518, hybridization of the probe DNA was low or absent. Two putative tetronasin synthase clones were identified on filter AC, at positions i4 and i12. The DNA hybridized strongly with actI on each of the duplicate filters. These were designated AC36 and AC108 respectively, and selected for further characterisation along with a further fifteen recombinants with homology to actI, and eleven recombinants with homology to monI.

Plasmid DNA prepared from 1.5ml of culture (STET preparation, Chapter 2.11.1.2) of each recombinant was digested with BamHI. The products were separated on a 0.8% (w/v) agarose gel and transferred to Hybond-N. Hybridization of actI and monI probes (Figures 5.11a and 5.11b respectively), identified several candidates with the desired homologues. Digestion of the same preparations with SalI indicated that eight of the ten isolates which hybridized strongly with actI contained identical inserts (XA169 and AA138 contained inserts which were both distinct from each other and the remaining recombinants and XB101 contained an additional BamHI fragment). Of the six recombinants which hybridized strongly with monI, five contained overlapping or identical inserts (MB9 and MA5 both contained an additional BamHI fragment of the same size and which probably originated from partial digestion of S. longisporoflavus DNA - see Chapter 6 for an extended restriction map of the MB74 locus in S. longisporoflavus - and MA155 contained an unusually small plasmid species of undetermined origin). AC36 and MB74 were chosen as likely homologues for actI and monI respectively. Confirmation that the correct fragments had

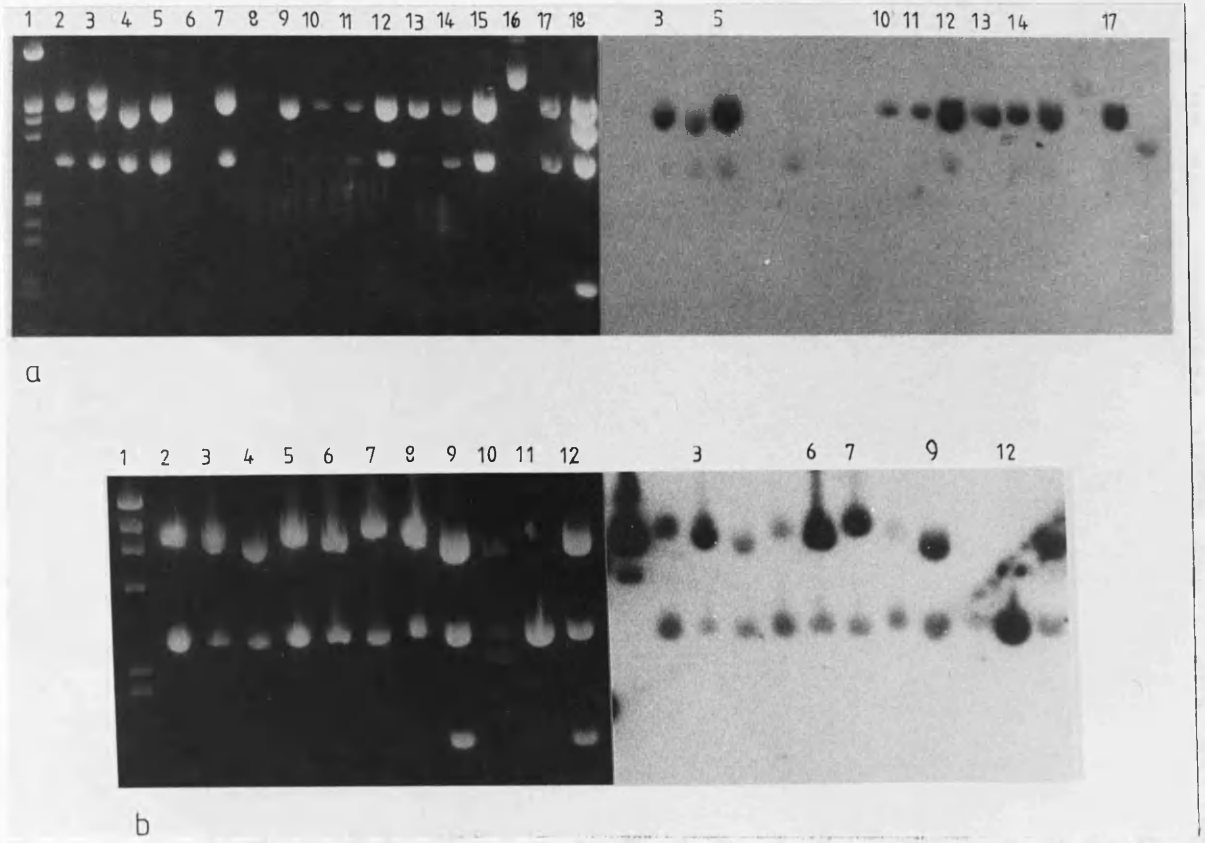


FIGURE 5.11; Hybridization Of actI (a) And monI (b) To Plasmid DNA Which Were Thought To Contain The actI or monI Homologues From S. longisporoflavus

a: Agarose gel (stained with ethidium bromide) and autoradiograph of STET DNA (digested with BamHI) prepared from recombinants thought to contain the actI homologue from S. longisporoflavus

Lane,

1. lambda HindIII/EcoRI
2. XB145
3. XB101
4. XA169
5. XA84
6. AD88
7. AD86
8. AD84
9. AD81
10. AD23
11. AC108
12. AC36
13. AB38
14. AB10
15. AA138
16. AA89
17. AA65
18. AA64

b: Agarose gel (stained with ethidium bromide) and autoradiograph of STET DNA (digested with BamHI) prepared from recombinants thought to contain the monI homologue from S. longisporoflavus

Lane,

1. lambda HindIII/EcoRI
2. MD118
3. MD6
4. MC165
5. MB82
6. MB74
7. MB64
8. MB28
9. MB9
10. MB6
11. MA155
12. MA5

been isolated was provided by hybridisation of radiolabelled AC36 and MB74 insert DNA with Southern blots of S. longisporoflavus total DNA. Clearly from Figure 5.12 the same KpnI, SalI and SstI fragments of S. longisporoflavus DNA with most homology to actI and monI, formed hybrids with AC36 and MB74 respectively.

5.6 HYBRIDIZATION OF AC36 AND MB74 WITH TOTAL DNA PREPARED FROM VARIOUS Streptomyces sp.

Hybridization of AC36 and MB74 with total DNA prepared from other streptomycetes which were known to produce polyketide metabolites was investigated. This was undertaken in order to identify any homology between the DNA isolated from S. longisporoflavus and DNA fragments suspected to be involved in polyketide metabolism.

From Figure 5.12 it was clear that AC36 did not share a high level of sequence homology with DNA from S. hygroscopicus or S. rimosus. This was also true for DNA from S. cinnamonensis and S. coelicolor (where a decrease in the stringency of the hybridization conditions - to 1xSSC at 65^{o}C - was required for hybridization to a 2.1kb BamHI fragment of S. coelicolor presumed to be actI). At the same stringency, hybridization to total DNA from S. rimosus was also possible (Figure 5.13b) and two high molecular weight fragments from a BamHI and BglII double digest and two SstI fragments (7.2 and 2.4kb) appeared to have sequence homology with AC36. Cross hybridization of MB74 with DNA from several streptomycetes was more profitable (Figure 5.13a). The sizes of restriction fragments with homology to MB74, and the expected size of fragment thought to encode the PKS endogenous to that strain, are summarised in Table 5.4. The general disagreement of the observed and expected results was exemplified by strong hybridization of MB74 to an 11kb BamHI fragment from S. cinnamonensis. Homology to the 4.3kb BamHI fragment used in cloning MB74 was not even represented as a faint band on the autoradiograph. Rearrangement of the sequences in vitro could be ruled out because the fragment obtained was in good agreement with the size expected from Southern analysis and the insert DNA also contained the expected KpnI and SphI restriction sites. Equally, actI, otcY and monI can all be shown to hybridize to a 4.3kb BamHI fragment in S. cinnamonensis total DNA (Figure 5.14), ruling out the

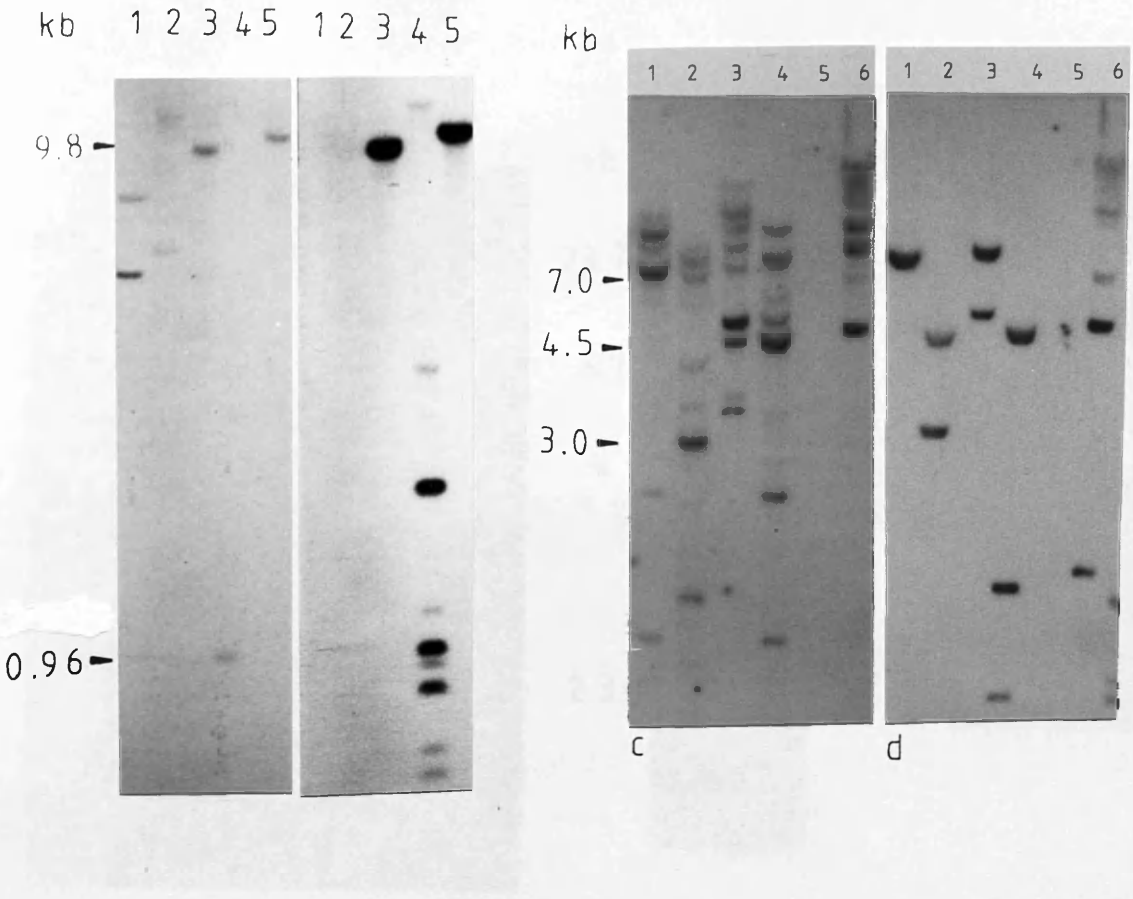


FIGURE 5.12; Cross Hybridization Of actI And AC36 (a And b Respectively), monI And MB74 (c And d Respectively), With Total DNA Prepared From S. longisporoflavus 83E6

a and b lane,
1. S. hygroscopicus total DNA digested with BamHI
2. S. rimosus total DNA digested with BamHI and BglII
3. S. longisporoflavus total DNA digested with SstI
4. S. longisporoflavus total DNA digested with SalI
5. S. longisporoflavus total DNA digested with KpnI

c and d lane,
1. S. longisporoflavus total DNA digested with BamHI
2. S. longisporoflavus total DNA digested with BamHI and KpnI
3. S. longisporoflavus total DNA digested with KpnI
4. S. longisporoflavus total DNA digested with BamHI and SphI
5. phage lambda DNA digested with HindIII
6. S. longisporoflavus total DNA digested with SphI

The s.a. of the probes were 2.4×10^9dpm ug^{-1} for actI (a), 9.0×10^8dpm ug^{-1} for AC36 (b), 1.5×10^9dpm ug^{-1} for monI (c) and 7.5×10^8dpm ug^{-1} for MB74 (d). Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate with 0.5xSSC at 65^oC for a, c and d, and with 1xSSC at 65^oC for b. Washing was performed at the same stringency.

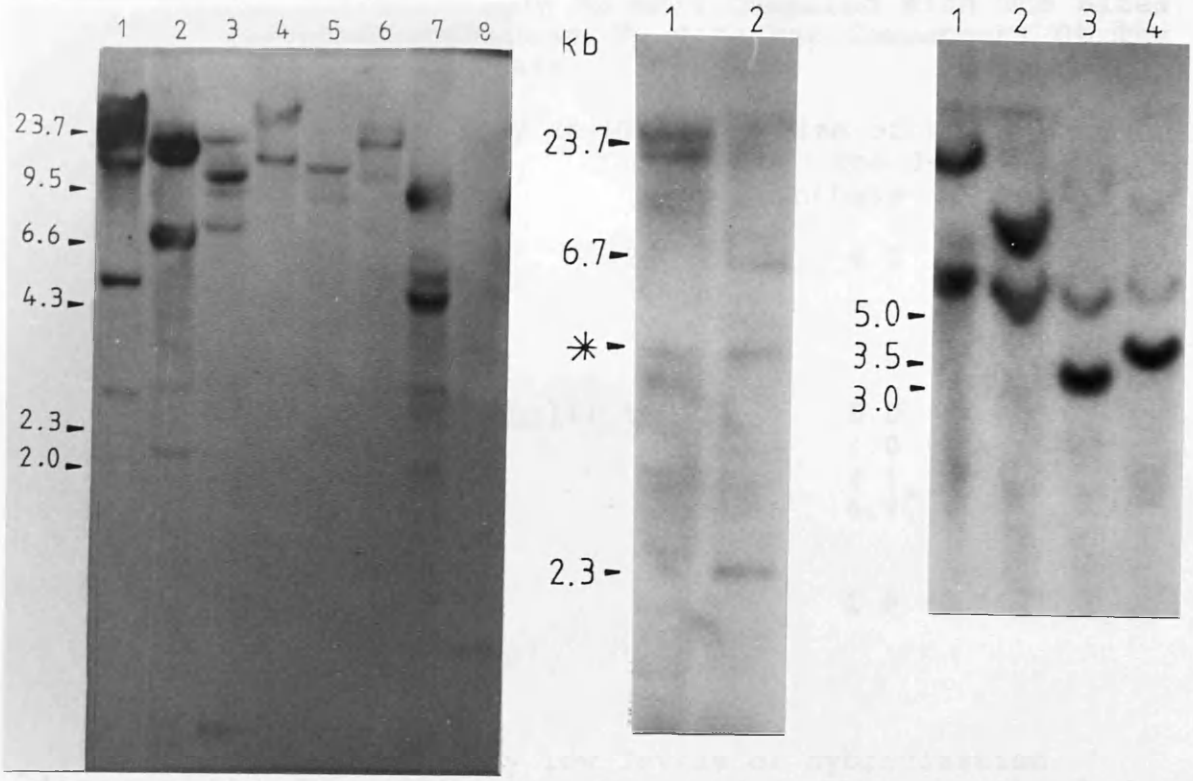


FIGURE 5.13; Cross Hybridization Of Insert DNA From AC36 (b) And MB74 (a And c) With Total DNA Prepared From S. rimosus And Various Streptomyces sp. Respectively

a lane,

1. S. coelicolor total DNA digested with PstI
2. S. coelicolor total DNA digested with BamHI
3. S. cinnamonensis total DNA digested with BamHI
4. S. cinnamonensis total DNA digested with EcoRI

(b1) 5. S. rimosus total DNA digested with BamHI and BglII

(b2) 6. S. rimosus total DNA digested with SstI

7. S. hygroscopicus total DNA digested with BamHI
8. S. hygroscopicus total DNA digested with KpnI

c lane,

1. S. coelicolor total DNA digested with BamHI
2. S. coelicolor total DNA digested with BamHI and XhoI
3. S. coelicolor total DNA digested with BamHI and KpnI
4. S. coelicolor total DNA digested with BamHI and SphI

*Hybridization artefact

The s.a. of the probes were 9.0×10^8dpm ug^{-1} for AC36 (b) and 7.5×10^8dpm ug^{-1} for MB74 (a and c). Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate with 0.5xSSC at 65°C. Washing was performed at the same stringency.

TABLE 5.4; The Sizes Of Restriction Fragments From Four Streptomycetes With Homology To MB74 Compared With The Sizes Of Restriction Fragments Known To Code For Components Of The Endogenous PKS In Each Strain

	homology to MB74 R.F.	size (kb)	size of R.F. encoding the B-ketoacyl- synthase	reductase
S. cinnamonensis*	BamHI	23[W]	4.3	4.3
		11.0		
		8.6[W]		
		6.3[W]		
		0.7[W]		
S. rimosus	BamHI/BglII	12.0	5.0	18.0
		8.2[W]	1.0	
S. coelicolor	BamHI	13.0	2.1	1.1
		6.7	6.7*	
		2.7[W]		
		2.1[W]		
S. hygroscopicus	BamHI	4.9[W]	1.8	6.0
		4.3		
		2.8[W]		
		2.0[W]		

[W]weak homology indicated by low levels of hybridization
*directs the biosynthesis of a polyketide involved in sporulation

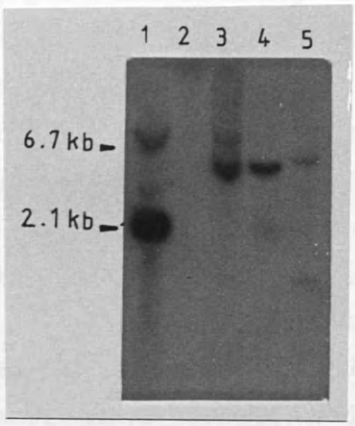


FIGURE 5.14; Hybridization Of actI To Total DNA Prepared From Several Streptomycetes

Lane,
1. S. coelicolor total DNA digested with BamHI
2. S. longisporoflavus total DNA digested with BamHI
3. S. hygroscopicus total DNA digested with BamHI
4. S. rimosus total DNA digested with BamHI and BglII

The s.a. of the probe was 7.5×10^8dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate with 0.2xSSC at 65°C. Washing was performed at the same stringency.

possibility of a restriction site polymorphism in the strain of S. cinnamonensis used. It therefore appears that the 7.0kb BamHI fragment isolated from S. longisporoflavus is more homologous to an 11kb BamHI fragment from S. cinnamonensis than the 4.3kb BamHI fragment used in its isolation. The sequences in MB74 with homology to the 11kb fragment do not necessarily have to overlap with the seqences with homology to the 4.3kb fragment. The observation could therefore be explained by close sequence homology of a gene encoded by MB74 (contiguous with sequences with homology to components of the putative monensin synthase) with a gene encoded on an 11kb BamHI fragment from S. cinnamonensis. The function of the gene in S. cinnamonensis is unknown, however a role in monensin biosynthesis cannot be ruled out. Similarly close linkage of the 11kb and 4.3kb BamHI fragments in the genome of S. cinnamonensis to form a gene cluster for monensin biosynthesis is a possibility which cannot be excluded.

The sequences homologous to MB74 in S. rimosus and S. hygroscopicus DNA could not be localised to the respective 20 or 30kb of known restriction map surrounding the actI/III homologues in these species. However MB74 did hybridize with four fragments in a BamHI digest of total DNA from S. coelicolor of which two were candidates for the biosynthesis of polyketides. The 2.1kb fragment with which MB74 has a low level of homology could not be assumed to be the actI fragment because cross hybridization could not be demonstrated with plasmid borne DNA (see section 5.7). More interestingly the probe DNA hybridized strongly with a BamHI fragment measured to be 6.7kb in length. In Southern analysis actI hybridized to two BamHI fragments from S. coelicolor measuring 2.1kb and 6.7kb in size (Malpartida et al., 1984; Figure 5.14). As described in Chapter 3.6, the 6.7kb BamHI fragment was recently shown to direct the biosynthesis of a pigmented polyketide (WhiE), which was involved in sporulation (see Chapter 3.6 and Figure 1.1). The 6.7kb BamHI fragment had been isolated previously by Horinouchi and Beppu, (1985), and restriction mapped. Further evidence that this was indeed the same fragment was obtained by Southern analysis of DNA from S. coelicolor which had been digested with both BamHI and one of the following three restriction endonucleases, XhoI, PstI or KpnI (Figure 5.13c). In each case MB74 hybridized with a fragment whose length

correlated well with the published restriction map of WhiE.

5.7 CROSS HOMOLOGY BETWEEN AC36, MB74, pIJ2330, pMONI AND pPZ508

The restriction maps of pAC36, pMB74 and pMon were determined (Figure 5.15). The relatedness of sequences involved in the biosynthesis of various polyketides was investigated by Southern analysis of recombinant plasmid DNA (Figure 5.16). All the plasmid constructs involved were based on related cloning vectors derived from colEI (except where probe template could be obtained from subclones in pLorist6). In most of the experiments spurious hybridization of the probe to vector sequences was evident and for reasons described earlier this "homology" could be discounted from interpretation of the results without infringing the validity of hybridization to fragments derived purely from insert DNA. The inter-relationship of actI, monI, AC36, MB74 and otcY was as shown in Figure 5.17. Although the low levels of hybridization observed between some of the constructs elicit equivocal conclusions, the consistent overlap of homology suggested that AC36 and MB74 could be involved in polyketide biosynthesis. In particular the BamHI/KpnI fragment of MB74 hybridized with the PstI/BamHI fragment from monI (and not the sequence with homology to actI - hence the lack of cross homology between actI and MB74). Furthermore MB74 contained other sequences which hybridized with AC36 and otcY.

MB74 therefore has homology with sequences (monI, otcY and WhiE) implacated in the biosynthesis of three different polyketides. This could be construed as evidence (albeit circumstanstial) for the involvement of MB74 in polyketide production. Given the curious stereochemistry of tetronasin, the tetronasin synthase may contain unusual components. For example the weak level of hybridization observed with the aforementioned DNA might be due to a conserved catalytic domain in a tetronasin synthase polypeptide which otherwise has evolved to recognise a polyketide intermediate with the opposite stereochemistry. Cross hybridization to other sequences in the genomic DNA of S. coelicolor, S. rimosus and S. hygroscopicus is more difficult to explain. However, it is possible that some components of the tetronasin synthase are more homologous to the fatty acid synthases of these species or that sequences closely linked to the tetronasin gene

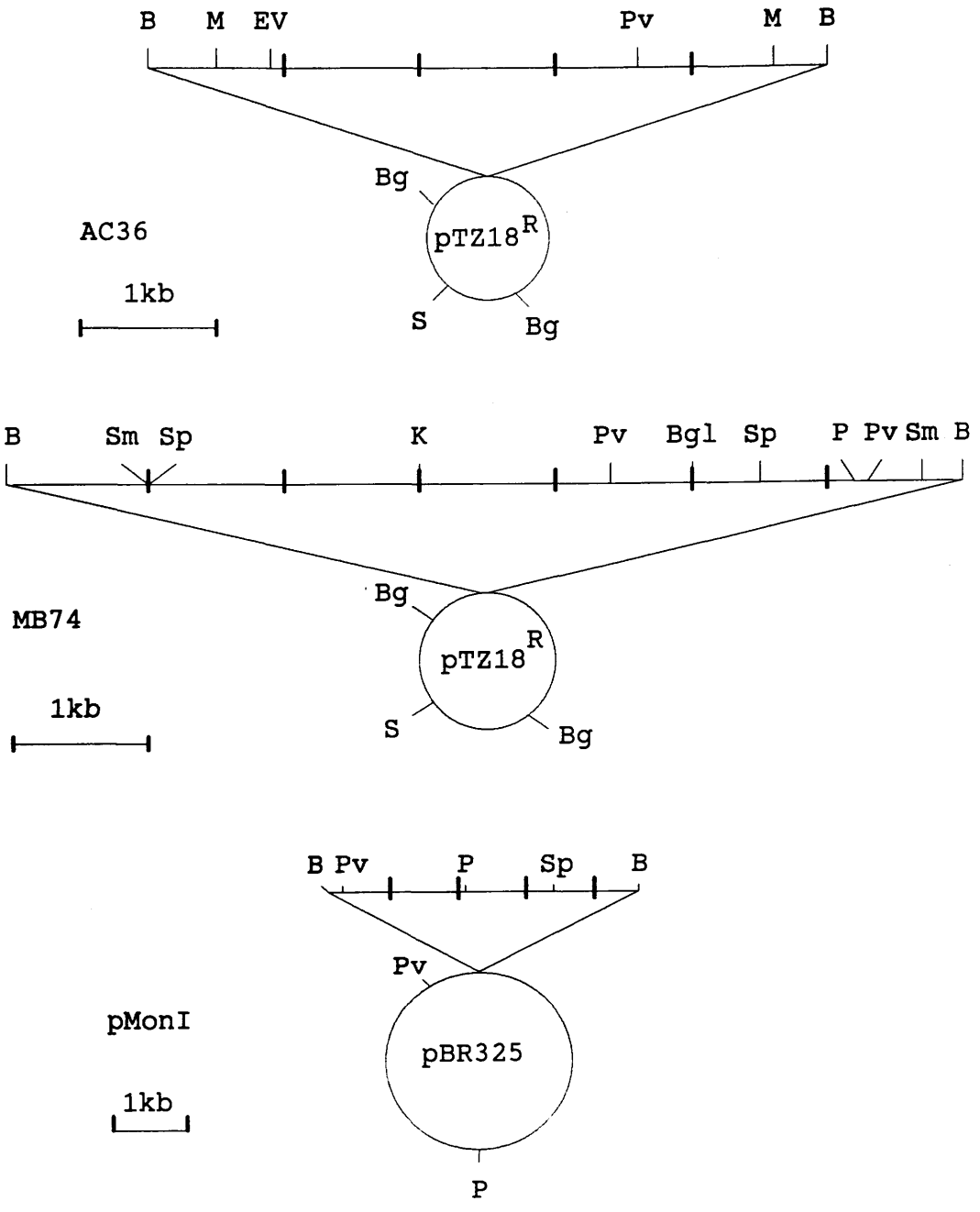


FIGURE 5.15; Restriction Endonuclease Maps Of AC36, MB74 And pMonI

Key,

B	= BamHI	P	= PstI
Bg	= BglII	Pv	= PvuII
Bgl	= BglI	S	= SalI
EV	= EcoRV	Sm	= SmaI
K	= KpnI	Sp	= SphI
M	= MluI	Ss	= SstI

FIGURE 5.16a; Cross Hybridization Of actI Prepared From pLoractI With pMonI And AC36

Lane,
1. pMonI digested with BamHI + PstI + SphI
2. phage lambda DNA digested with HindIII
3. AC36 digested with PvuII + EcoRV

FIGURE 5.16b; Cross Hybridization Of monI Prepared From pLormonI With MB74 And pIJ2330

Lane,
1. phage lambda DNA digested with HindIII + EcoRI
2. MB74 digested with PvuII + KpnI
3. phage lambda DNA digested with HindIII
4. pIJ2330 digested with PstI + BamHI + BglII
5. pIJ2330 digested with PstI + BamHI

FIGURE 5.16c; Cross Hybridization Of monI Prepared From pLormonI With AC36

Lane,
1. AC36 digested with PvuII + EcoRV
2. phage lambda DNA digested with HindIII + EcoRI

FIGURE 5.16d; Cross Hybridization Of AC36 With pIJ2330 And MB74

Lane,
1. pIJ2330 digested with BamHI
2. pIJ2330 digested with BglII
3. AC36 digested with PvuII + EcoRV
4. MB74 digested with PvuII + KpnI

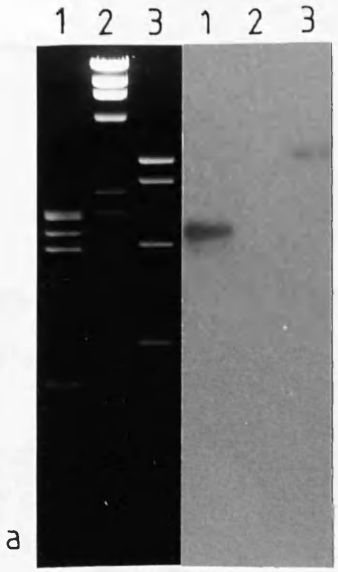
FIGURE 5.16e: Cross Hybridization Of MB74 With pLormonI

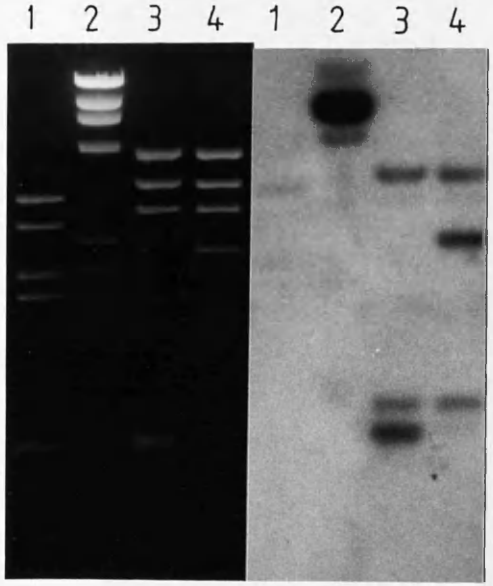
Lane,
1. pLormonI digested with BamHI
2. pLormonI digested with BamHI + PvuII + PstI

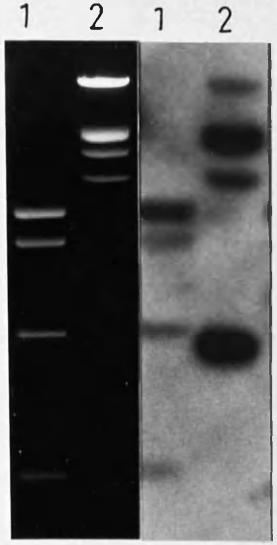
FIGURE 5.16f; Cross hybridization Of otcY With pMonI, pIJ2330, AC36 And MB74

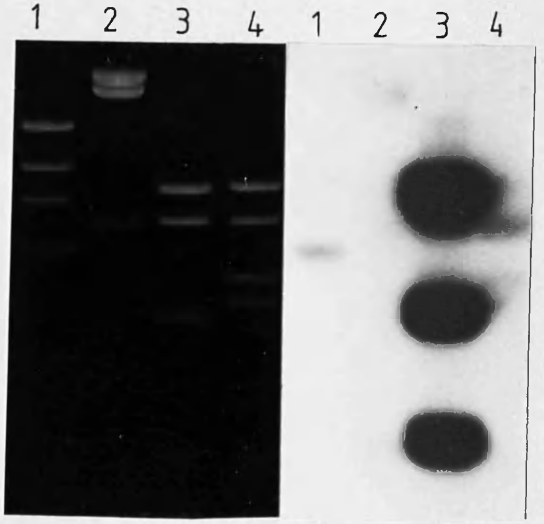
Lane,
1. pPZ508 and pPZ510 linearised
2. pMonI digested with PstI PvuII
3. pIJ2330 digested with BamHI
4. pIJ2330 digested with BglII
5. AC36 digested with PvuII + EcoRV
6. MB74 digested with PvuII + KpnI

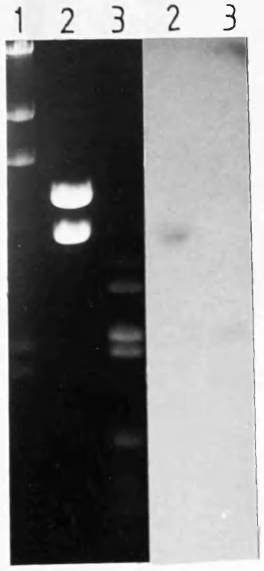
The s.a. of the probes were 7.0×10^8dpm ug^{-1} for actI, 1.2×10^9dpm ug^{-1} for MB74, 2.0×10^9dpm ug^{-1} for monI 7.5×10^8dpm ug^{-1} for AC36 and 1.0×10^9dpm ug^{-1} for otcY. Hybridization was carried out in Denhardts solution 10% (w\v) dextran sulphate with 0.5xSSC at 65°C except for b, 1xSSC at 70°C and c, 2xSSC at 60°C. Washing was performed at the same stringency as hybridization.


1 2 3 1 2 3
a


1 2 3 4 1 2 3 4
b


1 2 1 2
c


1 2 3 4 1 2 3 4
d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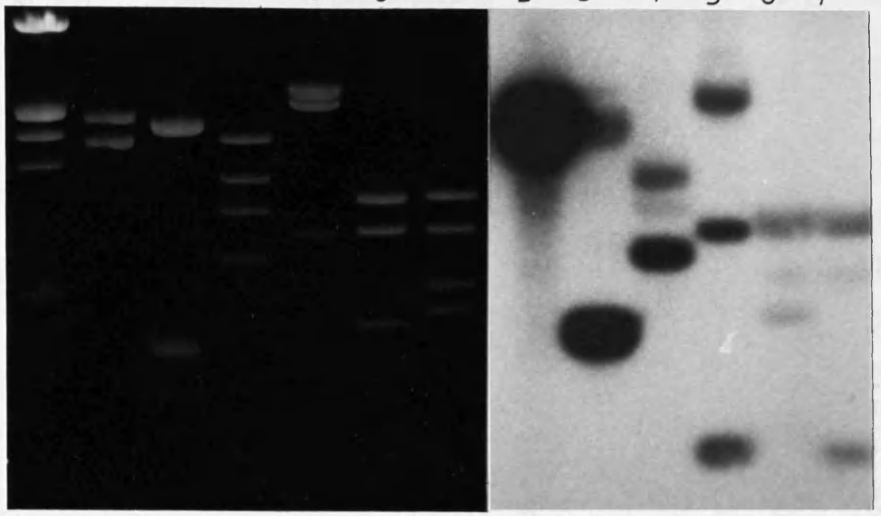
e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f

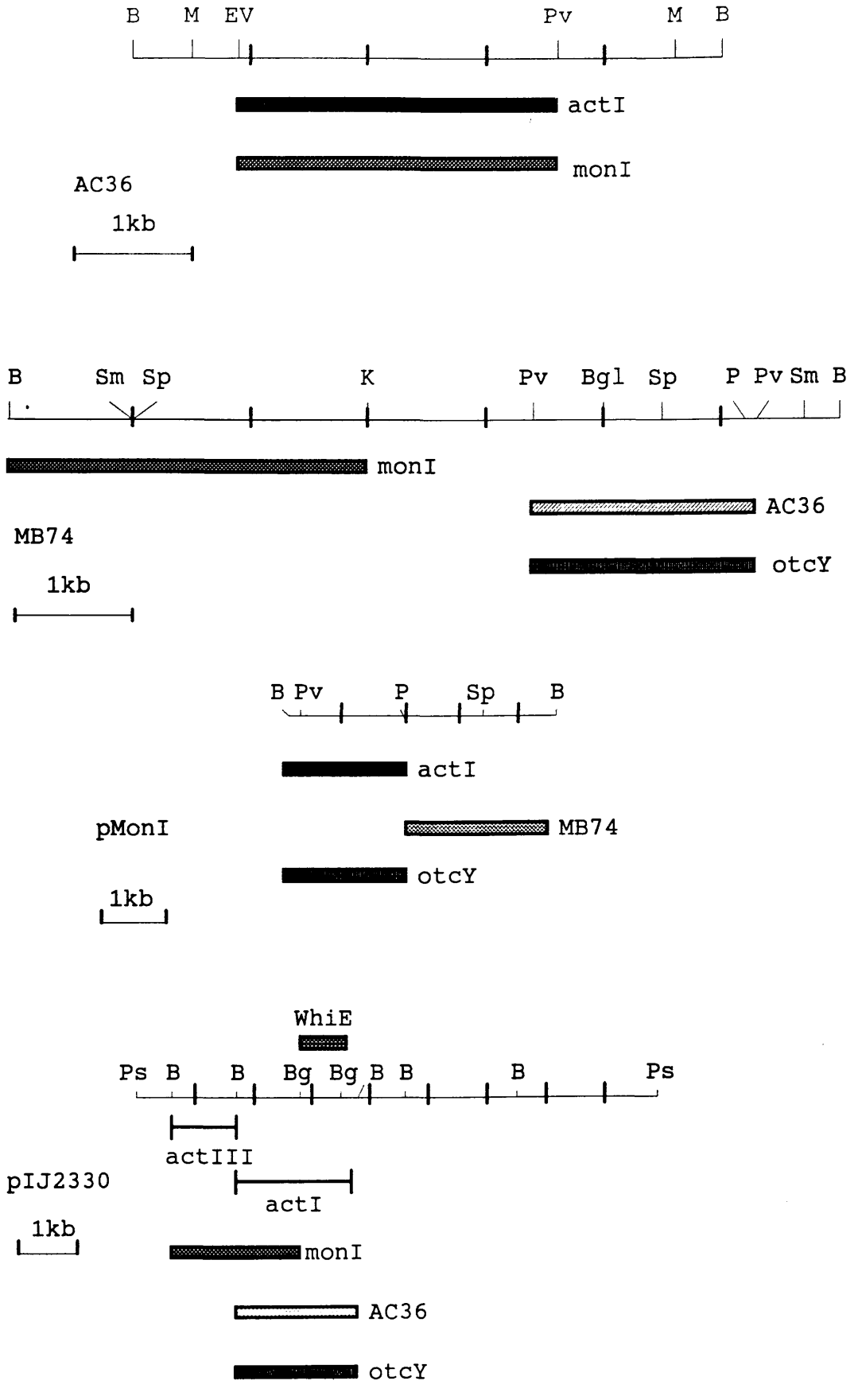


FIGURE 5.17; Inter-relationship Of AC36, MB74, monI, pIJ2330 (act) And otcY As Determined By Cross Hybridization

The positions of actI and III, and the sequences homologous to WhiE on pIJ2330 are indicated.

cluster are responsible.

The low levels of hybridization observed using AC36 as a probe might equally be explained by sequence divergence of polyketide biosynthetic genes.

A similar scenario was reported by (Stutzman-Engwell and Hutchinson, (1989) - see Chapter 1.6.6 - for five DNA fragments isolated from S. peucetius. All have homology to known PKS genes and can apparently influence polyketide biosynthesis, but they exhibit little homology to each other.

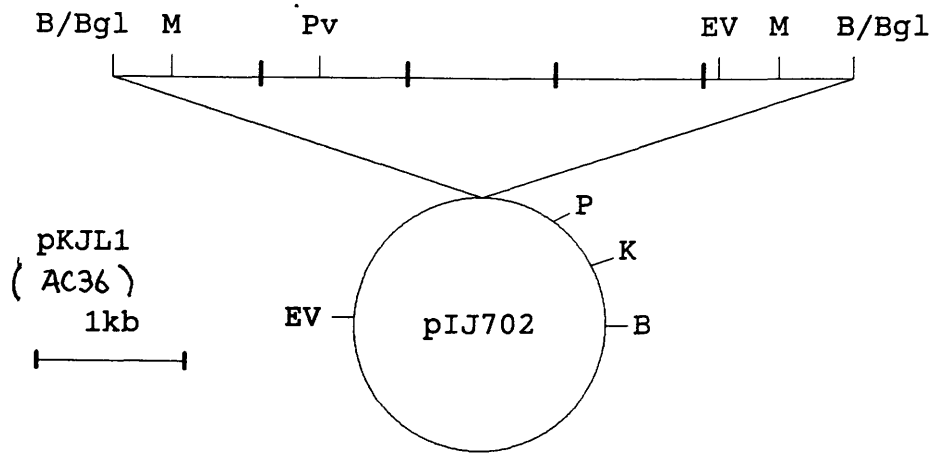
5.8 SUBCLONING OF AC36 AND MB74 INTO pIJ702

To facillitate complementation studies in mutants of S. longisporoflavus deficient in tetronasin biosynthesis and also to test for any resistance functions carried on AC36 or MB74 the insert DNA was subcloned from pTZ18^{R} into pIJ702. The respective BamHI insert fragments from AC36 and MB74, and pIJ702 digested with BglII and treated with CIAP were purified from an agarose gel using Gene Clean. Ligations were performed in 1xBRL ligation buffer with a total DNA concentration of 10ng ul^{-1} and an insert to vector molar ratio of 2:1. 1U of T4 DNA ligase was added to the 20ul reaction volume and ligation was allowed to continue for 2hrs at 22^{o}C. S. lividans TK64 protoplasts were transformed with 5ul aliquots of the DNA. Plasmid DNA was prepared and analysed from five mel^{-} transformants selected at random for each ligation experiment. Four recombinants containing MB74 (two each of either orientation) and three recombinants containing AC36 (in one orientation only) were recovered giving plasmids pKJL1, 2 and 3 (pKJL1 and 2 are shown in Figure 5.18. Neither pKJL1 nor pKJL2 were able to confer tetronasin resistance upon S. lividans or S. albus (the performance of these recombinant strains in plate assay for tetronasin resistance can be seen in Figure 4.12, p.158). Preliminary experiments to test pKJL1 and 2 in complementation studies are described in Chapter 7.

5.9 TRANSCRIPTIONAL ANALYSIS OF AC36 AND MB74

The transcriptional patterns of the two clones were investigated by S1 nuclease mapping to identify restriction fragments which were completely internal to transcription units for use in gene disruption.

The technique (performed essentially as described in



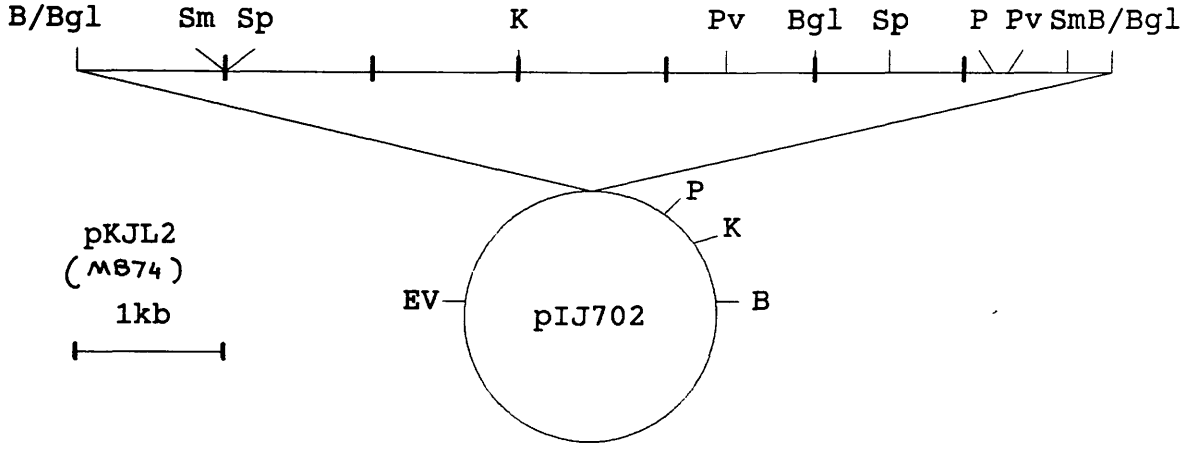


FIGURE 5.18; Restriction Maps Of pKJL1 And pKJL2

Key, as for Figure 5.16

Hopwood et al., 1985), involved hybridization of DNA restriction fragments with RNA prepared from S. longisporoflavus grown under conditions which gave tetronasin production. In the presence of 80% (v/v) formamide the stability of RNA/DNA hybrids is greater than the corresponding DNA duplex (Casey and Davidson, 1977). It was therefore possible to define empiric conditions in which RNA/DNA hybrids were formed preferentially, with unbound DNA remaining in ss form. S1 nuclease (Sigma type III) can then be used to degrade the ss molecules, leaving intact only regions of the stable duplex. The expediency of this technique allows the researcher to perform all the previous manipulations with unlabelled nucleic acid, and only now were the protected hybrids fractionated by alkaline agarose gel electrophoresis (thus also degrading the RNA component), and the transciptionally active sequences detected by Southern analysis.

The Tm of the duplex in hybridization buffer was determined by melting the double helix at 85^{o}C for 10 minutes, then allowing individual aliquots to equilibrate to one of a range of temperatures between 55^{o}C and 70^{o}C, before treatment with S1 nuclease. Using 50ng of DNA per reaction, and eliminating heterologous DNA from the digestion buffer, protected DNA (i.e. DNA in a ds form) can be visualised after gel electrophoresis (Figure 5.19). This result indicated that the restriction fragments, under the conditions used had a Tm between 60 and 65^{o}C. Restriction fragments from MB74 behaved similarly, so hybridization at 65^{o}C was used to select for the formation of heterodimers.

Total RNA was prepared from S. longisporoflavus 4584 in a time course experiment from 4, 5, 6 and on separate occasions from 7 day old cultures. The culture was grown from a 100ul dense spore inoculum in YEME which allows dispersed growth and tetronasin production. For the first 6 days of growth tetronasin was undetectable by bioassay. The antibiotic only appeared in the culture broth on day 7. The earlier time points were included to allow detection of pre-production phase, possibly transient, transcripts of the tetronasin biosynthetic genes. The potential importance of this was highlighted by Hershberger et al., (1988), in S. fradiae where mRNA from tylosin biosynthetic genes was detected only at the onset of production, being

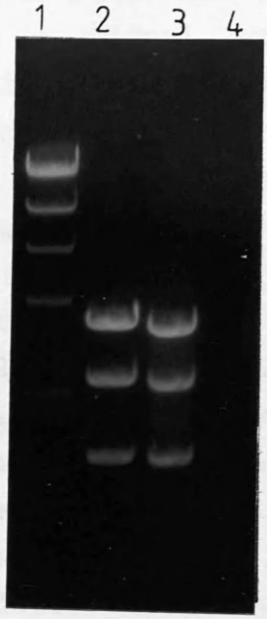


FIGURE 5.19; Determination Of The Duplex Melting Temperature For Restriction Fragments Of AC36

Lane,
1. phage lambda DNA digested with HindIII
2. AC36 digested with PvuII and EcoRV, renatured at 55°C
3. AC36 digested with PvuII and EcoRV, renatured at 60°C
4. AC36 digested with PvuII and EcoRV, renatured at 65°C

After renaturation the samples were treated with 100 units of S1 nuclease.

outlasted by the translation products. Figure 5.20a illustrates the recovery of fairly pure RNA from a 7 day old culture, by pelleting the RNA through a dense CsCl cushion. The RNA contained five prominent bands, representing the 23S, 16S and 5S rRNA's, and either their degradation intermediates, or unprocessed precursors. Baylis and Bibb, (1988), have established, at least for S. coelicolor, that the 23S, 16S and 5S rRNA genes are very closely linked in six gene sets, so it is a formal possibility that the rRNA species are co-ordinately transcribed. The pattern commonly found is shown in Figure 5.20b, where the two prominent bands are assumed to be the 23S and 16S rRNA subunits.

Restriction fragments of DNA from AC36 and MB74 were purified from agarose gels using a "Gene Clean" kit. This was an important preparatory step, because of the presence of linear molecules in the plasmid DNA - a problem encountered previously, (see section 5.5). As explained before, digestion of plasmid DNA containing both linear and ccc molecules, with restriction endonucleases will yield the expected fragments and also a continuous range of molecules smaller than the largest digestion product, which because of their indiscreet size may not be observed by ethidium bromide staining of an agarose gel. If the total DNA products were used in an S1 mapping experiment, the homologous RNA would hybridize, with equal probability, to any of the DNA fragments. This would result in a continuous range of protected fragments, determined in size, by the length of the sheared fragment, and therefore a smear of radioactivity upon Southern analysis would be observed. Obviously the proportion of linear molecules in any sample of plasmid DNA, will determine the intensity of the smear and the degree of the problem.

Figure 5.21 is the culmination of three experiments. In the first (Figure 5.21a), 50ng of PvuII/EcoRV digestion products (2.7kb, 1.4kb and 0.9kb) from the gel purified BamHI insert of AC36, were hybridized with 40ug total RNA (lane 2) prepared from mycelium cultured for 7 days in YEME (the RNA contained five presumptive rRNA fragments). An identical DNA sample was hybridized with 40ug of tRNA to provide a negative control (lane 3). The nucleic acid was treated with 100U of S1 nuclease. Comparison of lanes 2 and 3 indicated the two larger fragments to have been protected specifically by the total RNA. No conclusion could be drawn from protection of

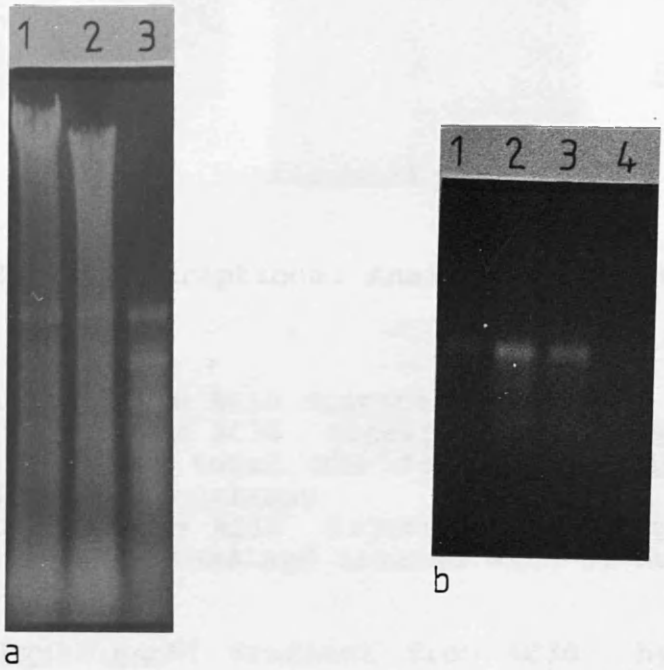


FIGURE 5.20; Total RNA Prepared From S. longisporoflavus 4584 Showing: a, Stages In Preparation And The Presence Of Multiple Species Of rRNA Species And b, The Yields Obtained From Cultures Of Different Ages

a; lane,
1. nucleic acid prepared from S. longisporoflavus
2. nucleic acid prepared from S. longisporoflavus treated with DNaseI
3. nucleic acid prepared from S. longisporoflavus and pelleted through a dense CsCl gradient

b; lane,
1. total RNA prepared from a 4 day culture
2. total RNA prepared from a 5 day culture
3. total RNA prepared from a 6 day culture
4. total RNA prepared from a 7 day culture

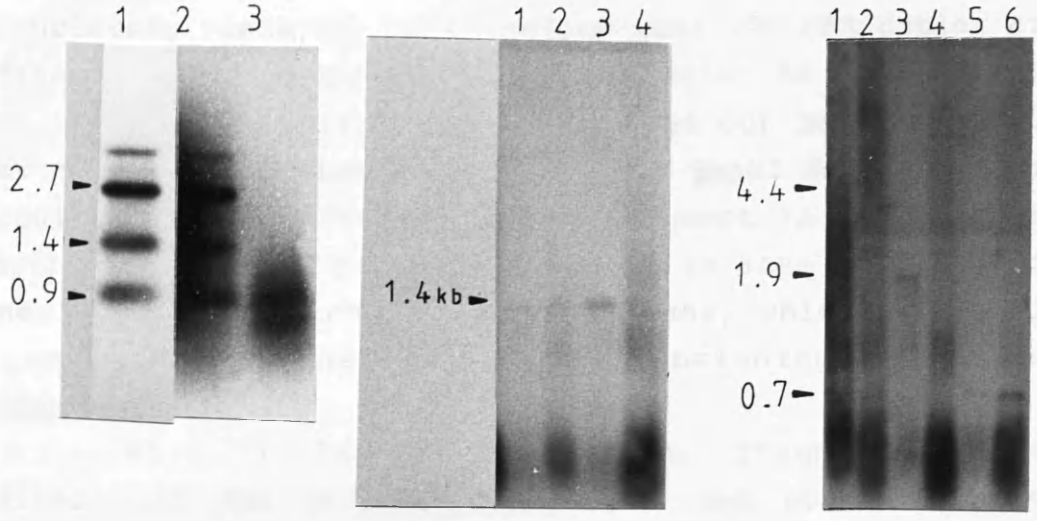


FIGURE 5.21; Transcriptional Analysis Of AC36 (a And b) And MB74 (c)

a: Lane,
1. BamHI insert from AC36 digested with PvuII and EcoRV
2. BamHI insert from AC36 digested with PvuII and EcoRV hybridized with total RNA from S. longisporoflavus and treated with S1 nuclease
3. BamHI insert from AC36 digested with PvuII and EcoRV hybridized with tRNA and treated with S1 nuclease

b: Lane,
1. 1.4kb PvuII\BamHI fragment from AC36 hybridized with total RNA from a 4 day culture of S. longisporoflavus and treated with S1 nuclease
2. 1.4kb PvuII\BamHI fragment from AC36 hybridized with total RNA from a 5 day culture of S. longisporoflavus and treated with S1 nuclease
3. 1.4kb PvuII\BamHI fragment from AC36 hybridized with total RNA from a 7 day culture of S. longisporoflavus and treated with S1 nuclease
4. 1.4kb PvuII\BamHI fragment from AC36 hybridized with tRNA and treated with S1 nuclease

c: Lane,
1. 4.4kb BamHI\PvuII fragment from MB74 hybridized with total RNA from S. longisporoflavus and treated with S1 nuclease
2. 4.4kb BamHI\PvuII fragment from MB74 hybridized with tRNA and treated with S1 nuclease
3. 1.9kb BamHI\PvuII fragment from MB74 hybridized with total RNA from S. longisporoflavus and treated with S1 nuclease
4. 1.9kb BamHI\PvuII fragment from MB74 hybridized with tRNA and treated with S1 nuclease
5. 0.7kb PvuII fragment from MB74 hybridized with total RNA from S. longisporoflavus and treated with S1 nuclease
6. 0.7kb PvuII fragment from MB74 hybridized with tRNA and treated with S1 nuclease

the 0.9kb EcoRV/BamHI fragment in lane 2, because of a band of corresponding size in the control experiment, undigested by S1 nuclease, (lane 3). This implied that the DNA duplex of this fragment was not denatured by incubation at 85°C for 10 minutes, or the hybridization was carried out below its Tm. However partial digestion by EcoRV of the BamHI fragment from AC36 could yield an additional 3.6kb fragment. A fragment of corresponding size was present, not only in lane 1, but also in lane 2 after treatment with S1 nuclease, which suggested full protection of the whole insert including the 0.9kb EcoRV\BamHI fragment.

When isolated restriction fragments were hybridized with RNA prepared from different points of the growth phase, (RNA isolated from 5 day old mycelia became degraded in storage and had to be eliminated from the study) no protection the 3.6kb PvuII/BamHI fragment from AC36 could be detected. However the 1.4kb PvuII/BamHI fragment was specifically protected by total RNA from S. longisporoflavus 4584 (Figure 5.21b). Furthermore, with equimolar amounts of RNA per experiment and uniform maintenance of hybridization parameters for the individual samples, it could be concluded tentatively that the transcript from this fragment accumulated late in the growth phase - a result consistent with the profile of a gene involved in antibiotic production.

Transciptional analysis of fragments from MB74 (Figure 5.21c) identified the 1.9kb PvuII fragment to be completely internal to a transcription unit. No protection of the 4.4kb BamHI/PvuII fragment by RNA from a six day old culture could be discerned, and the absence of degradation of the 0.7kb BamHI/PvuII fragment with the tRNA control, rendered that experiment inconclusive.

The findings of this analysis were summarised in five main points;

1. The whole 5kb insert of AC36 was found to be fully protected from S1 nuclease by hybridization to total RNA from S. longisporoflavus and may be completely internal to a single transcription unit.

2. The 1.4kb BamHI/PvuII fragment from AC36 was found to be completely internal to a transcription unit, and its relative abundance appeared to increase during the growth of the

culture.

3. The 1.9kb PvuII fragment of MB74 was found to be completely internal to a transcription unit.

4. Protection of DNA fragments greater than 2.8kb in length was observed in only one experiment (Figure 5.21a) and was not repeatable with different RNA preparations. The inability to protect large DNA fragments was also a feature S1 mapping analysis of the resistance determinants (data not shown, see Chapter 4.6). The level of fragment protection was not high, only a small fraction of the DNA used hybridized with its complementary message. If multiple transcriptional start or termination sites are located within the DNA fragment, or if RNA degradation is prevalent, then RNA:DNA duplexes of various sizes will be formed. This may be more pronounced for larger DNA fragments which are more likely to contain RNA processing sites or transcriptional start/termination sites. In such cases, the protected fragments are less likely to be detected because the number of surviving molecules of any one discreet size would be reduced and may fall outwith the sensitivity of this experiment. This hypothesis could explain the observed protection of the 2.7kb PvuII/EcoRV fragment of AC36 in Figure 5.21a, but absence of any discreet band when the overlapping 3.6kb PvuII/BamHI fragment was used.

5. Conditions were not optimal for S1 mapping DNA fragments less than 1kb in length. On both occasions when small DNA fragments were used (0.9kb EcoRV/BamHI fragment from AC36 and 0.7kb BamHI/PvuII fragment from MB74), protection by mRNA could not be confirmed due to non-specific protection by tRNA or failure to denature or prevent reassociation of, the complementary DNA strands.

5.8 SUMMARY

Conditions were defined for mixed phase hybridization, which allowed annealing of both actI (containing genes for early steps in actinorhodin biosynthesis), and monI (containing putative genes for the eqivalent steps in monensin biosynthesis) specifically to related sequences from S. longisporoflavus 83E6. Curiously, Southern analysis revealed that the actI and monI homologues (respectively contained on 5.0 and 7.5kb BamHI fragments), were not closely linked on the S. longisporoflavus genome.

Two gene libraries of S. longisporoflavus DNA, enriched for DNA fragments of the appropriate size, were constructed in the plasmid cloning vector pTZ18^{R} and contained in E. coli JM109. Colony hybridization identified seventeen recombinants with homology to actI and eleven with homology to monI. From several found to contain identical inserts of the correct size two, AC36 (actI homologue) and MB74 (monI homologue), were chosen to be characterised further.

Cross hybridization of MB74 to the DNA of four other streptomycetes, namely, S. coelicolor, S. rimosus, S. cinnamonensis and S. hygroscopicus, indicated the presence of closely related sequences in each case. Generally the sizes of the homologous fragments did not correspond to fragments known to be involved in polyketide biosynthesis, however evidence was obtained which suggested that MB74 contained sequence homology with WhiE. Curiously MB74 was more closely related to an 11kb BamHI fragment from S. cinnamonensis, than to the 4.3kb BamHI, monI fragment. Similarly, sequences that were related to AC36 were identified in S. rimosus. Cross hybridization of plasmid borne sequences indicated that MB74 (which hybridized to different sequences on monI than those with homology to actI) comprised different fragments which shared homology with monI or otcY (and AC36). AC36 shared homology with both actI and monI.

Using RNA prepared from S. longisporoflavus, S1 mapping experiments identified restriction fragments from both clones which were completely internal to transcription units. These were considered suitable for use in gene disruption. Furthermore, a time course experiment suggested that a transcript from AC36 accumulated late in the growth phase.

CHAPTER 6

CONSTRUCTION OF A DNA LIBRARY REPRESENTATIVE OF THE *S. longisporoflavus* 83E6 GENOME IN THE PHAGE lambda REPLACEMENT VECTOR EMBL3: ISOLATION OF RECOMBINANT PHAGES CONTAINING DNA CONTIGUOUS WITH EACH OF THE FOUR CLONES TETR1, TETR5, AC36 AND MB74

6.1 INTRODUCTION

A library of DNA representative of the S. longisporoflavus 83E6 genome was constructed in a bacteriophage lambda cloning vector in order to isolate DNA fragments contiguous with each of the four clones (AC36, MB74, tetR1 and tetR5) for two reasons;

1. If recombinants were obtained which contained sequences from two or more of the DNA fragments isolated previously, then close linkage of the fragments might be established. It would then not be unreasonable to suspect that a gene cluster involved in the biosynthesis of tetronasin had been isolated.
2. If it could be established that any one of the recombinants isolated previously encodes an enzyme of the tetronasin biosynthetic pathway, then contiguous DNA from the S. longisporoflavus genome might also be expected to contain genes for tetronasin biosynthesis.

6.2 CONSTRUCTION OF THE LIBRARY

6.2.1 CHOICE OF CLONING VECTOR

An in depth understanding of the biology of phage lambda, has resulted in the derivation of a large variety of cloning vectors. Phage lambda EMBL3 (Frischhauf et al., 1983), was chosen for the construction of a representative genomic library of S. longisporoflavus DNA for several reasons;

a, Facile identification of the desired clones by plaque hybridization.

b, The vector accepts DNA of a manageable size range (9 - 23kb).

c, Selection for recombinant phage is very strong.

The basis for the selective propagation of recombinant molecules relies on the DNA packaging constraint enforced by the inflexibility of the head structure of the virus. Only genomes of around 40kb to 52kb in length are packaged with high efficiency. The EMBL3 non-recombinant genome is 42.4kb long. It is flanked by cohesive ends (cos), required for circularisation, and comprises a left arm (cos to polylinker), of 19.4kb, and a right arm (polylinker to cos), of 9.3kb, separated by a 13.7kb stuffer fragment (Figure 6.1). EMBL3 cut with BamHI can generate concatomers of a 28.7kb molecule with complementary cos ends, after

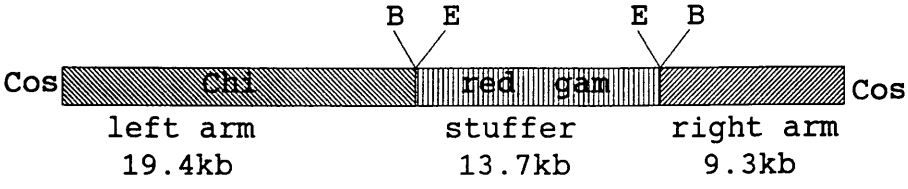


FIGURE 6.1; Functional Map Of Bacteriophage Lambda Replacement Vector EMBL3

ligation of the left and right arms of EMBL3. The 28.7kb molecule carries all the necessary information for completion of the phage life cycle. However the proximity of the cos ends renders packaging - both in vivo & in vitro - inefficient. Reconstitution of the original EMBL3 molecule can be prevented by phosphatasing the cut vector DNA, and double digestion of the vector with BamHI (generating arms with GATC overhangs, suitable for accepting donor DNA cut with Sau3A), and EcoRI (generating TTAA overhangs on the stuffer fragment, which are non-complementary to the arms). The 9bp BamHI/EcoRI fragments can be removed by selective precipitation of the larger DNA fragments with isopropanol.

Any parental EMBL3 genomes surviving this treatment were discriminated against biologically by constructing the library in an E. coli host lysogenic for phage P2. This selection is accomplished by virtue of a functional gam gene of phage lambda contained in the stuffer fragment of EMBL3. The gam gene product inactivates an endogenous E. coli protein, exonuclease V (ExoV), encoded by the recBC genes. ExoV prevents rolling-circle replication of the phage lambda genome. Although lambda can replicate via a bidirectional mechanism, the concatenated DNA generated by rolling-circle replication is required for efficient packaging of the genome into the icosahedral phage head. Phage P2 biology is also regulated by the recBC status of the host, such that lysogeny of a recB,C host is not possible. In a P2 lysogen, gam$^+$ phage cannot replicate. Although not fully understood, but presumably connected to the interaction with exonuclease V, the gam gene product in a P2 lysogen effects a discontinuation of protein and DNA synthesis. Constructing the library in a P2 lysogen thus provided strong selection against the survival of non-recombinant EMBL3. However, for efficient packaging a multimeric genome is required. This cannot be achieved by the recombination genes (red) of phage lambda, which are contiguous with gam in the genome and are also lost with the stuffer fragment. To circumvent the absence of gam & red in the recombinant phage, the EMBL3 arms are endowed with an octanucleotide Chi site. Intermolecular recombination via the host recABC pathway using the Chi site as a substrate, multimerises the recombinant phage genome, providing a suitable substrate for packaging.

EMBL3 can therefore be described as phenotypically Spi$^+$

(susceptible to P2 interference) by virtue of gam, whilst recombinant genomes, red$^-$, gam$^-$ and Chi$^+$ are Spi$^-$.

6.2.2 PREPARATION OF EMBL3 VECTOR DNA

EMBL3 DNA cut with BamHI and EcoRI, with the 9bp fragments removed, and treated with alkaline phosphatase was purchased from Promega Biotec (Cat. No. B1701).

6.2.3 PREPARATION OF DONOR DNA

In an experiment analogous to that performed in Chapter 4.2.3, except for an increase in the required DNA length (15 - 23kb), high molecular weight genomic DNA from S. longisporoflavus 83E6 was digested partially with restriction endonuclease Sau3A (0.1U ug^{-1}), for varying time periods (0 - 32 minutes). Having fractionated the digestion products by gel electrophoresis (0.3% (w/v) agarose), using phage lambda both uncut and digested with HindIII as size markers, a 2 minute exposure to Sau3A was found to generate most restriction fragments of length 15 - 23kb. The digestion parameters were exactly scaled up to accommodate 1mg of genomic DNA. The DNA was digested and fragments 15 - 23kb in length were purified by electroelution from a TAE agarose gel. Despite the packaging constraints imposed for viability of recombinants, isolation of DNA fragments of the desired length was necessary to avoid fulfilment of the genome size requirement by in vitro ligation of smaller molecules. Analysis of recombinants containing fragments which are not juxtaposed in the parental genome is complicated and can lead to misinterpretation of results.

6.2.4 LIGATION OF VECTOR ARMS WITH SIZE SELECTED DONOR DNA

Ligation was carried out following the recommendations of Promega Biotec. High concentrations of DNA were used (200ng ul^{-1}, 3:1 molar ratio of vector arms to insert) to promote the formation of large concatenates. PEG, irrespective of its enhancement of the formation of concatenates, was omitted from the BRL ligation buffer, because it was reported to adversely affect in vitro packaging of DNA and subsequent infection of the host strain.

6.2.5 IN VITRO PACKAGING OF THE LIGATED DNA

The packaging protocol recommended for

Stratagene Gigapack Gold (Cat. No. 200216), was followed to package 200ng of the ligated DNA. The method preferentially selects large genomes for packaging (Maniatis et al, 1982). Therefore recombinant phage with inserts around 20kb in length were expected to be recovered.

6.2.6 INFECTION OF *E. coli* WITH THE PACKAGED DNA: RECOVERY OF A GENE BANK OF *S. longisporoflavus* 83E6 DNA CONTAINED IN PHAGE lambda EMBL3

Plating bacteria, competent for efficient infection by phage lambda were prepared as described in Chapter 2.9.2. The infectivities of three strains (LE392, P2392 and NM621) were compared to determine the numbers of recombinants recovered. LE392 is a permissive strain for EMBL3. P2392 is a P2 lysogen of the former, and selects for the Spi^- phenotype of recombinant phage. NM621 was also tried as a cloning recipient as it lacks restriction enzymes for both methylated and unmethylated DNA, and has been reported to allow stable maintenance of palindromic DNA (Whittaker, et al., 1988).

The packaged ligation mix was infected into the three strains. The infected cells were grown in a 0.7% (w/v) agarose top layer, on an L-agar base. The rich media keeps the plaques small by ensuring fast growth of the host strain. Agarose is more cohesive than agar, and use of the former in the top layer eases the preparation of filter replicates and is reported to reduce non-specific binding of the radioactive probe. The efficiency of infection of each strain (Table 6.1) indicated that selection for recombinant phage, through extensive preparation of the vector DNA, was so strong that biological selection of the Spi^- phenotype was unnecessary.

Assuming the insert size to average 20kb, the size of library required to give a high probability (99%) of complete representation of the *S. longisporoflavus* genome can be calculated by the binomial theorem.

$$N = \frac{\ln(1-p)}{\ln(1-x/y)}$$

where, N = number of recombinants
p = probability
x = insert size (kb)
y = genome size (5848kb for *S. coelicolor*)

TABLE 6.1; Efficiency Of Infection Of *E. coli* Strains LE392 (permissive), P2392 (restrictive), And NM621 With *in vitro* Packaged, Control DNA And EMBL3 Arms Ligated To DNA From *S. longisporoflavus*

HOST	Spi^- SELECTION	DNA PACKAGED	pfu ug^{-1} DNA (x 10^5)
LE392	no	ligated	5.1
NM621	no	ligated	5.0
P2392	yes	ligated*	5.4
LE392	no	control*	55
NM621	no	control	53
P2392	yes	control	65

*control DNA (Spi^- Chi^+ concatenated phage lambda) was supplied with the Stratagene packaging kit.

therefore,

$$N = \frac{\ln(1-0.99)}{\ln(1-20/5848)} = 1344 \text{ clones}$$

The library comprised some 35 000 pfu, and assuming all were recombinant and contained a 20kb insert, this constituted 120 genome equivalents - well in excess of 99% probability of full representation.

6.3 HYBRIDIZATION OF RADIOLABELLED TETR1, TETR5, AC36 AND MB74 WITH THE DNA LIBRARY CONTAINED IN PHAGE lambda EMBL3

To test for representation of the S. longisporoflavus genome in the library, replicate impressions of the original undiluted (the plaque density was calculated as 1 pfu mm^{-2}) and ten fold dilution plates were taken. Free phage particles and any unpackaged phage DNA were adsorbed to Amersham Hybond-N. Both impressions were required to allow hybridization of the probe to duplicate filters for specific hybridization to be confirmed. Prehybridization of the 12 filters (duplicate filters from two plates of each of the three host strains, infected with undiluted packaged phage - 5000 plaques per plate - and similarly for the ten-fold dilution of packaged phage) was carried out in a large volume (100ml) for 12hrs. Radioactive probes were prepared independently by random primed DNA synthesis from the insert DNA of tetR1, tetR5, AC36 and MB74. The individual probes were mixed and hybridized to the 12 filter discs.

With 20kb inserts, the equivalent of one streptomycete genome should be contained in about 290 plaques. Assuming the four clones to be unlinked, around 70 clones in every 5000 plaques were expected to hybridize with the probe DNA. No allowance was included in the calculation for recombinants which contained only part of a DNA sequence isolated previously in a recombinant. Sau3A cleavage of the genomic DNA within the previously cloned DNA could yield two constructs, from a single genome, with homology to one of the probes. The insert size of tetR1, tetR5, AC36 and MB74 averages about 6kb. With inserts in EMBL3 expected to be around 20kb, around one third of the recombinants might be expected to begin or end within the previously isolated sequences. Therefore 90 clones per 5000 plaques were expected to hybridize with the probe DNA. Figure 6.2 shows the result of hybridization of all four radiolabelled probes to a

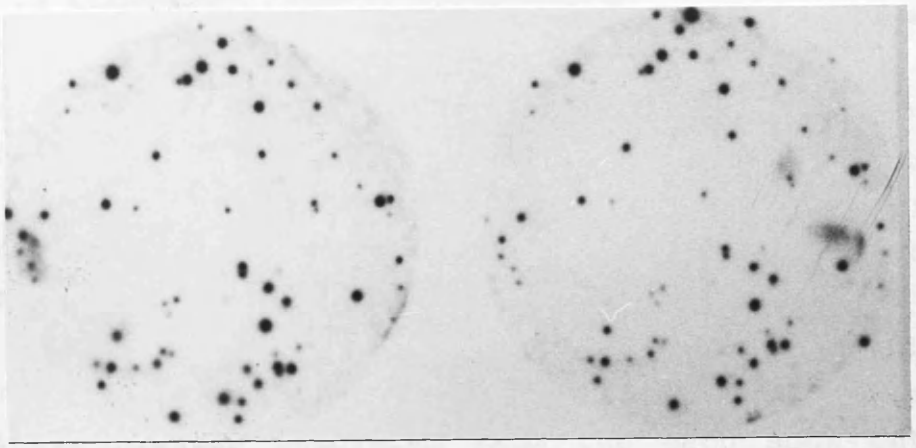


FIGURE 6.2; Hybridization Of Radiolabelled AC36, MB74, tetR1 And tetR5 With Duplicate Plaque Lifts From A lawn Of *E. coli* P2392 Infected With 5000pfu Of A Representative *S. longisporoflavus* 83E6 Gene Library Contained In Phage Lambda EMBL3

library containing 5000 plaques. 68 phage hybridized reproducibly on replicate filters, which was not inconsistent with earlier assumptions, i.e. virtually all plaques represented recombinant phage with around 20kb inserts. However the census of positive clones did not rule out the possibility of two of the probes being closely-linked on the S. longisporoflavus genome. Equal numbers of positive clones were identified irrespective of which E. coli strain was used as a host for the library.

Due to the high plaque density on the plates it was difficult to assign unequivocally a signal to an individual plaque. Plugs of agar, 4mm in diameter (determined by the thick end of a Pasteur pipette), centred around the point of hybridization were isolated for each positive signal on the P2392 host. Free phage particles were "soaked out" of the agar by incubation at room temperature in 2ml of phage buffer for two hours. Individual plaques were isolated from these preparations, using a sterile tooth-pick to streak a drop of the high titre phage suspensions on top of standard double layer phage plate seeded with E. coli P2392.

The area sampled by the plug was roughly $12mm^2$ and should represent on average, phage from 12 plaques. Ten individual plaques recovered from the each soak out, were picked and spotted onto a double layer phage plate, again seeded with strain P2392. To quickly generate an aesthetic array of plaques and confidently avoid the confusion resulting from double infection of the same spot, a plaque applicator was used. The plaque applicator - an array of holes drilled through a solid plastic block - was designed by Chris Mackenzie. When placed above an agar plate, phage carried on the tip of a thin glass rod, can be applied to the surface of the plate through the holes. By leaving the rods in the holes until completion of the experiment, the researcher can avoid cross contamination of the phage and ensure an even distribution of plaques on the plate - a more extensive description of the technique can be found in Mackenzie et al., (1989).

Using Amersham Hybond-N, eight plaque lifts were taken from each plate. This allowed independent probing with each of the four clones in duplicate. Figure 6.3 shows the pattern produced when radiolabelled insert DNA from MB74 was hybridized to 225 plaques, at $65^{o}C$, 0.5xSSC. The other three

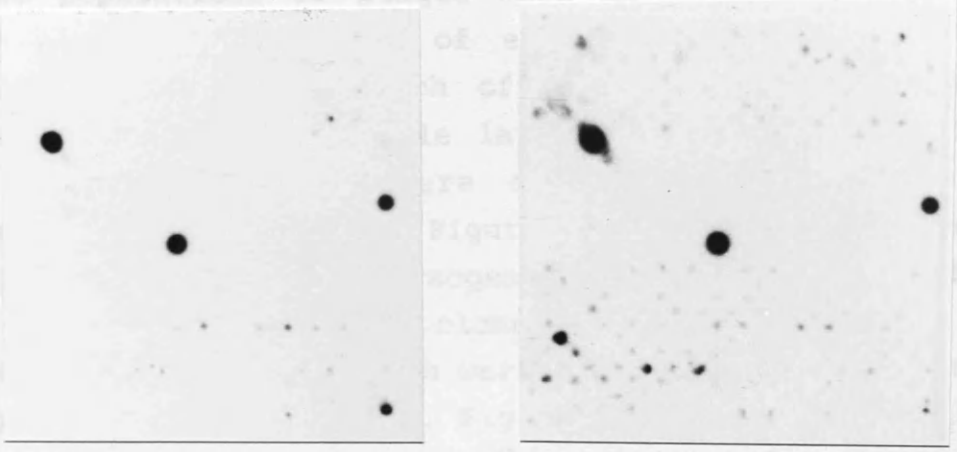


FIGURE 6.3; Hybridization Of Radiolabelled MB74 With Duplicate Plaque Lifts Of 225 Recombinant Phage Lambda Thought To Contain Inserts Homologous To Either AC36, MB74, tetR1 Or tetR5

probes produced similar hybridization patterns. However where a particularly strong signal was obtained (the three central clones in Figure 6.3), it was unique to an individual probe and unique within the series of ten plaques isolated from the one soak out. In contrast, plaques giving a less striking signal (but nevertheless above "background"), tended to hybridize with more than one probe and were not unique in their series of ten. These recombinants which were potentially most interesting, were possibly comprised of the intervening sequences between two linked probes, with the poor signal due to only partial representation of probe DNA in the phage genome. For further analysis, one positive plaque in a series of ten from each soak out, was picked and streaked out to isolate single plaques. In any series which had two populations of plaque with differing hybridization patterns, a representative of each form was chosen. Five individual plaques from each of the twenty seven streaked out, were spotted onto double layer phage plates as before. Four negative controls were added to the pattern, as indicated in the legend to Figure 6.4; EMBL4 which did not form plaques on the P2 lysogen (P2392) used as a host, EMBL3,A which was an EMBL3 clone of non-streptomycete DNA, and EMBL3,B and EMBL3,C which were EMBL3 clones containing S. rimosus DNA picked at random. Figure 6.4a-d was the result of independent hybridization, at 65^{o}C, 0.5xSSC, of each probe to duplicate plaque lifts. Clearly this identified two recombinants homologous to AC36, four homologous to MB74, three homologous to tetR1 and one homologous to tetR5. In addition some recombinants hybridized less efficiently with the probe DNA. Thirteen recombinants were selected for further analysis on the basis of their homology to the four probes used. The level of hybridization of each of the recombinants with the probe DNA to which they annealed is given in Table 6.2.

6.4 ANALYSIS OF DNA FROM RECOMBINANTS WHICH HYBRIDIZED WITH AC36, MB74, TETR1 OR TETR5

DNA was prepared from each and the size of the insert DNA was estimated (Table 6.2) from products of restriction enzyme digests after gel electrophoresis. Phage DNA cut with BamHI was transferred from an agarose gel to Amersham Hybond-N. Radiolabelled probes, made from each of

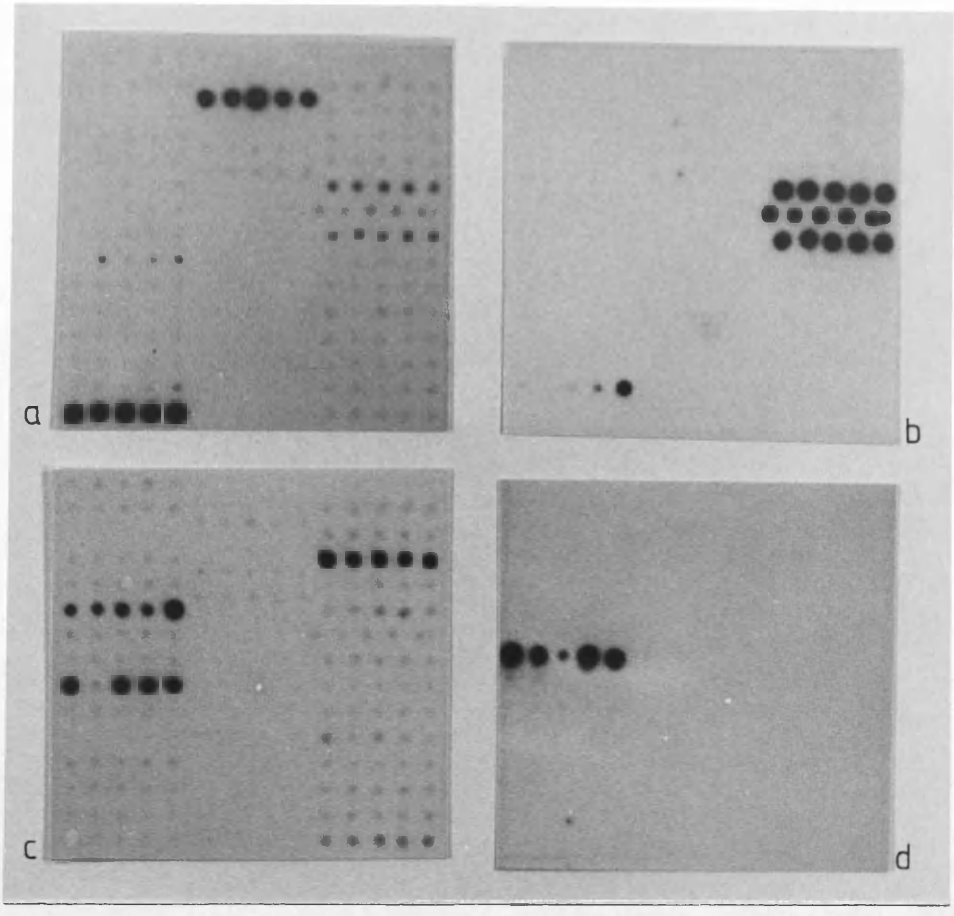


FIGURE 6.4; Hybridization Of Radiolabelled AC36 (a), MB74 (b), tetR1 (c) And tetR5 (d) To Putative Phage Lambda Clones

Pattern of clones;

phage 1		EMBL4
phage 2		phage 3
EMBL4	phage 4	phage 5A
phage 8		phage 5B
phage 12	phage10	phage 11
phage 13B	EMBL3A	phage 13A
EMBL3B		phage 20
phage 22		phage 24
phage 31		phage 33
phage 34		phage 37
phage 38		phage 39
phage 40		phage 41
phage 42		phage 44
phage 55		phage 56
phage 57		EMBL3C

The s.a. of the probes were $1x10^{9}$dpm ug^{-1} for AC36, $7.8x10^{8}$dpm ug^{-1} for MB74, $5.0x10^{8}$dpm ug^{-1} for tetR1 and $1.5x10^{9}$dpm ug^{-1} for tetR5. Hybridization was carried out in Denhardt's solution, 10% (w/v) dextran sulphate, 0.5xSSC at $65^{o}C$. Washing was performed at the same stringency.

TABLE 6.2; Phage Selected For DNA Preparation

lambda clone	position*	hybridization signal strong	weak	weak	insert size (kb)
4	1	AC36			15.6
5B	1	tetR1			19.5
13A	1	MB74	AC36		19.4
13B	1	tetR1			20.7
20	4	MB74	AC36	tetR1	16.1
22	1	tetR5			11.8
24	1	MB74	AC36		18.0
31A	1	tetR1	AC36	tetR5	
31B	3	tetR1	tetR5		15.9
31C	4		AC36	tetR5	14.0
55	1	MB74	AC36		15.5
56	1		tetR1		20.8
57	1	AC36			19.3

*The position of the lambda clone indicates the plaque selected from the series of five, numbered one to five (left to right), from Figure 6.4.

the four clones (tetR1, tetR5, AC36 and MB74) were hybridized independently to identical blots at high stringency (70°C, 0.2xSSC). Quite clearly, Figure 6.5 showed that the insert DNA of each of twelve recombinants (phage 55 DNA had not yet been purified) hybridized uniquely with only one of the four probes used. This left the observed cross reactivity of some of the phage, to more than one of the probes, unexplained.

The cross homology of phage 13A, 20, 24, 55 and 57 to both MB74 and AC36 (Figure 6.4) may be explained by sequence homology of the two unlinked probes. This was confirmed by low stringency hybridization (60°C, 0.5xSSC), of AC36 to DNA from phage 55 (Figure 6.6 lane 3). Clearly, phage 55 spans the genomic locus of MB74 (Figure 6.6 lane 2), and shares homology with AC36 which can be detected in low stringency Southern analysis of the phage clones. The cross homology of AC36 to MB74 was contained exclusively within the 7kb insert of MB74, and this can be demonstrated in relaxed stringency cross hybridization of the plasmid DNA (see Chapter 5.7). The observed hybridization of tetR1 to phage 20 can also be explained by homology of the resistance gene overlapping with the 7kb insert of MB74. Figures 6.7a and b show hybridization of MB74 (at high stringency) and tetR1 (at low stringency) to DNA around the MB74 locus of the S. longisporoflavus genome. Again, phage 55 was used as an example. From restriction enzyme digests of the purified DNA and a detailed restriction map of EMBL3 arms (Frischhauf et al., 1983), the recognition sites of infrequently cutting enzymes were located within the insert DNA of phage 55 (Figure 6.8). The restriction map derived from these data was in agreement with the hybridization pattern of radiolabelled MB74 to phage 55 DNA. A comparison of the hybridization patterns of tetR1 and MB74 to phage 55 DNA (Figure 6.7) indicated that in general, the probes hybridized to the same DNA fragments which were completely internal to the insert DNA of phage 55. However, tetR1 was also found to have homology with an additional SmaI fragment measuring 4.5kb (located to the right of the MB74 sequences and including 3kb of the right arm of EMBL3). Similarly, homology with a KpnI fragment located to the right of MB74 and including the

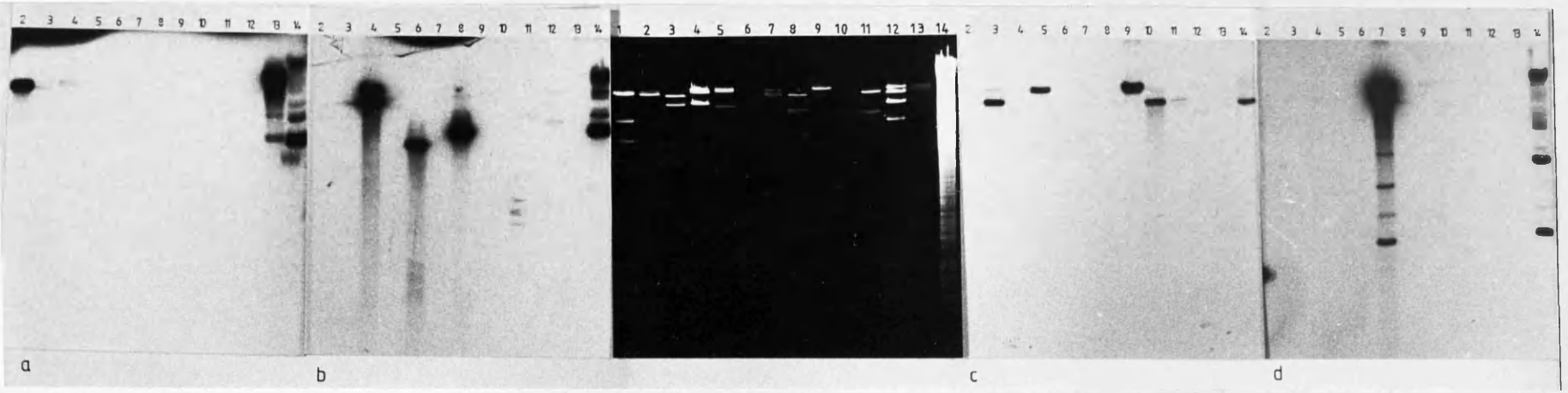


FIGURE 6.5; Hybridization Of AC36 (a), MB74 (b), tetR1 (c) And tetR5 (d) With DNA Prepared From Thirteen Phage Lambda Clones Of Interest

Lane,

1. phage lambda DNA digested with HindIII
2. phage 4 digested with BamHI
3. phage 5B digested with BamHI
4. phage 13A digested with BamHI
5. phage 13B digested with BamHI
6. phage 20 digested with BamHI
7. phage 22 digested with BamHI
8. phage 24 digested with BamHI
9. phage 31A digested with BamHI
10. phage 31B digested with BamHI
11. phage 31C digested with BamHI
12. phage 56 digested with BamHI
13. phage 57 digested with BamHI

The s.a. of the probes were all above 1.0×10^{9} dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, 0.2xSSC at $70^{o}C$.

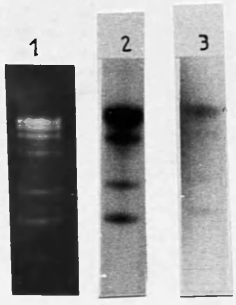


FIGURE 6.6; Hybridization Of MB74 At High Stringency (lane 2) And AC36 At Low Stringency (lane 3) To DNA Prepared From Phage Lambda 55

Phage 55 DNA was digested with BamHI. The fragments larger than 7kb which hybridized with the probe DNA were derived from partial digestion of the DNA by the restriction enzyme.
The s.a. of the probes were $1x10^{9}$dpm ug^{-1} for MB74 and $6.5x10^{8}$dpm ug^{-1} for AC36. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, with 0.2xSSC at 70^{o}C for MB74, and 0.5xSSC at 60^{o}C for AC36.

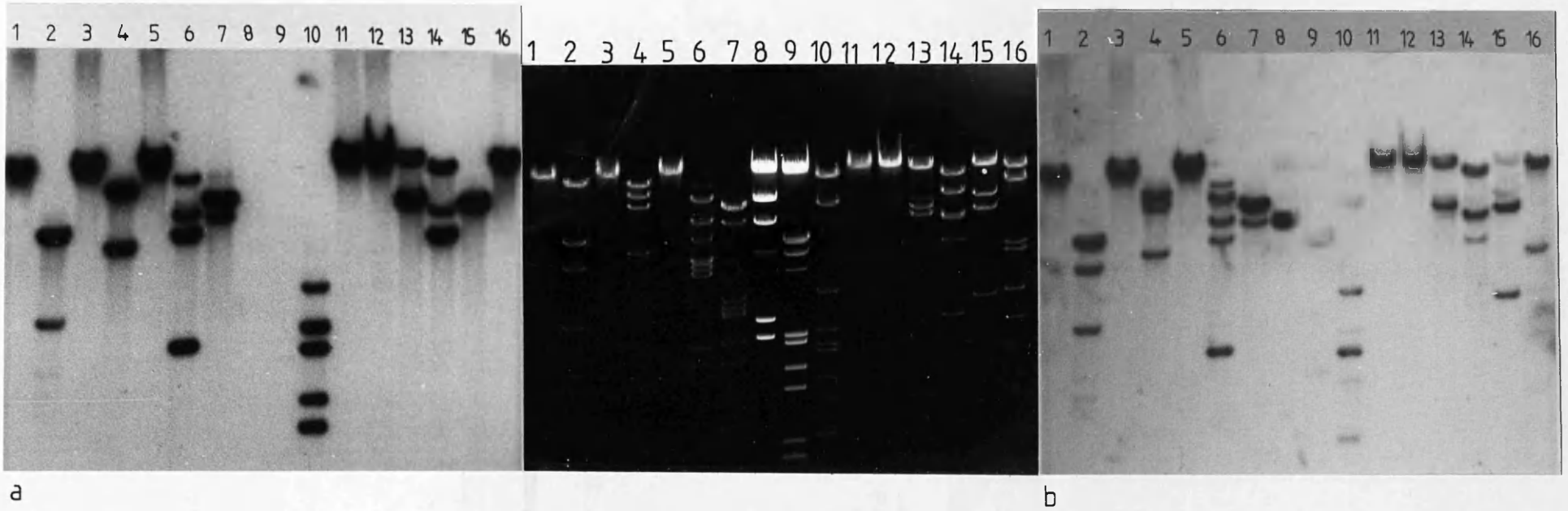


FIGURE 6.7; Hybridization Of MB74 At High Stringency (a) And tetR1 At Low Stringency (b) To Phage 55 DNA Digested With Various Restriction Endonucleases

Lane,

1. phage 55 digested with HindIII
2. phage 55 digested with SmaI
3. phage 55 digested with EcoRI
4. phage 55 digested with KpnI
5. phage 55 digested with XbaI
6. phage 55 digested with PvuII
7. phage 55 digested with PstI
8. phage lambda DNA digested with HindIII
9. phage lambda DNA digested with HindIII and EcoRI
10. phage 55 digested with SalI
11. phage 55 digested with EcoRV
12. phage 55 undigested
13. phage 55 digested with BglII
14. phage 55 digested with SphI
15. phage 55 digested with BamHI
16. phage 55 digested with ClaI

The s.a. of the probes were $1x10^{9}$dpm ug^{-1}. Hybridization was carried out in Denhardts solution, 10% (w/v) dextran sulphate, with 0.2xSSC at 70°C for MB74 and with 0.5xSSC at 65°C for tetR1.

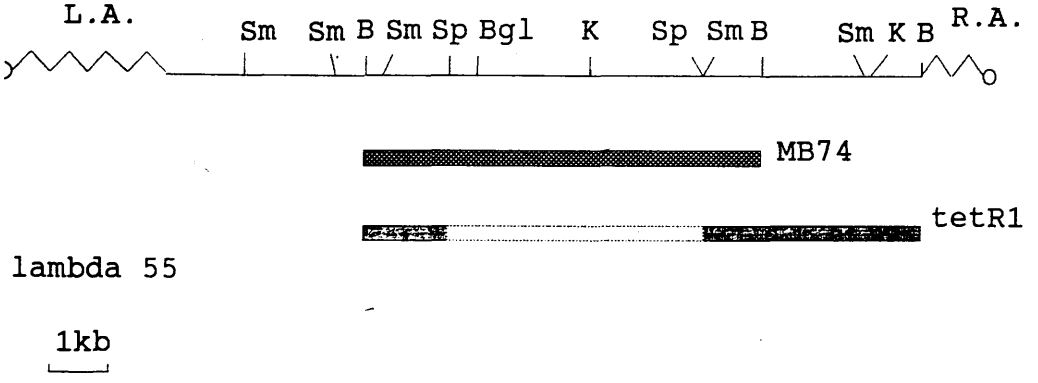


FIGURE 6.8; Restriction Map Of The MB74 Locus Of S. longisporoflavus, As Determined From Phage 55, And Showing The Overlapping Sequence Homology To tetR1

The shaded blocks indicate the position of the 7kb BamHI fragment of MB74 and the DNA fragments from the insert of phage 55 to which radiolabelled tetR1 hybridized. The lightly shaded box indicates the region which hybridized poorly to the probe.

whole of the right arm of EMBL3 was observed. The region of homology to tetR1 was confined specifically within the insert DNA of phage 55. This was shown by hybridization of the probe with the 2.5kb BamHI fragment (lane 15) extending from the right end of MB74 to the end of the insert DNA (giving the relationship shown in Figure 6.8). In addition annealing of the tetR1 probe to the right arm of EMBL3 could not be ruled out in view of hybridization to the 6.6kb phage lambda HindIII fragment which remains unexplained (lane 8).

Curiously the tetR1 probe hybridized only weakly to the 4.5kb SphI fragment which was central to the MB74 sequences (lane 14). Although tetR1 hybridized strongly to the 5.7kb SmaI fragment (which spans the 4.5kb SphI fragment) the low level of hybridization to the SphI fragment in question was registered in Figure 6.8 by the lightly shaded region indicated with homology to tetR1. It was therefore possible that tetR1 shares homology with two regions of phage 55 separated by a region of low homology.

6.5 SUMMARY

This chapter describes the construction of a representative library of genomic DNA from S. longisporoflavus 83E6 contained in the bacteriophage lambda replacement vector EMBL3. Recombinants which contained DNA isolated previously, were identified by hybridization to radiolabelled probes prepared from AC36, MB74, tetR1 and tetR5. Eleven phage were recovered; two contained sequences from AC36, three contained sequences from MB74, four contained sequences from tetR1 and one contained sequences from tetR5. The average insert size measured 17.3kb. Hybridization of AC36 and tetR1 at relaxed stringency to DNA prepared from phage 55 was demonstrated. The low level of homology to AC36 was located within the 7kb BamHI fragment common to MB74. In contrast, two separate regions appeared to share most homology with tetR1. These were located in the left end of MB74 (as shown in Figure 6.8) and the rightmost 3.5kb of the insert DNA of phage 55 (which includes 0.9kb of MB74). This result demonstrated the close linkage of MB74 and a tetR1 homologue in S. longisporoflavus.

CHAPTER 7

ANALYSIS OF DNA ISOLATED FROM S. longisporoflavus FOR SEQUENCES WHICH COULD BE INVOLVED IN TETRONASIN BIOSYNTHESIS - CONCLUDING REMARKS AND FUTURE EXPERIMENTS

7.1 INTRODUCTION

In the previous chapters the isolation of DNA fragments from S. longisporoflavus which either conferred resistance to tetronasin in S lividans (tetR1 and tetR5 - Chapter 4), or contained sequences with homology (determined by cross hybridization to actI or monI (AC36 and MB74 respectively - Chapter 5) were described. Evidence for the involvement of sequences carried on any of these clones in the biosynthesis of tetronasin in S. longisporoflavus was largely circumstantial.

Efforts to identify likely candidates for the "tetronasin synthase" genes by cross-hybridization with sequences involved in the biosynthesis of other polyketide antibiotics did not identify a unique candidate from AC36 or MB74 (Chapter 5.7). The isolation of phage lambda EMBL3 recombinants containing flanking DNA from each of the four loci did not establish close linkage between any of the sequences cloned previously, although MB74 was shown to overlap with sequences which hybridized at low stringency with tetR1. Neither the insert DNA from AC36 nor MB74 when subcloned into the streptomycete vector pIJ702 (pKJL1 and pKJL2 respectively - Chapter 5.8) could confer resistance to tetronasin in S. lividans or S. albus. However, future experiments should test whether DNA flanking MB74 functions as a tetronasin resistance determinant.

In the absence of good evidence regarding the function of AC36, or MB74 and additional sequences present on tetR1 and tetR5, experiments were designed to determine their roles (if any) in tetronasin biosynthesis. Preliminary attempts to answer this question tested both the ability of the recombinant plasmid DNA to rescue mutants of S. longisporoflavus deficient in the biosynthesis of tetronasin and whether disruption of the endogenous AC36 or MB74 sequences resulted in loss of tetronasin biosynthesis.

Although the data reported in sections 7.2 and 7.3 were inconclusive, it nevertheless illustrated the logical progression of the work and what would be required to be repeated if the study were to be continued.

7.2 ATTEMPTS TO COMPLEMENT S. longisporoflavus MUTANTS DEFICIENT IN THE BIOSYNTHESIS OF TETRONASIN

The isolation and phenotypes of S.

longisporoflavus mutants deficient in the biosynthesis of tetronasin were described in Chapter 3. The introduction of plasmid DNA into wild type S. longisporoflavus strain 4584 by PEG-mediated transformation of protoplasts and regeneration of transformants on R9 agar was also established and described in Chapter 3.

In an initial experiment, ptetR- 11, 51 (see Chapter 4.5), pKJL- 1 and 2 (see Chapter 5.8), and pAA- 2, 6 and 8 (see Chapter 4.2.5) were to be introduced into six S. longisporoflavus mutants which were impaired in tetronasin when grown on Emersons agar (TNP mutants). Protoplasts were prepared from each of the TNP strains and S. longisporoflavus 4584 as described in Chapter 3.7.

Unfortunately no transformants were obtained of TNP12, TNP38 and 4584, and where transformants were recovered (with TNP- 44, 56, 61 and 62) the numbers obtained were much lower than expected (10-70 transformants ug^{-1} DNA). The poor efficiency of transformation might reflect differences in regeneration media or individual preparations of protoplasts. However, mutated strains often exhibit lowered competence for plasmid DNA uptake (M. Warren, pers. comm.). This phenomenon has not been investigated but may simply be due to alterations in the cellular physiology of these strains. In S. longisporoflavus it could be imagined that any mutation which alters the growth of the strain might adversely effect the parameters important to the preparation, survival or regeneration of protoplasts.

Where numbers permitted, twelve individual transformants from each experiment were patched onto Emersons agar plates (four per plate), incubated for 7 days at $30^{o}C$ before tetronasin production was assayed (Table 7.1). Thiostrepton selection for maintenance of the plasmid DNA was relaxed for the bioassay because it inhibited differentiation and possibly tetronasin production in S. longisporoflavus (this was not tested because B. subtilis was sensitive to thiostrepton).

Conclusions could not readily be drawn from these data without repetition and further investigation. However some of the observations were considered worth pursuing. In particular it would be interesting to ascertain whether the observed rescue of the tet^{-} mutation in TNP61 and TNP62 after transformation with pAA6 and pAA8 respectively, represented

TABLE 7.1; Phenotypes Of Some S. longisporoflavus TNP Strains Containing Various Plasmid Constructs

TNP strain	Plasmid	Bioactivity No. producing / No. tested	titre*	Description
44	---@	0/12	0	as wild type
44	AA2	0/1	0	as wild type
56	---	1/12	1	as wild type
56	AA8	0/12	0	all synthesized brown pigment and were bald
61	---	1/12	2	as wild type
61	AA6	1/1	5	producer was very slow growing
62	---	0/12	0	as wild type
62	KJL1	0/12	0	as wild type
62	KJL2	0/12	0	as wild type
62	AA8	1/4	5	producer was very slow growing
62	AA2	0/2	0	as wild type

*titre was estimated by the size of the zone of inhibition in mm from the colony edge.
@--- represents plasmid free colony.

plasmid borne complementation or spontaneous reversion. The phenotype of all TNP56 transformants containing pAA8 certainly testified to the presence of some biologically-active sequence carried on this plasmid, which although perhaps unlikely to be directly involved in tetronasin biosynthesis was nevertheless interesting for its pleiotropic effects.

It was clear from this experiment that pKJL- 1 and 2 were unable to rescue the deficiency in TNP62 which disrupted tetronasin biosynthesis. This was probably because the genomic mutation in TNP62 did not correspond to the DNA insert carried on pIJ702. However, poor expression of the insert DNA in the high copy number plasmid vector cannot be ruled out.

Transformants containing pKJL- 1 or 2 were not obtained even when sufficient DNA was added to the transformation to expect their recovery. It is possible, therefore, that high copy-numbers of tetR11 or tetR51 are lethal to S. longisporoflavus. Efforts to subclone both this DNA and AC36 and MB74 into low copy-number Streptomyces vectors for use in complementation studies faltered after pXEΔ13 was chosen as a cloning recipient. pXEΔ13 is maintained in Streptomyces at low copy-number by virtue of SCP2 origin of replication but the 17kb plasmid also contains an E. coli replicon (from colE1) enabling propagation and manipulation in E. coli. Unfortunately efforts to subclone the insert DNA from AC36, MB74, tetR1 and tetR5 into pXEΔ13 resulted only in the recovery of non-recombinant pXEΔ13 or deletion derivatives of the parental plasmid which suggested that such constructs may have been unstable in E. coli. It may therefore be necessary to subclone these inserts into a simplified SCP2 based vector (e.g. pIJ916) using S. lividans as the primary cloning recipient prior to re-introduction into S. longisporoflavus strains.

7.3 ATTEMPTS TO DISRUPT THE ENDOGENOUS COPIES OF AC36 AND MB74 IN S. longisporoflavus 4584

A preliminary investigation showed that infection of S. longisporoflavus by ϕKC515 was rather inefficient and both high titre phage stocks and pre-germinated spores of S. longisporoflavus were required to recover plaques. This suggested that mutational cloning would be ineffective and

indeed it proved impossible to efficiently transfer phage from a primary library constructed in S. lividans to S. longisporoflavus 4584. Very few plaques were recovered on S. longisporoflavus from a velvet impression of a primary phage library and where mycelial fragments of S. lividans were also transferred (even after exposure to chloroform vapour) growth of the primary cloning host inhibited germination of S. longisporoflavus spores. Efforts to circumvent this problem by using pre-germinated S. longisporoflavus spores to propagate phage released from transfectants of S. lividans protoplasts also failed to produce plaques, presumably for the same reasons. An alternative to construction of a mutational cloning library was to subclone small DNA fragments from putative tetronasin synthase genes into ϕKC515. High titre stocks of the recombinant phages could be prepared and it might then be possible to infect S. longisporoflavus 4584. It was hoped that lysogeny of S. longisporoflavus, by integration of the recombinant phage genome into the recipient cell genome at the homologous site, would disrupt transcription through these sequences and confirm the role of some or all of the DNA in tetronasin biosynthesis.

7.3.1 SUBCLONING OF FRAGMENTS FROM AC36 AND MB74 INTO ϕKC515

To be confident of creating lysogens which disrupt a transcription unit, the fragments of AC36 and MB74 shown to be completely transcribed (the 2.7kb EcoRV/PvuII and 1.4kb PvuII/BamHI fragments from AC36 and the 1.9kb PvuII fragment from MB74 - see Chapter 5.9) were chosen to be subcloned into ϕKC515. Unfortunately, cleavage of ϕKC515 DNA by PvuII was problematic with only an estimated 50% of the DNA digested. This could not be improved by either phenol extraction of the DNA or digestion with a ten-fold excess of restriction enzyme. Recombinant phage were not identified by hybridization of radiolabelled AC36 or MB74 with transfectant plaques of S. lividans TK64 obtained from ligation experiments using partially digested DNA.

It was therefore decided to attempt to subclone larger DNA fragments from AC36 and MB74 into ϕKC515. This would hopefully enhance the chance of success at two stages in the experiment:

1. By increasing the recovery of recombinants through

targeting "sticky end" fragments for ligation.

2. By increasing the frequency of the integration event through provision of extended sequence homology between the recombinant phage and recipient genome.

Ligation of insert and ϕKC515 DNA;

The 5kb BamHI fragment from AC36 and the 2kb and 5kb BamHI/BglII fragments from MB74 were chosen for subcloning into ϕKC515 and each was purified from an agarose gel. From S1 mapping experiments (Chapter 5.9) a preliminary transcription map was derived for AC36 which was consistent with complete transcription of the whole 5kb insert although, protection of the full size fragment was not tested. The BglII restriction site in MB74 is located within the 1.9kb PvuII fragment which was shown to be completely transcribed. However, S1 mapping of the remainder of the BamHI/BglII fragments of MB74 was inconclusive.

ϕKC515 DNA will not efficiently package genomes greater than 42kb. Therefore ϕKC515 digested with both BamHI and BglII was prepared and phosphatased (the CIAP was removed by phenol extraction rather than gel purification of the large phage replicon). Double digestion with these enzymes excised a 1.85kb fragment containing the tsr gene from ϕKC515 generating a derivative which could accept inserts of up to 6kb. Although tsr would be useful for selection of lysogens, thiostrepton was thought to prevent differentiation in S. longisporoflavus and might inhibit tetronasin biosynthesis pleiotropically. Selection for viomycin resistance (ϕKC515 carries the vph resistance determinant) was used to identify and maintain lysogens (B. subtilis was resistant to viomycin thus enabling tetronasin production to be assayed whilst selection for lysogens was maintained).

100ng of prepared vector DNA was ligated to each of 50ng of the 5kb AC36 or MB74 DNA fragments or 20ng of the 2kb MB74 fragment, in 20ul 1xBRL ligation buffer with 1U T4 DNA ligase for 2hrs at 22°C. The ligation mix was used to transfect S. lividans TK64 protoplasts.

Transfection of S. lividans TK64 protoplasts;

Transfection of S. lividans TK64 protoplasts was stimulated 10-fold by including positively-charged DNA-free, liposomes in the transfection mix (Table 7.2). The ligated DNA was introduced into S. lividans TK64 protoplasts by PEG-mediated, liposome-assisted transfection.

TABLE 7.2; Transfection Of *S. lividans* TK64 Protoplasts

Protoplasts	Liposome fraction added	DNA used	Efficiency of transfection (pfu ug^{-1})
Fresh/Frozen	No	ØKC515	$2.2x10^2$
Fresh/Frozen	Yes	ØKC515	$3.7x10^3$
Frozen Stock@	Yes	ØKC515	$7.3x10^5$
Frozen Stock	Yes	self ligated vector*	$3.5x10^4$
Frozen Stock	Yes	vector + insert A*	$3.8x10^4$
Frozen Stock	Yes	vector + insert B*	$3.3x10^4$
Frozen Stock	Yes	vector + insert C*	$4.0x10^4$

@Frozen Stock protoplasts were prepared earlier and were transformed with pIJ702 with an efficiency giving $8.0x10^7$ transformants ug^{-1}

*vector DNA = ØKC515 digested with BamHI + BglII and treated with CIAP.

insert DNA; A = 5kb BamHI fragment from AC36
B = 5kb BamHI/BglII fragment from MB74
C = 2kb BamHI/BglII fragment from MB74

Ligation conditions for vector + insert DNA were given in the text. Ligation conditions for vector self ligation were as for vector + insert except for substitution of the insert fraction with sterile H_2O.

Lysogeny of S. longisporoflavus 4584;

A comparison of the numbers of plaques recovered with vector ligated to insert and self-ligation of vector DNA was inconclusive (Table 7.2). However, because the viomycin phosphotransferase gene provides strong selection for lysogens in S. longisporoflavus a high titre phage suspension was prepared for each of the sub-cloning experiments from a plate containing around 100 plaques. 5ul aliquots of these stocks were spotted onto pre-germinated spores of S. longisporoflavus. Any colonies arising from within a plaque were streaked onto DNA agar containing 10-30ug ml^{-1} viomycin. Two viomycin resistant isolates, S. longisporoflavus GD1 and GD2, were particularly interesting because they both failed to produce aerial mycelia or synthesize tetronasin. GD1 and GD2 might be lysogenic for recombinant phage carrying the whole AC36 insert or the 5kb BamHI/BglII fragment from MB74 respectively. To test this, radiolabelled AC36 and MB74 were used to probe total DNA prepared from the two strains. Both probes hybridized with the same size BamHI and KpnI fragments as found in the tetronasin producing strains 4584 and 83E6. After over-exposure of the autoradiograph, hybridization to larger "satellite" fragments was also observed (see Figure 7.1 for an example). Without further investigation it is impossible to deduce firm conclusions from these data. However it is possible - even likely - that these strains were lysogenic for recombinant phage given that they were both resistant to viomycin at 20ug ml^{-1} (Southern analysis of chromosomal DNA using radiolabelled ϕKC515 could be used to confirm this). The additional fragments which hybridized with the probe DNA therefore could have originated from cleavage of recombinant genomes.

It is also possible that a spontaneous mutation was responsible for the pleiotropic phenotype of GD1 and GD2. Without being able to complete the life cycle it was likely that neither of these strains was derived from a single spore. So even if they were lysogens they were likely to be heterogenotes (the phage DNA would be integrated into a genome within a mycelial fragment which contained more than one chromosome). This would explain the persistence of the original wild type sequences in these two strains. If this is the case then the phenotype of these heterogenotes would, in most situations, be expected to remain wild type. It could

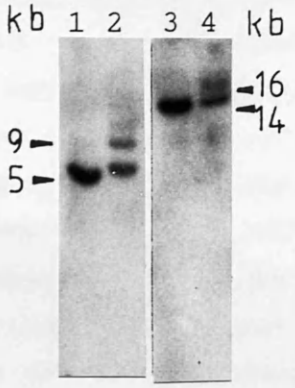


FIGURE 7.1; Hybridization Of AC36 To Total DNA Prepared From S. longisporoflavus Strains 83E6 And GD1

1. total DNA from strain 83E6 digested with BamHI
2. total DNA from strain GD1 digested with BamHI
3. total DNA from strain 83E6 digested with KpnI
4. total DNA from strain GD1 digested with KpnI

The s.a. of the probe was 7.5×10^8dpm ug^{-1}. Hybridization was carried out in Denhardt's solution, 10% (w/v) dextran sulphate, with 0.2xSSC at 70^{o}C. Washing was performed at the same stringency.

therefore be argued that the pleiotropic mutations in GD1 and GD2 were unrelated to integration events at the AC36 or MB74 loci.

In the event that the phenotype of either, or both strains was a consequence of disruption of tetronasin biosynthesis with a pleiotropic developmental deficiency, it may be expected that tetronasin biosynthesis was intrinsically associated with the differentiation pathway. This was considered unlikely both for the reasons given above and because mutants with normal morphology which failed to produce tetronasin had been obtained (Chapter 3). It nevertheless remained an attractive hypothesis because of the observed homology between WhiE and MB74 (Chapter 5.6). The possible inter-relationship of tetronasin and differentiation was investigated by testing whether sub-lethal concentrations of the antibiotic - both in the presence (0.01M and 0.1M) and absence of NaCl - would restore the development of aerial mycelium and sporulation in GD1 and/or GD2. Although this was not apparent in either case, the possible inter-relationship of these traits should not be over-looked in future experiments.

7.4 CONCLUDING REMARKS AND FUTURE EXPERIMENTS

This thesis describes the isolation and preliminary characterisation of sequences (Chapters 4, 5 and 6) from S. longisporoflavus which are probably involved in the biosynthesis of, and/or resistance to the polyketide, polyether tetronasin.

Two non-identical DNA fragments (tetR1 and tetR5) were isolated from S. longisporoflavus and found to increase the tolerance of S. lividans to the antibiotic tetronasin (Chapter 4). tetR5 also conferred resistance upon a tetronasin-super-sensitive strain of S. albus to a level comparable with that found in wild type S. longisporoflavus 4584. Although the functions of these sequences in the parental strain were undetermined, they may by virtue of their activity in heterologous hosts, represent the first examples of the isolation of resistance determinants to an ionophore antibiotic. S. lividans/ptetR1 and S. lividans/ptetR5 are being tested (J. A. Robinson, pers. comm.) to determine whether cross resistance to a closely related antibiotic (the polyketide, polyether monensin) is

conferred. Sequences with homology to tetR1 or tetR5 were also identified by Southern analysis of total DNA from S. cinnamonensis (J. A. Robinson, pers. comm.). The isolation of these sequences is in progress to study their possible role in resistance to monensin. The results of these experiments were not available at the time of submission of the thesis.

Cross hybridization identified a 5kb BamHI fragment (AC36) from total DNA of S. longisporoflavus with homology to actI. Paradoxically a 4.3kb BamHI fragment from S. cinnamonensis (monI) with good sequence homology to actI (J. A. Robinson, pers. comm.) failed to exhibit sequence homology with the 5kb fragment but did hybridize with a 7kb BamHI fragment (MB74) from S. longisporoflavus. Both fragments were cloned. Cross hybridization of AC36 and MB74 with total DNA from several Streptomyces sp. which were known to produce polyketide antibiotics was investigated. At 65^{o}C in 0.5xSSC both probes failed to hybridize with DNA fragments which correlated with restriction fragments known to be involved in polyketide biosynthesis. MB74 was also found to have greater sequence homology with an 11kb BamHI fragment from S. cinnamonensis rather than the 4.3kb monI. Cross hybridization with isolated sequences involved in oxytetracycline (otcY), actinorhodin (actI) and putatively in monensin (monI) biosynthesis was investigated. Not surprisingly MB74 hybridized to a different restriction fragment from monI than actI and otcY (Chapter 5.7). Interestingly a fragment with a low level of homology to AC36 and otcY was discovered in MB74 which did not overlap with the sequences which hybridized to monI. A 2.7kb EcoRV/PvuII fragment from AC36 hybridized with both actI and monI. These data suggested that both AC36 and MB74 could be involved in polyketide biosynthesis but did not uniquely indicate sequences encoding the tetronasin synthase. More specific probes for polyketide synthase genes could be tested. All four clones described above have been released to P. F. Leadlay for Southern analysis using sequences purported to be specific for genes encoding ACP's.

Recombinant bacteriophages which contained flanking DNA of each of the clones (AC36, MB74, tetR1 and tetR5) were identified by hybridization from a representative library of S. longisporoflavus DNA. Close linkage between any of the four clones was not established. However, sequences with

homology to tetR1 were found, overlapping with and in the DNA flanking, MB74. By itself MB74 was unable to confer resistance to tetronasin upon S. lividans or S. albus.

If this work were to be continued initial experiments would be required to prove that the DNA isolated previously was involved in tetronasin resistance and biosynthesis in S. longisporoflavus. Once a role in tetronasin metabolism has been unequivocally established for a fragment of DNA more fundamental and biotechnological questions can be addressed. The remaining two sections discuss the experiments which would determine the role of the DNA in the parental and strain, and assuming involvement in tetronasin production can be unequivocally established, future studies which could be undertaken.

7.4.1 CHARACTERISATION OF TETRONASIN RESISTANCE DETERMINANTS

Further characterisation should initially determine the extent of the tetronasin resistance functions by deletion analysis of the respective 5.0kb and 5.5kb inserts of ptetR11 and ptetR51 and/or subcloning of the relevant sequences. The role of the sequences in S. longisporoflavus and the mechanism by which resistance to tetronasin is mediated can then by examined. This may be best approached by several strategies:

(a) Disruption of the genomic copies of tetR1 and tetR5 in the parental strain: This could be carried out using the mutational cloning vectors in analogous experiments to those described in section 7.3 and might provide useful information of the role of both or either sequences with regard to tetronasin resistance and production in S. longisporoflavus. To guard against auto-lethality where self-resistance to tetronasin may be impaired, the experiments could be carried out in parallel in strains which are deficient in the production of tetronasin but resistant to added antibiotic. Lethal mutations can also be indicated by incorporating additional (reporter) sequences from S. longisporoflavus in the phage vector. The expression of the chosen reporter DNA must be non-essential to the host (under non-selective conditions), but detection of mutants created by integration of the phage into the genomic copy of the reporter gene must be possible. For example it should be possible to use fragments from the DNA involved in proline biosynthesis which

was isolated from S. longisporoflavus (Chapter 4). A phage vector containing fragments of both the pro and tetronasin resistance genes could therefore integrate at either loci. If all lysogens were found to be proline auxotrophs it could be surmised that expression of the "test" DNA was essential to the survival of the strain.

(b) Analysis of the direct effect of tetR1 and tetR5 on production of tetronasin: Tetronasin resistance determinants may function by active efflux or covalent modification of the antibiotic as suggested in Chapter 4.1.3. Evidence for either mechanisms could be found by re-purification of tetronasin which has been added to cultures of strains which contain tetR1 and/or tetR5. For example if unmodified tetronasin was recovered from mainly the culture broth of tetronasin resistant, recombinant strains but was found to accumulate in the mycelia of sensitive strains then an efflux mechanism could be postulated. Similarly if modification of tetronasin correlated with the presence of either, or both tetR1 and tetR5 then obvious conclusions could be drawn.

(c) Determination of the DNA sequence: Determining the DNA sequence of genes encoded by tetR1 and tetR5 could be useful for comparison with sequences of known function. Sequence homology between resistance genes with efflux or phosphotransferase mechanisms has been observed (Chapter 4.1.1.3 and Brenner, 1987; respectively). It might therefore be possible to postulate the specific function of putative proteins from the DNA sequence of tetR1 and tetR5.

7.4.2 A PROVEN LINK BETWEEN TETRONASIN BIOSYNTHESIS AND THE DNA ISOLATED FROM S. longisporoflavus

The primary aim of a continuation of this work must be to determine a functional link between the DNA isolated and tetronasin biosynthesis in the parental strain. Evidence can be accumulated from DNA/DNA hybridization studies with genes for the biosynthesis of other polyketides or more specific probes for particular components of the PKS which could strengthen the case for involvement of AC36 and/or MB74 in tetronasin biosynthesis. Similarly, complementation of mutants deficient in the biosynthesis of tetronasin would provide additional evidence for a role in tetronasin biosynthesis. However, to demonstrate uneqivocally a

functional relationship, the coding sequences in the genome of the wild type strain should be disrupted with an accompanying loss of production observed. Repetition of the experiments described in section 7.3 is therefore proposed to finally elucidate the function of sequences carried on AC36 and MB74.

Once this has been established for one or more of the cloned DNA fragments the isolation and identification of the remainder of the putative tetronasin gene cluster by chromosome walking can begin. This assumes clustering of the genes for antibiotic biosynthesis in S. longisporoflavus. With most or all of the DNA encoding the biosynthetic pathway to tetronasin isolated, the fundamental questions posed in Chapter 1.4 regarding the biochemistry and molecular genetics of tetronasin production by S. longisporoflavus can be addressed. The pathway would also be amenable to the molecular biologist and it can be hoped that by the strategies described in Chapter 1.5 further biotechnological advances will be made.

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